# DEVELOPMENT OF MICROBIAL ECOLOGICAL THEORY: STABILITY, PLASTICITY, AND EVOLUTION OF MICROBIAL ECOSYSTEMS

EDITED BY : Shin Haruta, Yasuhisa Saito and Hiroyuki Futamata

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DEVELOPMENT OF MICROBIAL ECOLOGICAL THEORY: STABILITY, PLASTICITY, AND EVOLUTION OF MICROBIAL ECOSYSTEMS

Topic Editors:
Shin Haruta, Tokyo Metropolitan University, Japan
Yasuhisa Saito, Shimane University, Japan
Hiroyuki Futamata, Shizuoka University, Japan

As Antoni Gaudi skillfully employed beautiful mosaic tiles to represent nature and creatures, we are trying to assemble a variety of research articles into a comprehensive picture representing general rules of microbial ecosystems.
Photo credit: Sawa Arai & Miwa Arai

"How can we develop microbial ecological theory?" The development of microbial ecological theory has a long way to reach its goal. Advances in microbial ecological techniques provide novel insights into microbial ecosystems. Articles in this book are challenging to determine the central and general tenets of the ecological theory that describes the features of microbial ecosystems. Their achievements expand the frontiers of current microbial ecology and propose the next step. Assemblage of these diverse articles hopefully helps to go on this long journey with many avenues for advancement of microbial ecology.

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Theoretical frameworks

Editorial: Development of Microbial Ecological Theory: Stability, Plasticity, and Evolution of Microbial Ecosystems

Shin Haruta[1]*, Yasuhisa Saito[2] and Hiroyuki Futamata[3, 4]

[1] Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Japan, [2] Department of Mathematics, Shimane University, Matsue, Japan, [3] Research Institute of Green Science and Technology, Shizuoka University, Shizuoka, Japan, [4] Department of Applied Chemistry and Biochemical Engineering, Shizuoka University, Hamamatsu, Japan

Editorial on the Research Topic

Development of Microbial Ecological Theory: Stability, Plasticity, and Evolution of Microbial Ecosystems

We initiated this research topic based on the question "how can we develop microbial ecological theory?" We incorporated three important aspects, stability, plasticity, and evolution, which are key areas for progress in microbial ecological theory. Advances in microbial ecological techniques have allowed us to meticulously consider the dynamics of community structures, interspecies networks, and metabolic flux of microbial ecosystems. Studies using these techniques have demonstrated the stability (i.e., resistance, resilience, and persistence), dynamic equilibrium, and self-organizing ability of micro-ecosystems. Here, the fine contributors in this volume aimed to determine the central and general tenets of the ecological theory that describes the features of microbial ecosystems.

Analyses of the microflora in static-state conditions are insufficient to understand microbial ecosystems even if advanced omics techniques are used. Studies described in the included articles in this volume have tested several factors to elucidate how they affect the structures and functions of microbial communities. As external factors, the effects of temperatures (Melendrez et al.), nutrients (Aoyagi et al.; Aziz et al.; Itoh V et al.; Kato et al.), and viruses (Kashiwagi et al.; Thingstad et al.) were intensively investigated. Internal factors, such as dormancy (Chihara et al.; Thingstad et al.), genetic modifications (Kashiwagi et al.; Melendrez et al.; Thingstad et al.), distribution (Chihara et al.; Melendrez et al.; Stegen et al.; Thingstad et al.), and interspecies interactions (Aoyagi et al.; Fatma et al.; Hanajima et al.; Hasegawa et al.; Kato et al.) were widely examined and discussed. Kashiwagi et al. and Melendrez et al. enlightened us about the genetic and physiological plasticity of microbes in microbial communities. The articles mentioned above successfully provided information that has not been observed in microbial communities, suggesting unknown characteristics of microbial ecosystems.

To unveil complex microbial ecosystems, it is desirable to strategically apply stimuli (i.e., perturbation) and comprehensively examine the microbial response. Mathematical modeling and simulations are powerful tools to develop a strategy to interpret comprehensive data and predict unknown characteristics of ecosystems (Chihara et al.; Fatma et al.; Haruta et al., 2013; Melendrez et al.; Stegen et al.; Thingstad et al.).

Relationships between the "*diversity*" and "*stability*" of ecosystems have been extensively discussed so far. Some researchers stated that high *diversity* is beneficial for high *stability*, whereas others stated that higher *diversity* reduces *stability*. Clear definition and evaluation scale for these claims are still lacking due to technical limitations. Omic analysis cannot completely grasp all elements of microbial communities, and the stability of the community must depend on the strength and duration of perturbations. Theoretical frameworks are important to generalize microbial ecological observations. Song et al. systematically redefined "resilience" as an intrinsic property of complex microbial ecosystems by integrating ecological and engineering concepts in their perspective article. Renslow et al. developed a new method that broadens the use of spatial data for assessing "resilience" in general and reported its applicability using a simulated microbial community. Ke and Miki comparatively summarized theoretical frameworks for close interactions between organisms and environments by reviewing studies on plant-soil feedback. Their review article provides various aspects for the development and applicability of ecological theories. In other words, these theoretical frameworks hopefully help to interpret big data from multi-omics approaches.

The development of sophisticated microbial ecological theory still has a long way to reach its goal. We believe that this volume is a constructive step toward expanding the frontiers of current microbial ecology. Furthermore, the articles in this volume propose the next step we will need for this long journey with many avenues for advancement. SH said the following; this volume reminds us that microorganisms are totally different from macro-organisms, i.e., microorganisms are physiologically versatile, show high heterogeneity even in clonal population, frequently change genetic elements, and have close cell-to-cell interactions. It may be necessary to recognize microbial ecosystems as a huge gene network, as proposed by Song et al. and refurbish ecological theories that have been established for macro-ecosystems. To discover a novel theory, I would like to put another question "are there any ecological theories applicable to complex microbial ecosystems?" YS said the following; it is crucial to study how parts of a system give rise to the collective behaviors of the system and how the system interacts with its environment to understand the features of microbial ecosystems. Developing mathematical something helpful (or as powerful as possible) for this problem has a quite long journey and is more challenging than developing those for macrobial ecosystems. HF said the following; the phrases of "population dynamics," "community succession," and "interspecies interactions" always make me imagine the breath of microbial ecosystems. Microbial ecosystems seemingly change with utter abandon; however, there should be any yet-to-be-discovered rules in their behavior. Through this volume, I recognize that consideration of time scales is indispensable to exactly understand the dynamics of microbial ecosystems. Mathematical modeling research of the resilience focusing on recovery time and length by Renslow et al. breaks through to approach to ecological theory. It has been developing to understand microbial ecosystems integrally by linking gene expression, metabolisms, and interspecies interactions with the aid of mathematics. I believe that the microbial ecological theory emerges through these integral analyses.

We acknowledge the talented group of researchers who have contributed to this volume. They have aggressively addressed the critical issues in microbial ecology and have presented research, which can be a foundation for future studies. While initiating this research topic, we aimed to gather diverse research articles and assemble all of them into a comprehensive picture representing the general rules of microbial ecosystems. Actually, however, it was difficult for us to sufficiently assemble the wide variety of the great achievements. We hope you enjoy this unfinished but vivid and entrancing picture and this volume will help to find other pieces of the puzzle obtaining complete picture of microbial ecosystems.

AUTHOR CONTRIBUTIONS

SH drafted the manuscript. All authors wrote, revised, and approved the final version.

FUNDING

REFERENCES

Haruta, S., Yoshida, T., Aoi, Y., Kaneko, K., and Futamata, H. (2013). Challenges for complex microbial ecosystems: combination of experimental approaches with mathematical modeling. *Microb. Environ.* 28, 285–294. doi: 10.1264/jsme2.ME13034

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Dynamic transition of chemolithotrophic sulfur-oxidizing bacteria in response to amendment with nitrate in deposited marine sediments

Tomo Aoyagi[1][†], Makoto Kimura[1][†], Namiha Yamada[1], Ronald R. Navarro[1], Hideomi Itoh[2], Atsushi Ogata[1], Akiyoshi Sakoda[3], Yoko Katayama[4], Mitsuru Takasaki[5] and Tomoyuki Hori[1]*

[1] Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, [2] Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Sapporo, Japan, [3] Institute of Industrial Science, The University of Tokyo, Tokyo, Japan, [4] Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan, [5] Department of Food and Environmental Sciences, Faculty of Science and Engineering, Ishinomaki Senshu University, Ishinomaki, Japan

Although environmental stimuli are known to affect the structure and function of microbial communities, their impact on the metabolic network of microorganisms has not been well investigated. Here, geochemical analyses, high-throughput sequencing of 16S rRNA genes and transcripts, and isolation of potentially relevant bacteria were carried out to elucidate the anaerobic respiration processes stimulated by nitrate (20 mM) amendment of marine sediments. Marine sediments deposited by the Great East Japan Earthquake in 2011 were incubated anaerobically in the dark at 25°C for 5 days. Nitrate in slurry water decreased gradually for 2 days, then more rapidly until its complete depletion at day 5; production of N_2O followed the same pattern. From day 2 to 5, the sulfate concentration significantly increased and the sulfur content in solid-phase sediments significantly decreased. These results indicated that denitrification and sulfur oxidation occurred simultaneously. Illumina sequencing revealed the proliferation of known sulfur oxidizers, i.e., *Sulfurimonas* sp. and Chromatiales bacteria, which accounted for approximately 43.5% and 14.8% of the total population at day 5, respectively. These oxidizers also expressed 16S rRNA to a considerable extent, whereas the other microorganisms, e.g., iron(III) reducers and methanogens, became metabolically active at the end of the incubation. Extinction dilution culture in a basal-salts medium supplemented with sulfur compounds and nitrate successfully isolated the predominant sulfur oxidizers: *Sulfurimonas* sp. strain HDS01 and *Thioalkalispira* sp. strain HDS22. Their 16S rRNA genes showed 95.2–96.7% sequence similarity to the closest cultured relatives and they grew chemolithotrophically on nitrate and sulfur. Novel sulfur-oxidizing bacteria were thus directly involved in carbon fixation under nitrate-reducing conditions, activating anaerobic respiration processes and the reorganization of microbial communities in the deposited marine sediments.

Keywords: marine sediment, sulfur-oxidizing bacteria, environmental stimuli, high-throughput sequencing, the Great East Japan Earthquake in 2011

Introduction

Environmental stimuli affect the structure and function of microbial communities in natural environments. Numerous investigations have employed artificial stimulations by temperature, pH, NaCl, and electron donors and acceptors to clarify which microorganisms play important roles in the development and functioning of microbial communities (Hori et al., 2006; Kotsyurbenko et al., 2007; Lydmark et al., 2007; Ontiveros-Valencia et al., 2012). For instance, in terrestrial ecosystems, the supplementation with inorganic electron acceptors, e.g., nitrate, iron(III), and sulfate, has been shown to change the metabolic pathway and/or strengthen the degradation capability of indigenous microbial communities (Lueders and Friedrich, 2002; Labrenz et al., 2005; Hori et al., 2010), which in turn provides an opportunity to study the relationship among biogeochemical cycles of nitrogen, iron, sulfur, and carbon. However, the microbial community adaptation and development in response to environmental stimuli remain to be delineated because of the limited information concerning microbial population dynamics, rather than the dynamics of a particular taxon or a specific trophic group.

Marine sediment is commonly found on the sea floor below fish farms and in closed water areas throughout the world (Pearson and Rosenberg, 1978; Kemp et al., 1990; Armenteros et al., 2010; Kunihiro et al., 2011). A large quantity of marine sediment was deposited by the tsunami originating from the Great East Japan Earthquake in 2011, and the study of this material has shed light on the vast accumulation of marine sediments in not only closed sea areas but also coastal marine regions facing the open ocean (Inui et al., 2012). The geochemical cycles of elements in marine sediments have attracted much attention because of their possible association with coastal biotic activities. In a recent study, we reported that only limited degradation of organic matter occurred in association with the sulfate reduction in deposited marine sediments, even though the 16S rRNA transcripts detected were mainly from sulfate reducers (Hori et al., 2014). In the same report, we found that ferric iron amendment had little effect on the structure and activity of the sediment microorganisms. Marine sediments have been accumulating in the past few decades, possibly due to their resistance to the degradation abilities of sulfate and iron reducers. For this reason, biostimulation with energetically more favorable electron acceptors such as nitrate is considered more effective in facilitating degradation processes in the marine sediments. This is particularly relevant because nitrate pollution from farm run-off and/or other sources may currently be contributing to the nitrate amendment of marine sediments. However, the precise effects of such biostimulation and the responsible microorganisms are largely unknown.

The advent of high-throughput DNA sequencing technologies has revolutionized approaches to the study of microbial community structure and function (Caporaso et al., 2011; Miller et al., 2013). One of these technologies, the Illumina platform for 16S rRNA amplicon sequencing, has made it possible to comprehensively screen microbial communities at high resolution and high sensitivity (Caporaso et al., 2012). In addition, the comparative analysis of 16S rRNA genes and transcripts has enabled the fine-scale identification of metabolically active microorganisms. The resulting phylogenetic information is useful for the isolation of key microorganisms, which is one of the exclusive means of accessing their ecophysiological roles.

The objective of this study was to amend deposited marine sediments with an energetic electron acceptor, nitrate, and investigate the impact of this amendment on the structure and activity of microbial communities by using a combination of geochemical analyses, high-throughput Illumina sequencing of 16S rRNA genes and transcripts, and isolation.

Materials and Methods

Anaerobic Incubation of the Deposited Marine Sediments

Marine sediments deposited by the tsunami that originated from the Great East Japan Earthquake in 2011 were collected on September 2011. The sampling site was located on the coast of Higashi-matsushima, Miyagi, Japan (38°25′N, 141°14′E). The samples were stored at 4°C prior to experimental use. Sediment slurry was prepared by mixing the deposited sediment with artificial seawater (Daigo; Nihon Pharmaceutical) at a ratio of 1:4 (v/v). Aliquots (20 ml) of the homogenized slurry were transferred anaerobically into 50-ml serum vials, which were then sealed with butyl rubber septa. The samples were pre-incubated in the dark at 25°C for more than 30 days in order to activate the sediment microorganisms. After pre-incubation, the headspace of each sample vial was flushed with N_2. Three sediments were produced: (i) a non-autoclaved sediment amended with nitrate to a final concentration of 20 mM, (ii) a non-autoclaved sediment with addition of sterilized water in place of nitrate (control sediment), and (iii) an autoclaved sediment amended with nitrate to a final concentration of 20 mM. Sterilization of the sediment microorganisms was performed three times at 121°C for 1 h. All treatments were run in triplicate with static incubation for 5 days at 25°C in the dark.

Geochemical Analyses for Gas, Liquid, and Solid Phases of the Sediment Incubation

Samples of the headspace gas, slurry water and solid-phase sediment were taken at day 0, 2, and 5 from each vial of each of the triplicate replications of the two treatments (i.e., the nitrate and non-amended control treatments) performed in triplicate. Total CO_2, N_2O, and CH_4 in the headspace gas were analyzed by a gas chromatograph (GC-14B; Shimadzu) equipped with a packed column (ShinCarbon ST; Shinwa). Concentrations of NO_3^-, NO_2^-, NH_4^+, PO_4^{2-}, and SiO_2 in the slurry water were determined by the colorimetric method (Hansen and Koroleff, 2007) with an auto-analyzer (QuA Atro 2-HR; BLTEC). This colorimetric analysis was also conducted on the nitrate-amended sterilized sediments after a 5-day incubation. The sulfate concentration in the slurry water was determined by using an ion chromatograph (DX-500; Dionex) equipped with

an IonPac AS11 column (Dionex) and an ED40 electrochemical detector (Dionex). The total organic carbon (TOC) concentration from the slurry water was determined by the non-purgeable organic carbon method with a TOC analyzer (TOC-L; Shimadzu). The concentration of volatile fatty acids (VFAs) from the slurry water was measured by a high-pressure liquid chromatograph (Alliance e26951; Waters) equipped with an RSpak KC-811 column (Shodex) and a photodiode array (2998; Waters). Ferrous iron (Fe^{2+}) and total iron in the slurry were determined using 6N HCl extraction and the ferrozine method as described previously (Braunschweig et al., 2012). The content ratio of elements (i.e., carbon, hydrogen, nitrogen, and sulfur) in the solid-phase sediment was determined by using a CHNS analyzer (FLASH 2000 Organic Elemental analyzer; Thermo Scientific). The thermogravimetry (TG) and differential thermal analysis (DTA) curves of the solid-phase sediment were determined by using a simultaneous differential thermo gravimetric analyzer (DTG-60; Shimadzu). The sediment slurry samples were stored at –80°C for subsequent molecular analyses.

Nucleic Acid Extraction and Amplification of 16S rRNA Genes and Transcripts

Nucleic acids were extracted from each sample from one replication of the nitrate and control treatments at day 0, 2, and 5 using a direct lysis protocol involving bead beating (Noll et al., 2005). Total DNA and RNA were prepared by digestion with RNase (Type II-A; Sigma-Aldrich) and DNase (RQ1; Promega), respectively. The PCR was performed with a high-fidelity DNA polymerase (Q5; New England Biolabs). Reverse transcription PCR (RT-PCR) was carried out using a one-step RT-PCR system (Access Quick; Promega). The universal primer set 515F/806R was modified to contain an Illumina adaptor region, and the reverse primer was encoded with 6-bp barcodes (Caporaso et al., 2012). The thermal profile of PCR was as follows: an initial denaturation at 98°C for 90 s; followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s; and a final extension step at 72°C for 2 min. For RT-PCR, RT was performed at 48°C for 45 min, and the thermal profile of the subsequent PCR consisted of a denaturation at 94°C for 3 min; followed by 30 cycles of 30 s at 94°C, 45 s at 52°C, and 90 s at 72°C; with a final extension of 5 min at 72°C. The absence of the DNA contamination was confirmed by RT-PCR without reverse transcriptase.

Illumina Sequencing and Data Processing

The amplicons were purified first with an AMPure XP Kit (Beckman Coulter) and then a second time with a QIAquick gel extraction kit (QIAGEN). The barcode-encoded DNA library and an initial control (PhiX; Illumina) were subjected to paired-end sequencing with a 300-cycle MiSeq Reagent kit (Illumina) on a MiSeq sequencer (Illumina). The PhiX, low-quality (Q < 30), and chimeric sequences were removed and the paired-end sequences were assembled as described previously (Itoh et al., 2014). The sequences in each library were characterized phylogenetically using the QIIME software package version 1.7.0 (Caporaso et al., 2010). The operational taxonomic units (OTUs) were grouped

using a 97% sequence identity cut-off. Representative sequences for each OTU were assigned with the BLAST program in the DDBJ nucleotide sequence database. Using the program QIIME (Caporaso et al., 2010), α-diversity indices (i.e., Chao1, Shannon, and Simpson reciprocal) and the weighted UniFrac distances for principal coordinate analysis (PCoA) were calculated.

Isolation and Phylogenetic Analysis of the Dominant Sulfur Oxidizers

For isolation and cultivation of the dominant sulfur-oxidizing bacteria from the deposited marine sediments, the incubated slurry was transferred to a slightly modified Widdel medium (pH 7.0; Kanno et al., 2013) containing NaCl, $MgCl_2 \cdot 6H_2O$, KH_2PO_4, and NH_4Cl at concentrations of 20.5, 3, 0.136, and 0.005 g/l, respectively. In this protocol, 2 ml of the incubated slurry and 18 ml of basal medium were first distributed into 50 ml glass serum vials with butyl rubber septa. Then the solutions were further amended with nitrate (20 mM) as an electron acceptor and elemental sulfur (20 mM) or thiosulfate (20 mM) as an electron donor. After flushing the vial headspace with N_2/CO_2 (80: 20, v/v), enrichment was performed at 25°C in the dark. After 1 week, 5% of the enrichment cultures were transferred to fresh media, and the subculturing process was carried out four times. Thereafter, extinction dilution culture was conducted for the isolation of sulfur oxidizers. The purification of isolates was checked by microscopic observation and Illumina sequencing of 16S rRNA genes.

For the phylogenetic analysis of isolates, nucleic acids were extracted from the pure cultures as described above. Nearly the full length of the 16S rRNA gene sequences was amplified with PCR using a Q5 DNA polymerase (New England Biolabs) and the primer set B27f/B1525r (Lane, 1991). The thermal conditions of PCR were as described above, except that an annealing temperature of 52°C and an extension time of 1 min were employed. The PCR products were purified and ligated into the plasmid vector pGEM-T Easy (Promega), and the ligation mixture was used to transform *Escherichia coli* JM109 supercompetent cells (Nippon Gene) according to the manufacturer's instructions. The plasmid DNA was sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and a 3730xl DNA Analyzer (Applied Biosystems). A phylogenetic tree was constructed by the neighbor-joining and maximum likelihood methods using the program MEGA, version 5.2 (Tamura et al., 2011). The robustness of the tree topology was assessed by the bootstrap value based on 1,000 replications. For physiological characterization, the sulfate concentrations during the incubation with sulfur compounds (i.e., elemental sulfur and thiosulfate) and nitrate were determined to assess the chemolithotrophic growth of the isolates.

Nucleotide Sequence Accession Numbers

The nucleotide sequence data obtained from Illumina sequencing analyses based on the 16S rRNA genes and transcripts have been deposited in the MG–RAST database[1] under the project title "Dynamic transition of chemolithotrophic sulfur oxidizers

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[1]http://metagenomics.anl.gov/

in deposited marine sediments in 2015" with the ID numbers 4620652.3–4620661.3 (10 libraries), and those obtained from the phylogenetic analysis of isolates have been deposited in the DDBJ nucleotide sequence database[2] under accession numbers LC029406 to LC029408 (three isolates).

Results

Biogeochemical Activities in the Nitrate-Amended Marine Sediments

After amending with nitrate at a concentration of 20 mM, marine sediment slurries were anaerobically incubated for 5 days; this was designated the nitrate treatment. In parallel, the incubation of non-amended slurries was conducted as a control. Geochemical analysis showed significant differences in the biological activity under the different incubation conditions. First, based on the gaseous phase analysis of the samples (**Figure 1A**), N_2O was produced from day 2 in the nitrate-amended sediment but it was not detected in the control. Under both conditions, CO_2 was generated from the start of the incubations. In both cases the CO_2 concentrations increased to 258–378 μM at day 2, suggesting that the CO_2 production was caused by degassing and establishment of a CO_2-bicarbonate equilibrium. After 2 days, the CO_2 concentration stabilized at 255–285 μM under the nitrate treatment, while it increased to 434 μM at day 5 in the control. CH_4 was not detected under either condition throughout the incubation (data not shown).

With respect to the analysis of slurry water in the nitrate-amended samples, the nitrate concentration decreased to 15.5 mM at day 2 and was completely depleted at the end of the incubation (**Figure 1B**). Nitrite was not detected during the 5-day incubation (data not shown). The ammonium concentration increased slightly to 1.25 mM at day 2, and then decreased to 0.78 mM at day 5 (Supplementary Figure S1A). Sulfate concentrations were maintained at 14.6–15.5 mM during the first 2 days, but they increased remarkably to 26.0 mM at day 5 (**Figure 1C**). This suggests that sulfur components originally present in the sediment were gradually being oxidized to sulfate. TOC was kept at a constant level of 22.6 mg l^{-1} during the incubation (Supplementary Figure S1B). Since VFAs were absent in the slurry water, they were considered not to have contributed to the TOC content. The concentrations of silicate and phosphate, which are known nutrients for microbial growth, decreased throughout the incubation period (Supplementary Figures S1C,D). It was interesting to note that these geochemical parameters did not change in the control (**Figure 1** and Supplementary Figure S1). In addition, decreases in nitrate, silicate, and phosphate were also not observed in the nitrate-amended sterilized sediments (data not shown). These findings indicate that the apparent denitrification and sulfur oxidation under the nitrate treatment were microbially mediated processes.

With respect to the analysis of solid-phase sediments, the content ratio of the sulfur moiety decreased to 1.1 wt% at day 5 under the nitrate treatment, and this value was significantly lower
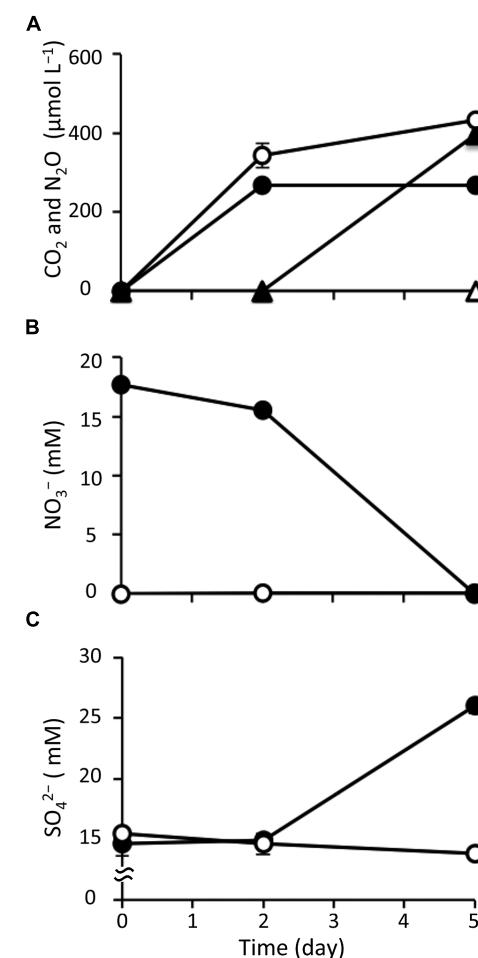
FIGURE 1 | Changes in geochemical parameters during anoxic incubation of nitrate-amended marine sediments (closed symbols) and the control (opened symbols). The panels show the concentrations of CO_2 (circle) and N_2O (triangle) **(A)**, nitrate **(B)**, and sulfate **(C)**. The error bars indicate the standard deviations of three replications.

than that in the control ($P < 0.05$; **Figure 2**). Combined with the results from the slurry water analyses, this strongly suggests that the reduced sulfur components in the nitrate-amended sediments were oxidized to sulfate which was then released to the liquid phase. No significant differences in the content ratios of the other elements (i.e., carbon, hydrogen, and nitrogen) were observed between the nitrate treatment and the control (Supplementary Figure S2). The TG/DTA curves also showed that there were no significant differences in these ratios from the beginning (Supplementary Figure S3A) until the end of the incubation under either set of conditions (Supplementary Figures S3B,C), which suggests that the major components of the sediments were little changed by nitrate amendment.

Changes in Microbial Communities Based on 16S rRNA Genes and Transcripts

Illumina sequencing on the basis of the 16S rRNA genes and transcripts demonstrated the dynamic transition of whole
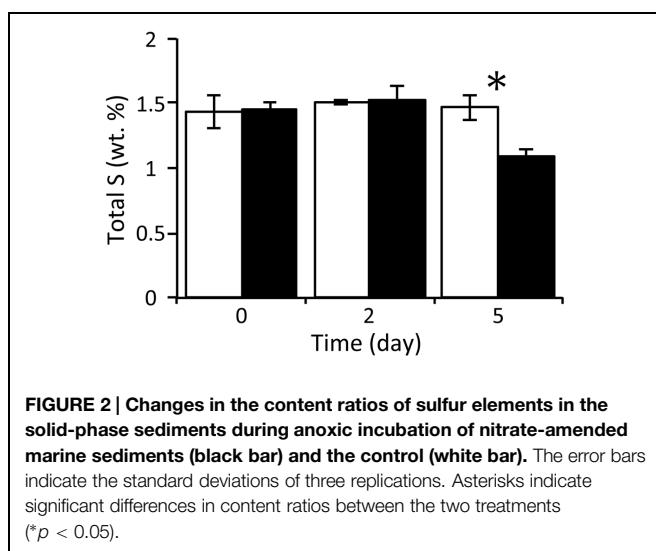
FIGURE 2 | Changes in the content ratios of sulfur elements in the solid-phase sediments during anoxic incubation of nitrate-amended marine sediments (black bar) and the control (white bar). The error bars indicate the standard deviations of three replications. Asterisks indicate significant differences in content ratios between the two treatments (*$p < 0.05$).

and metabolically active microbial communities in the nitrate-amended sediments (**Figure 3** and Supplementary Table S1). A total of 10 Illumina sequence libraries were constructed from 16S rRNA genes ('G') and transcripts ('T') at day 2 and 5 for both the nitrate ('N') treatment and the control ('C'). For instance, the library built from 16S rRNA genes at day 2 of the nitrate treatment was designated as the NG2 library. The NG5, NT2, NT5, CG2, CG5, CT2, and CT5 libraries were defined in the same manner. The libraries from 16S rRNA genes and transcripts at day 0 were presented as a consensus between the two conditions, and were designated as the G0 and T0 libraries, respectively. A total of 280,651 sequences (i.e., a mean of 28,065 sequences per library) were characterized phylogenetically. The numbers of sequences in all the libraries are shown in Supplementary Table S1.

Alpha-diversity indices (i.e., Chao1, Shannon, and Simpson reciprocal) of the Illumina libraries were determined on the basis of equal numbers of sequences ($n = 7,374$; Supplementary Table S1). The Chao1 values in the NG2, NG5, NT2, and NT5 libraries were significantly lower than those in the CG2, CG5, CT2, and CT5 libraries, respectively, which indicates that the richness of microbial communities decreased in response to nitrate amendment. Further, the values of the indices Shannon and Simpson reciprocal were in the order G0 > NG2 > NG5 libraries, while those for the G0, CG2, and CG5 libraries were at similar levels. A decreasing trend was also observed in the T0, NT2, and NT5 libraries but not in the T0, CT2, and CT5 libraries. These facts show that the even distribution of microbial communities significantly decreased with time following the nitrate treatment. The decrease in the evenness of the distribution was more obvious in the whole microbial populations (i.e., at the gene level) than in metabolically active microorganisms (at the transcript level). Taken together, these results showed that the microbial diversity was remarkably lowered by nitrate amendment and thus indicated that particular species dominated the microbial communities. In order to compare the community structures in all the libraries, a PCoA plot was generated from the weighted UniFrac

distance analysis using equal numbers of sequences ($n = 9,336$; Supplementary Figure S4). In the nitrate treatment, distance was maintained between all the libraries on the plot, while in the control treatment, the libraries were in close proximity. This suggests that the microbial communities shifted progressively with time in the nitrate-amended sediments. A considerably greater degree of change in the community was observed from days 2–5 compared to days 0–2. In addition, the libraries of 16S rRNA genes were located far from those of the transcripts, implying that only selected members of the microbial communities expressed 16S rRNA.

Phylogenetic analyses of the Illumina sequence libraries revealed the drastic changes in microbial community structures following the nitrate treatment (**Figure 3**). Notably, the Epsilon- and Gamma-proteobacteria classes increased drastically from 2% and 5% of the whole microbial population at day 0 to 44% and 17% at day 5, respectively (G0 and NG5 libraries in **Figure 3A**). Significant increases in these classes were also found in the metabolically active microbial communities (T0 and NT5 libraries in **Figure 3B**). The most abundant OTUs of these classes in the NG5 library are shown in **Figure 4**. Within the class Epsilonproteobacteria, OTU 4053 became the most predominant, accounting for 42.7% and 14.7% of the total NG5 and NT5 libraries, respectively (**Figures 4A,B**). This OTU was phylogenetically related to *Sulfurimonas denitrificans* (NR074133; 98.4% sequence similarity). Concerning the Gammaproteobacteria, the Chromatiales OTUs 5722 and 3944 accounted for 12.5% and 1.9%, respectively, of the total in the NG5 library (**Figure 4C**). The closely related species of these OTUs were *Thioalkalispira microaerophila* (NR025239; 96.8% sequence identity) and *Thioalbus denitrificans* (NR122087; 100% sequence identity), respectively. On the other hand, the epsilon- and gamma-proteobacterial OTUs are listed in descending order of relative abundance in the NT5 library (Supplementary Figure S5). Only small differences in the abundant epsilonproteobacterial OTUs were observed between the NT5 and NG5 libraries (**Figure 4A** and Supplementary Figure S5B), indicating that 16S rRNA expression was mostly originated from the major members, more specifically the OTU 4053, within the Epsilonproteobacteria. Meanwhile, the abundant gammaproteobacterial OTUs in the NT5 library were remarkably distinct from those in the NG5 library (**Figure 4C** and Supplementary Figure S5D). The metabolic activity of the community members appeared not to be linked with the population size, and a variety of minor microorganisms within the Chromatiales highly expressed 16S rRNA. Consequently, the high-resolution dynamics of microbial communities on the basis of the 16S rRNA genes and transcripts revealed that the specific members within the genus *Sulfurimonas* and the order Chromatiales, hitherto known as sulfur-oxidizing bacteria (Friedrich et al., 2001; Nakagawa et al., 2005; Campbell et al., 2006; Nakagawa and Takai, 2008), drastically proliferated and/or were metabolically activated in the nitrate-amended sediments. Moreover, additional quantification assays, e.g., quantitative PCR, of 16S rRNA genes and transcripts are effective for verifying the relationship between the population sizes and metabolic activities of the sulfur-oxidizing bacteria.
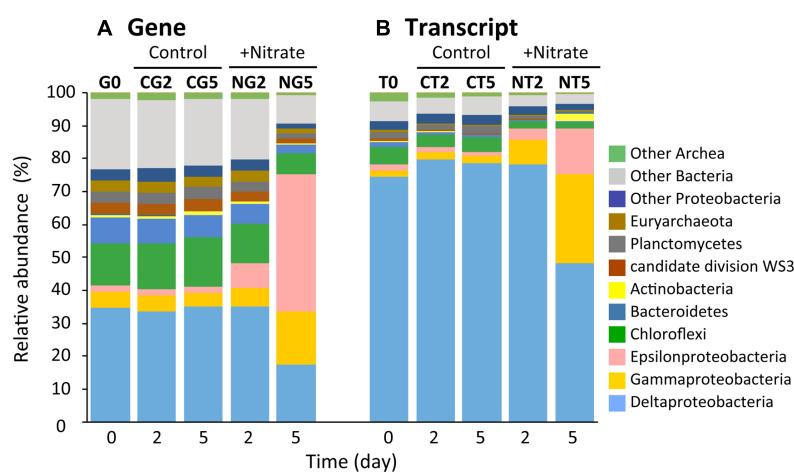
FIGURE 3 | Dynamic transition of microbial communities during anoxic incubation of marine sediments as determined by Illumina sequencing of 16S rRNA genes (A) and transcripts (B). Relative abundances of 16S rRNA genes ('G') and transcripts ('T') defined phylogenetically are represented by the colors shown at the right side of the graph. The sequence libraries were obtained from the incubation at day 0 (designated the G0 and T0 libraries) and at days 2 and 5 in the nitrate-amended sediment ('N'; designated the NG2, NG5, NT2, and NT5 libraries) and in the control ('C'; designated the CG2, CG5, CT2, and CT5 libraries). The sequence library is indicated at the top of each bar.

Some OTUs outside the Epsilon- and Gamma-proteobacteria exhibited higher levels of 16S rRNA expression in the NT5 library than in the CT5 library. The OTUs with more than five-fold increases in relative abundance are shown in **Table 1**. The metabolic activation of anaerobically respiring microorganisms other than sulfur oxidizers was observed at the end of the incubation. In particular, the 16S rRNA expression level of the fermentative and heterotrophically nitrate-reducing *Cellulomonas* sp. (OTU: 566) in the NT5 library was 430-fold higher than that in the CT5 library. The iron(III)-reducing bacteria such as the *Geobacter* spp. (OTUs: 8007, 425, and 3917), *Pelobacter* sp. (OTU: 2865), and *Geothrix* sp. (OTU: 2401) showed 13–94-fold increases in relative abundance. In addition, the 16S rRNA expressions from the syntrophically VFA-oxidizing Syntrophobacteriaceae bacterium (OTU: 11860) and the aceti-clastic methanogen *Methanosaeta* sp. (OTU: 3178) increased to some extent in the NT5 library compared to those in the CT5 library (data not shown).
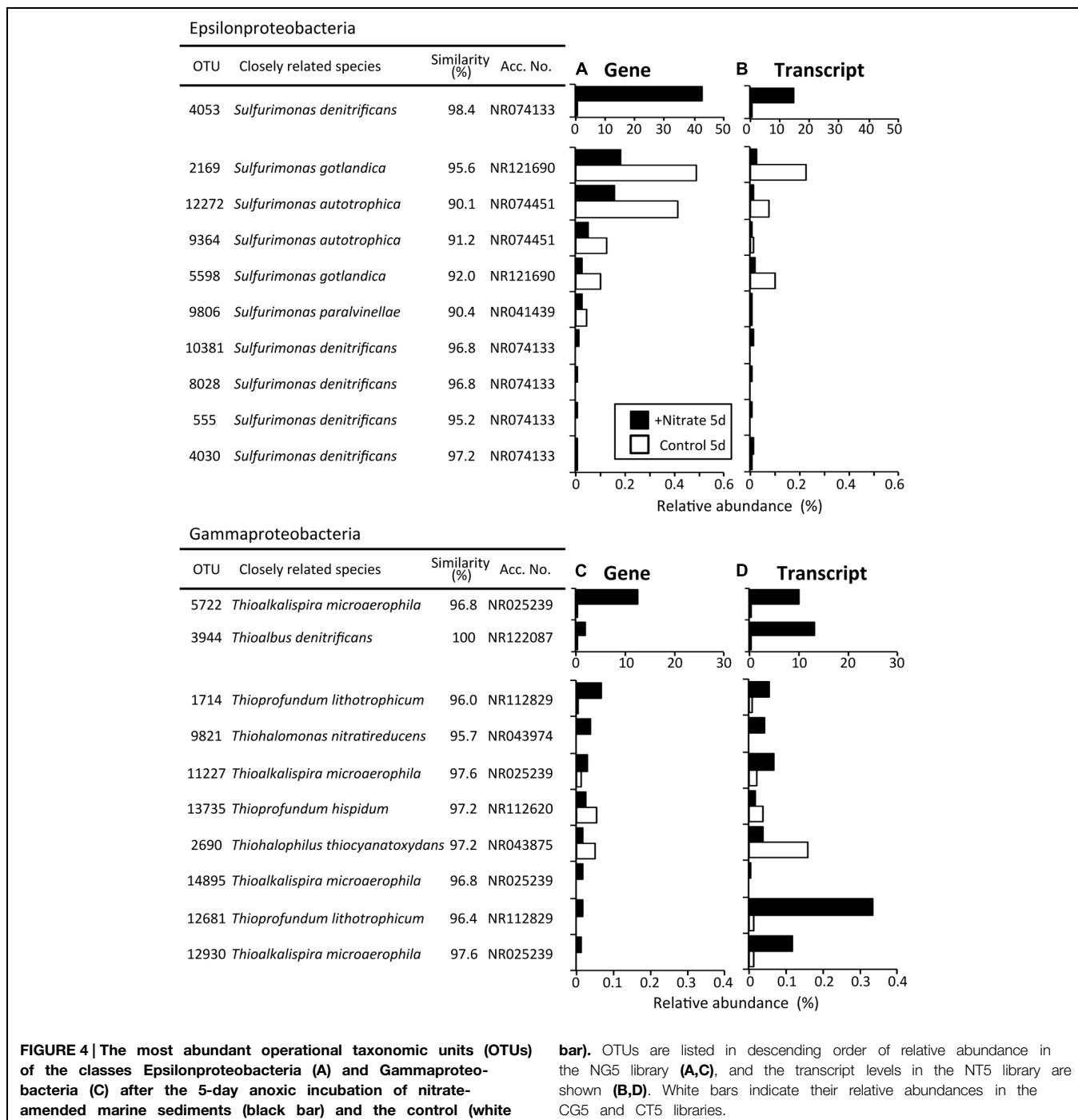
Regarding the composition of microbial communities in the control, the relative abundances in the main phyla or classes were not changed throughout the incubation (G0, CG2, CG5, T0, CT2, and CT5 libraries in **Figure 3**). Deltaproteobacterial sulfate reducers, i.e., Desulfobacteraceae and Desulfobulbaceae bacteria, were dominant. They occupied 11.5% and 7.7% of the CG5 library and 6.3% and 50.3% of the CT5 library, respectively. The dominance of sulfate reducers was consistent with the results from previous studies (Holmer and Kristensen, 1992; Habicht and Canfield, 1997). This suggests that these sulfate-reducing bacteria were originally present in the marine sediments and maintained their predominance throughout the control incubation. Although they expressed most of 16S rRNA under in situ conditions (at day 0) and at days 2 and 5, their contribution to sulfate reduction was small, as indicated by the constant sulfate concentration over time (**Figure 1C**).

Isolation of the Dominant Sulfur Oxidizers and Their Chemolithotrophic Growth

Through extinction dilution culture by using a liquid medium supplemented with nitrate and sulfur compounds, three strains, HDS01, HDS22, and HDSN4, were successfully isolated from the deposited sediments. The phylogenetic analysis based on the nearly full-length 16S rRNA genes indicated that the isolates formed two novel clusters in the phylogenetic tree and the strains HDS01 and HDNS4 were related to *S. denitrificans* (NR074133; 96.7% and 95.8% sequence similarities, respectively), while the strain HDS22 had 95.2% sequence similarity to *Thioalkalispira microaerophila* (NR025239; **Figure 5**). The strains HDS01 and HDS22 corresponded to the OTUs 4053 and 5722, and were identified as the most predominant sulfur oxidizers in the nitrate-amended sediments (**Figures 4A,C**). During the incubation of these two strains (HDS01 and HDS22) with elemental sulfur and thiosulfate as electron donors and nitrate as electron acceptor, the sulfate concentration significantly increased, which indicates that both strains were able to grow chemolithotrophically on these compounds. Because of their low sequence similarities, we consider that these three isolates are novel species in the genera *Sulfurimonas* and *Thioalkalispira*. Further characterizations of these isolates are in progress.

Discussion

In this study, we investigated microbial community succession stimulated by nitrate amendment of deposited marine sediments. Geochemical analyses were used to monitor the change in the physicochemical parameters of the nitrate-amended sediments (e.g., consumption of nitrate, and production of N_2O and sulfate). High-throughout Illumina sequencing based on the 16S rRNA genes and transcripts revealed the proliferation

FIGURE 4 | The most abundant operational taxonomic units (OTUs) of the classes Epsilonproteobacteria (A) and Gammaproteobacteria (C) after the 5-day anoxic incubation of nitrate-amended marine sediments (black bar) and the control (white bar). OTUs are listed in descending order of relative abundance in the NG5 library **(A,C)**, and the transcript levels in the NT5 library are shown **(B,D)**. White bars indicate their relative abundances in the CG5 and CT5 libraries.

and metabolic activation of sulfur-oxidizing bacteria. In addition, isolation of the dominant sulfur oxidizers demonstrated their chemolithotrophic growth on nitrate, which suggests that the biotically fixed carbon was further available for growth of the other anaerobic microorganisms. Actually, at the end of the incubation, Illumina sequencing detected the expression of 16S rRNA from fermentative bacteria, ferric iron reducers, and aceticlastic methanogens. The combination of geochemical analyses, high-throughput sequencing, and isolation was proven to be a

powerful approach to comprehensively characterize microbial communities affected by environmental stimuli and their trophic interaction in the marine sediments.

The amendment with nitrate initiated the co-occurrence of sulfur oxidation and denitrification during anoxic incubation of marine sediment slurries (**Figure 1**). Since the concentration of TOC in slurry waters changed little during the incubation (Supplementary Figure S1B), it was suggested that little or no heterotrophic denitrification occurred. There have been

TABLE 1 | Operational taxonomic units (OTUs) highly expressing 16S rRNA after the 5-day anoxic incubation of nitrate-amended marine sediments.

OTU ID	Closely related species	Similarity (%)	Accession number	Phylum/Class	Family	Relative abundance (%)[a]	Increasing ratio (fold)[b]	Putative function[c]
4053	Sulfurimonas denitrificans	98.4	NR121690	Epsilonproteobacteria	Helicobacteraceae	14.64	2421.7	Sulfur oxidation
566	Cellulomonas oligotrophica	98.8	KF817659	Actinobacteria	Cellulomonadaceae	2.60	430.2	Fermentation, nitrate reduction
13536	Thioalkalispira microaerophila	97.6	NR025239	Gammaproteobacteria	Thioalkalispiraceae	0.82	269.8	Sulfur oxidation
11183	Thioalbus denitrificans	96.8	NR122087	Gammaproteobacteria	Ectothiorhodospiraceae	0.43	143.1	Sulfur oxidation
8007	Geobacter bremensis	100	KF800712	Deltaproteobacteria	Geobacteraceae	6.78	93.5	Iron(II) reduction
3944	Thioalbus denitrificans	100	NR122087	Gammaproteobacteria	Ectothiorhodospiraceae	13.37	38.8	Sulfur oxidation
2401	Geothrix fermentans	99.2	NR036779	Acidobacteria	Holophagae	0.21	34.7	Iron(III) reduction
14834	Thioalbus denitrificans	96.8	NR122087	Gammaproteobacteria	Ectothiorhodospiraceae	0.20	33.7	Sulfur oxidation
7917	Thioprofundum lithotrophicum	95.2	NR112829	Gammaproteobacteria	Thioalkalispiraceae	0.09	30.7	Sulfur oxidation
2865	Pelobacter carbinolicus	95.2	NR075013	Deltaproteobacteria	Pelobacteraceae	0.09	28.6	Iron(III) reduction, sulfate reduction
425	Geobacter luticola	99.6	NR114303	Deltaproteobacteria	Geobacteraceae	6.02	26.5	Iron(III) reduction
20	Thioprofundum lithotrophicum	94.5	NR112829	Gammaproteobacteria	Thioalkalispiraceae	0.07	22.5	Sulfur oxidation
12681	Thioprofundum lithotrophicum	96.4	NR112829	Gammaproteobacteria	Thioalkalispiraceae	0.33	22.1	Sulfur oxidation
5722	Thioalkalispira microaerophila	96.8	NR025239	Gammaproteobacteria	Thioalkalispiraceae	10.17	21.4	Sulfur oxidation
15348	Thioprofundum lithotrophicum	95.3	NR112829	Gammaproteobacteria	Thioalkalispiraceae	0.31	17.4	Sulfur oxidation
13687	Desulfovibrio butyratiphilus	95.3	NR112679	Deltaproteobacteria	Desulfovibrionales	0.09	15.3	Butyrate oxidation, sulfate reduction
3917	Geobacter pelophilus	97.6	NR026077	Deltaproteobacteria	Geobacteraceae	0.12	12.9	Iron(III) reduction
14604	Desulfocapsa sulfexigens	93.3	KF952439	Deltaproteobacteria	Desulfobacterales	0.06	10.2	Sulfur disproportionation
12930	Thioalkalispira microaerophila	97.6	NR025239	Gammaproteobacteria	Thioalkalispiraceae	0.12	9.7	Sulfur oxidation
17265	Desulfomonile tiedjei	98.4	NR074118	Deltaproteobacteria	Syntrophaceae	0.09	7.7	Sulfate reduction
9626	Desulfobulbus mediterraneus	94.5	NR025150	Deltaproteobacteria	Desulfobulbaceae	4.05	6.2	Sulfate reduction
1714	Thioprofundum lithotrophicum	96.0	NR112829	Gammaproteobacteria	Thioalkalispiraceae	0.06	6.1	Sulfur oxidation

[a]The relative abundance of the 16S rRNA transcripts in the NT5 library. More than 0.05% of the abundance is shown.
[b]The increasing ratio was calculated from the relative abundance in the NT5 library to that in the CT5 library.
[c]The putative function was determined by the reported physiology of the closely related species.
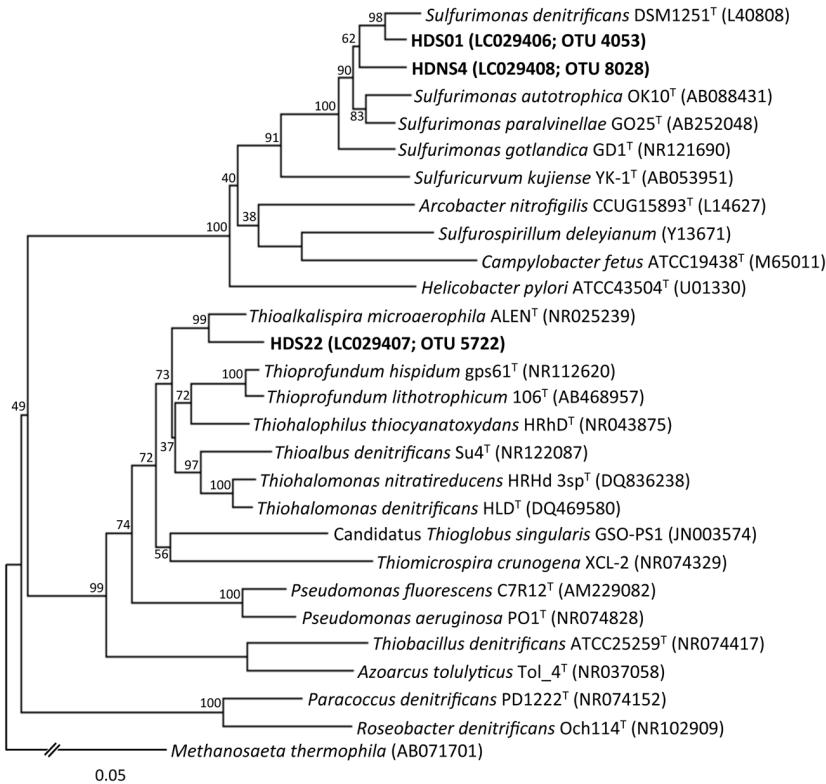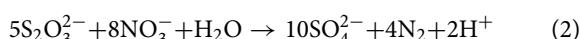
FIGURE 5 | Phylogenetic tree showing the taxonomic distribution of 16S rRNA genes from isolates (the strains HDS01, HDNS4, and HDS22), and their known cultivated relatives. The tree was constructed by the neighbor-joining method using the nearly full-length 16S rRNA gene sequences. Bootstrap values were obtained from 1,000 replications. The scale bar represents 5% sequence divergence.

reports showing that, in the absence of organic matter as a carbon source, denitrification was coupled with sulfur oxidation in anoxic marine sediments in the Black Sea and the Baltic Sea (Böttcher and Lepland, 2000; Zopfi et al., 2004; Thang et al., 2013). Sulfur compounds that were originally present in the sediments may have served as the electron donor. In this study, the content ratios of sulfur significantly decreased with time in solid-phase sediments amended with nitrate (**Figure 2**). This strongly suggests that certain sulfur compounds such as zerovalent sulfur, thiosulfate, and hydrosulfide ion were oxidized to sulfate in association with denitrification. The oxidation of these compounds and its coupling with denitrification are expressed by the following equations (Cardoso et al., 2006; Zhang et al., 2015):

$$5S^0 + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+ \qquad (1)$$

$$5S_2O_3^{2-} + 8NO_3^- + H_2O \rightarrow 10SO_4^{2-} + 4N_2 + 2H^+ \qquad (2)$$

$$5HS^- + 8NO_3^- + 3H^+ \rightarrow 5SO_4^{2-} + 4N_2 + 4H_2O \qquad (3)$$

Indeed, throughout the incubation of nitrate-amended sediments, the concentration of nitrate decreased by 17.7 mM, while that of sulfate increased by 11.3 mM (**Figures 1B,C**). The ratio of NO_3/SO_4 was 1.57, which is close to the expected stoichiometric value from the equilibrium (3). The accumulation of hydrogen sulfide, the end product of sulfate reduction, has frequently been observed in anoxic marine sediments (Holmer and Kristensen, 1992; Zopfi et al., 2004). This implies that hydrogen sulfide was most likely the electron donor utilized for the redox reaction with denitrification in such environments.

The sulfur oxidizers affiliated with the classes Epsilon- and Gamma-proteobacteria were drastically proliferated in the nitrate-amended sediments, as revealed by Illumina sequencing of 16S rRNA genes and transcripts (**Figure 3**). The predominant OTUs 4053 and 5722 were successfully isolated and were designated as *Sulfurimonas* sp. strain HDS01 and *Thioalkalispira* sp. strain HDS22, respectively. The 16S rRNA genes of these isolates had 95.2–96.7% sequence similarities to those of cultured relatives and were able to grow chemolithotrophically on nitrate and sulfur compounds such as elemental sulfur and thiosulfate. These chemolithotrophic sulfur oxidizers drastically increased during the incubation; the relative abundances of the OTU 4053 (strain HDS01) and the OTU 5722 (strain HDS22) increased from 0.03% and 0.5% at day 0 to 42.7% and 12.5% at day 5, respectively. In addition, the expression levels of the bacterial rRNA were clearly enhanced by nitrate (**Figures 4B,D**). Furthermore, lower CO_2 concentrations were found in the nitrate-amended

sediments than in the non-amended controls, suggesting the occurrence of CO_2 fixation in the former (**Figure 1A**). These results strongly suggest that the OTUs 4053 and 5722 (i.e., the strains HDS01 and HDS22, respectively) were directly involved in chemolithotrophic denitrification-dependent sulfur oxidation in the marine sediments. High-resolution microbial analysis further indicated that, aside from these two isolates, the order Chromatiales proliferated and became metabolically active (**Figure 4** and Supplementary Figure S5). This phylogenetic group may have played a complementary role in the geochemical cycles of sulfur and nitrogen in the marine sediments. On the other hand, the filamentous sulfur-oxidizing Desulfobulbaceae bacteria, which originally dominated the sediment microbial communities, did not increase under nitrate amendment, indicating that they played only minor roles in the redox process.

Notably, anaerobic microorganisms in addition to the chemolithotrophic sulfur oxidizers were metabolically activated at the end of the nitrate-amended incubation (**Table 1**). Sulfate, iron(III), and CO_2 were originally present in the marine sediments (**Figures 1A,C** and Supplementary Figure S1E) and thus were available as electron acceptors for anaerobic respiration processes. Fine-scale analyses of Illumina sequence data successfully detected the increases in 16S rRNA transcripts from fermentative and nitrate-reducing bacteria (i.e., *Cellulomonas* sp.), iron(III) reducers (e.g., *Geobacter* spp., *Pelobacter* sp., and *Geothrix* sp.), and aceticlastic methanogens (i.e., *Methanosaeta* sp.) at day 5 (**Table 1**), although no changes in geochemical parameters such as the concentrations of TOC, iron(II), and CH_4 were found (Supplementary Figures S1B,F). It is tempting to speculate that the carbon fixed by the chemolithotrophic sulfur oxidizers might be utilized as an electron donor by these anaerobically respiring microorganisms. In order to clarify the trophic relationships of anaerobic microorganisms in the marine sediments, isotope-tracing assays such as ultra-high-sensitivity stable isotope probing (Aoyagi et al., 2015) should be applied.

It is also noteworthy that two different types of chemolithotrophic sulfur-oxidizing bacteria (i.e., the genus *Sulfurimonas* and the order Chromatiales) coexisted in our experiments (**Figure 4**). Niche differentiation of these physiologically similar microorganisms developed during the incubation, and was a potential driving force in shaping the sediment microbial communities. A single species [the OTU 4053 (strain HDS01)] of the genus *Sulfurimonas* increased exclusively (**Figures 4A,B**), while a wide variety of species [e.g., OTUs 5722 (strain HDS22), 3944, and 1143] within the order Chromatiales became metabolically active. Almost all of the top Chromatiales bacteria in terms of 16S rRNA transcript expression levels, with the exception of the OTUs 5722 and 6560 (Supplementary Figure S5D), accounted for quite low percentages of the total microbial populations at the gene level (Supplementary Figure S5C), suggesting that these minor Chromatiales members actively dissimilated sulfur and nitrate but only marginally assimilated carbon for their growth in the sediments. Recently,

genomics-based studies have reported that bacteria belonging to the genus *Sulfurimonas* and those belonging to the order Chromatiales employed different metabolic pathways for sulfur oxidation, nitrate reduction, and carbon fixation (Sievert et al., 2008; Kovaleva et al., 2011; Grote et al., 2012; Handley et al., 2014; Thomas et al., 2014). Further, some Chromatiales bacteria have been reported to be capable of growing specifically at low concentrations (i.e., 1–2 mM) of nitrate, and thereby adapting to energy-limiting conditions (Sorokin et al., 2006). These metabolic types and capacities might have triggered the distinct responses of these two sulfur oxidizers to nitrate amendment. More detailed physiological characterization and investigation into the competitive mechanism of these two isolates (the strains HDS01 and HDS22) may lead to a better understanding of the niche differentiation of sulfur-oxidizing bacteria in marine sediments.

Conclusion

The results of this study demonstrated that the novel microorganisms *Sulfurimonas* sp. strain HDS01 and *Thioalkalispira* sp. strain HDS22 were directly involved in the chemolithotrophic denitrification-dependent sulfur oxidation in nitrate-amended marine sediments. In addition to these two sulfur-oxidizers, a variety of the Chromatiales bacteria, including minor but metabolically active microorganisms, might also play important roles in the geochemical cycles of sulfur, nitrogen, and carbon. High-resolution analyses of 16S rRNA transcripts implicated trophic interaction and niche differentiation of key microorganisms as the ecological principles underlying the reorganization of microbial communities. The two different types of sulfur oxidizers stably co-existed in the sediments and complementarily fixed carbon, leading to the metabolic activation of fermentative bacteria, ferric iron reducers, and aceticlastic methanogens. The mechanisms underlying the community reorganization should be studied to gain deeper insight into the stability and flexibility of microbial ecosystems.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00426/abstract

References

Aoyagi, T., Hanada, S., Itoh, H., Sato, Y., Ogata, A., Friedrich, M. W., et al. (2015). Ultra-high-sensitivity stable-isotope probing of rRNA by high-throughput sequencing of isopycnic centrifugation gradients. *Environ. Microbiol. Rep.* 7, 282–287. doi: 10.1111/1758-2229.12243

Armenteros, M., Pérez-García, J. A., Ruiz-Abierno, A., Díaz-Asencio, L., Helguera, Y., Vincx, M., et al. (2010). Effects of organic enrichment on nematode assemblages in a microcosm experiment. *Mar. Environ. Res.* 70, 374–382. doi: 10.1016/j.marenvres.2010.08.001

Böttcher, M. E., and Lepland, A. (2000). Biogeochemistry of sulfur in a sediment core from the west-central Baltic Sea: evidence from stable isotopes and pyrite textures. *J. Mar. Syst.* 25, 299–312. doi: 10.1016/S0924-7963(00)00023

Braunschweig, J., Bosch, J., Heister, K., Kuebeck, C., and Meckenstock, R. U. (2012). Reevaluation of colorimetric iron determination methods commonly used in geomicrobiology. *J. Microbiol. Methods* 89, 41–48. doi: 10.1016/j.mimet.2012.01.021

Campbell, B. J., Engel, A. S., Porter, M. L., and Takai, K. (2006). The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nat. Rev. Microbiol.* 4, 458–468. doi: 10.1038/nrmicro1414

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108(Suppl. 1), 4516–4522. doi: 10.1073/pnas.1000080107

Cardoso, R. B., Sierra-Alvarez, R., Rowlette, P., Flores, E. R., Gomez, J., and Field, J. A. (2006). Sulfide oxidation under chemolithoautotrophic denitrifying conditions. *Biotechnol. Bioeng.* 95, 1148–1157. doi: 10.1002/bit.21084

Friedrich, C. G., Rother, D., Bardischewsky, F., Quentmeier, A., and Fischer, J. (2001). Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? *Appl. Environ. Microbiol.* 67, 2873–2882. doi: 10.1128/aem.67.7.2873-2882.2001

Grote, J., Schott, T., Bruckner, C. G., Glockner, F. O., Jost, G., Teeling, H., et al. (2012). Genome and physiology of a model Epsilonproteobacterium responsible for sulfide detoxification in marine oxygen depletion zones. *Proc. Natl. Acad. Sci. U.S.A.* 109, 506–510. doi: 10.1073/pnas.1111262109

Habicht, K. S., and Canfield, D. E. (1997). Sulfur isotope fractionation during bacterial sulfate reduction in organic-rich sediments. *Geochim. Cosmochim. Acta* 61, 5351–5361. doi: 10.1016/S0016-7037(97)00311-6

Handley, K. M., Bartels, D., O'Loughlin, E. J., Williams, K. H., Trimble, W. L., Skinner, K., et al. (2014). The complete genome sequence for putative H - and S-oxidizer Candidatus *Sulfuricurvum* sp., assembled de novo from an aquifer-derived metagenome. *Environ. Microbiol.* 16, 3443–3462. doi: 10.1111/1462-2920.12453

Hansen, H. P., and Koroleff, F. (2007). "Determination of nutrients," in *Methods of Seawater Analysis*, eds K. Grasshoff, K. Kremling, and M. Ehrhardt (Weinheim: Wiley-VCH), 159–228. doi: 10.1002/9783527613984.ch10

Holmer, M., and Kristensen, E. (1992). Impact of marine fish cage farming on metabolism and sulphate reduction ofunder-lying sediment. *Mar. Ecol. Progr. Ser.* 80, 191–201. doi: 10.3354/meps080191

Hori, T., Haruta, S., Ueno, Y., Ishii, M., and Igarashi, Y. (2006). Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. *Appl. Environ. Microbiol.* 72, 1623–1630. doi: 10.1128/aem.72.2.1623-1630.2006

Hori, T., Kimura, M., Aoyagi, T., R. Navarro, R., Ogata, A., Sakoda, A., et al. (2014). Biodegradation potential of organically enriched sediments under sulfate- and iron-reducing conditions as revealed by the 16S rRNA deep sequencing. *J. Water Environ. Technol.* 12, 357–366. doi: 10.2965/jwet.2014.357

Hori, T., Muller, A., Igarashi, Y., Conrad, R., and Friedrich, M. W. (2010). Identification of iron-reducing microorganisms in anoxic rice paddy soil by ^{13}C-acetate probing. *ISME J.* 4, 267–278. doi: 10.1038/ismej.2009.100

Inui, T., Yasutaka, T., Endo, K., and Katsumi, T. (2012). Geo-environmental issues induced by the 2011 off the Pacific Coast of Tohoku Earthquake and tsunami. *Soils Found.* 52, 856–871. doi: 10.1016/j.sandf.2012.11.008

Itoh, H., Aita, M., Nagayama, A., Meng, X. Y., Kamagata, Y., Navarro, R., et al. (2014). Evidence of environmental and vertical transmission of *Burkholderia* symbionts in the oriental chinch bug, *Cavelerius saccharivorus* (Heteroptera: Blissidae). *Appl. Environ. Microbiol.* 80, 5974–5983. doi: 10.1128/aem.01087-4

Kanno, M., Katayama, T., Tamaki, H., Mitani, Y., Meng, X. Y., Hori, T., et al. (2013). Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. *Appl. Environ. Microbiol.* 79, 6998–7005. doi: 10.1128/aem.02900-3

Kemp, W. M., Sampou, P., Caffrey, J., Mayer, M., Henriksen, K., and Boynton, W. R. (1990). Ammonium recycling versus denitrification in Chesapeake Bay sediments. *Limnol. Oceanogr.* 35, 1545–1563. doi: 10.4319/lo.1990.35.7.1545

Kotsyurbenko, O. R., Friedrich, M. W., Simankova, M. V., Nozhevnikova, A. N., Golyshin, P. N., Timmis, K. N., et al. (2007). Shift from acetoclastic to H$_2$-dependent methanogenesis in a west Siberian peat bog at low pH values and isolation of an acidophilic *Methanobacterium* strain. *Appl. Environ. Microbiol.* 73, 2344–2348. doi: 10.1128/aem.02413-6

Kovaleva, O. L., Tourova, T. P., Muyzer, G., Kolganova, T. V., and Sorokin, D. Y. (2011). Diversity of RuBisCO and ATP citrate lyase genes in soda lake sediments. *FEMS Microbiol. Ecol.* 75, 37–47. doi: 10.1111/j.1574-6941.2010.00996.x

Kunihiro, T., Takasu, H., Miyazaki, T., Uramoto, Y., Kinoshita, K., Yodnarasri, S., et al. (2011). Increase in Alphaproteobacteria in association with a polychaete, *Capitella* sp. I, in the organically enriched sediment. *ISME J.* 5, 1818–1831. doi: 10.1038/ismej.2011.57

Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habbena, W., and Jurgens, K. (2005). Impact of different in vitro electron donor/acceptor conditions on potential chemolithoautotrophic communities from marine pelagic redoxclines. *Appl. Environ. Microbiol.* 71, 6664–6672. doi: 10.1128/aem.71.11.6664-6672.2005

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acid Techniques in Bacterial Systematics*, eds E. Stackebrandt M. Goodfellow (Chichester: John Wiley and Sons), 115–175.

Lueders, T., and Friedrich, M. W. (2002). Effects of amendment with ferrihydrite and gypsum on the structure and activity of methanogenic populations in rice field soil. *Appl. Environ. Microbiol.* 68, 2484–2494. doi: 10.1128/AEM.68.5.2484-2494.2002

Lydmark, P., Almstrand, R., Samuelsson, K., Mattsson, A., Sorensson, F., Lindgren, P. E., et al. (2007). Effects of environmental conditions on the nitrifying population dynamics in a pilot wastewater treatment plant. *Environ. Microbiol.* 9, 2220–2233. doi: 10.1111/j.1462-2920.2007.01336.x

Miller, C. S., Handley, K. M., Wrighton, K. C., Frischkorn, K. R., Thomas, B. C., and Banfield, J. F. (2013). Short-read assembly of full-length 16S amplicons reveals bacterial diversity in subsurface sediments. *PLoS ONE* 8:e56018. doi: 10.1371/journal.pone.0056018

Nakagawa, S., and Takai, K. (2008). Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance. *FEMS Microbiol. Ecol.* 65, 1–14. doi: 10.1111/j.1574-6941.2008.00502.x

Nakagawa, S., Takai, K., Inagaki, F., Hirayama, H., Nunoura, T., Horikoshi, K., et al. (2005). Distribution, phylogenetic diversity and physiological characteristics of epsilon-Proteobacteria in a deep-sea hydrothermal field. *Environ. Microbiol.* 7, 1619–1632. doi: 10.1111/j.1462-2920.2005.00856.x

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* 7, 382–395. doi: 10.1111/j.1462-2920.2005.00700.x

Ontiveros-Valencia, A., Ziv-El, M., Zhao, H. P., Feng, L., Rittmann, B. E., and Krajmalnik-Brown, R. (2012). Interactions between nitrate-reducing and sulfate-reducing bacteria coexisting in a hydrogen-fed biofilm. *Environ. Sci. Technol.* 46, 11289–11298. doi: 10.1021/es302370t

Pearson, T. H., and Rosenberg, R. (1978). Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. *Oceanogr. Mar. Biol.* 16, 229–311. doi: 10.2983/035.034.0121ů1.10

Sievert, S. M., Scott, K. M., Klotz, M. G., Chain, P. S., Hauser, L. J., Hemp, J., et al. (2008). Genome of the epsilonproteobacterial chemolithoautotroph

Sulfurimonas denitrificans. Appl. Environ. Microbiol. 74, 1145–1156. doi: 10.1128/aem.01844-7

Sorokin, D. Y., Tourova, T. P., Lysenko, A. M., and Muyzer, G. (2006). Diversity of culturable halophilic sulfur-oxidizing bacteria in hyper-saline habitats. *Microbiology* 152, 3013–3023. doi: 10.1099/mic.0.29106-0

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Thang, N., Brüchert, V., Formolo, M., Wegener, G., Ginters, L., Jørgensen, B., et al. (2013). The impact of sediment and carbon fluxes on the biogeochemistry of methane and sulfur in littoral Baltic Sea Sediments (Himmerfjärden, Sweden). *Estuaries Coasts* 36, 98–115. doi: 10.1007/s12237-012-9557-0

Thomas, F., Giblin, A. E., Cardon, Z. G., and Sievert, S. M. (2014). Rhizosphere heterogeneity shapes abundance and activity of sulfur-oxidizing bacteria in vegetated salt marsh sediments. *Front. Microbiol.* 5:309. doi: 10.3389/fmicb.2014.00309

Zhang, Z., Lo, I. C., Zheng, G., Woon, K., and Rao, P. (2015). Effect of autotrophic denitrification on nitrate migration in sulfide-rich marine sediments. *J. Soils Sediments* 15, 1019–1028. doi: 10.1007/s11368-015-1078-6

Zopfi, J., Ferdelman, T. G., and Fossing, H. (2004). Distribution and fate of sulfur intermediates—sulfite, tetrathionate, thiosulfate, and elemental sulfur—in marine sediments. *Geol. Soc. Am. Special Pap.* 379, 97–116. doi: 10.1130/0-8137-2379-5.97

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Bacterial population succession and adaptation affected by insecticide application and soil spraying history

Hideomi Itoh[1], Ronald Navarro[2], Kazutaka Takeshita[1], Kanako Tago[3], Masahito Hayatsu[3], Tomoyuki Hori[2] and Yoshitomo Kikuchi[1]*

[1] Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo, Japan
[2] Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan
[3] Environmental Biofunction Division, National Institute for Agro-Environmental Sciences, Tsukuba, Japan

Although microbial communities have varying degrees of exposure to environmental stresses such as chemical pollution, little is known on how these communities respond to environmental disturbances and how past disturbance history affects these community-level responses. To comprehensively understand the effect of organophosphorus insecticide application on microbiota in soils with or without insecticide-spraying history, we investigated the microbial succession in response to the addition of fenitrothion [O,O-dimethyl O-(3-methyl-p-nitrophenyl) phosphorothioate, abbreviated as MEP] by culture-dependent experiments and deep sequencing of 16S rRNA genes. Despite similar microbial composition at the initial stage, microbial response to MEP application was remarkably different between soils with and without MEP-spraying history. MEP-degrading microbes more rapidly increased in the soils with MEP-spraying history, suggesting that MEP-degrading bacteria might already exist at a certain level and could quickly respond to MEP re-treatment in the soil. Culture-dependent and -independent evaluations revealed that MEP-degrading *Burkholderia* bacteria are predominant in soils after MEP application, limited members of which might play a pivotal role in MEP-degradation in soils. Notably, deep sequencing also revealed that some methylotrophs dramatically increased after MEP application, strongly suggesting that these bacteria play a role in the consumption and removal of methanol, a harmful derivative from MEP-degradation, for better growth of MEP-degrading bacteria. This comprehensive study demonstrated the succession and adaptation processes of microbial communities under MEP application, which were critically affected by past experience of insecticide-spraying.

Keywords: fenitrothion, organophosphorus insecticide, soil microbes, deep sequencing, *Burkholderia*, methylotroph

INTRODUCTION

Biological communities are exposed to varying levels of environmental stresses or disturbances such as global warming, typhoon, drought, and bush fire (Phillips et al., 2009; O'Connell and Nyman, 2011; Peñuelas et al., 2013; Rota et al., 2014), and little is known about community succession and adaptation processes under such environmental stimuli. In addition to naturally occurring environmental disturbances, chemical insecticides have been developed and used worldwide to control agricultural, hygienic, and household pest insects. Although insecticides have revolutionized modern agriculture and sanitation in terms of pest management, abuse of usage sometimes causes serious problems including environmental pollution from its residues, health hazard, unexpected effects on non-targeted organisms, and evolution of insecticide-resistant insects (Whalon et al., 2008; Diez, 2010). Thus, insecticide-spraying is thought to be a man-made, yet strong, environmental stress on natural organisms.

Organophosphorus insecticide, a general name referring to insecticides containing a phosphoester bond, is one of the most widely used insecticides. It includes a number of commercially available chemicals such as diazinon, malathion, and dichlorvos (Singh and Walker, 2006; Singh, 2009). The chemical compounds found in organophosphorus insecticide show high mammalian toxicity as well as insecticidal activity by inhibiting acetylcholinesterase (AchE), causing overstimulation of the cholinergic synapses by the overaccumulation of acetylcholine (da Silva et al., 2013). Fenitrothion [O,O-dimethyl O-(3-methyl-p-nitrophenyl) phosphorothionate, abbreviated as MEP] is one of the organophosphorus compounds commonly used because of its broad-spectrum activity and less mammal toxicity. In natural fields, MEP can be degraded photochemically, but is mainly degraded by soil bacteria. Biodegradation pathways of MEP were first investigated in forest soils using C^{14}-labeled MEP (Spillner et al., 1979) and are currently described in the International Programme on Chemical Safety IPCS (1992). In

microorganisms, MEP is hydrolyzed to 3-methyl-4-nitrophenol and then finally decomposed to CO_2 through a phenol ring cleavage pathway (IPCS, 1992). Previous culture-dependent studies reported that members of the genera *Burkholderia*, *Pseudomonas*, *Cupriavidus*, *Corynebacterium*, *Arthrobacter*, and *Sphingomonas* have been identified as MEP-degraders, some of which use the compound as a sole carbon source (Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009). Considering that only a tiny fraction of environmental bacteria is culturable (Wilson and Piel, 2013), culture-independent as well as culture-dependent approaches are pivotal to comprehensively understand microbial dynamics in soils after spraying with MEP, as previously mentioned (Jacobsen and Hjelmsø, 2014).

Recent advances in sequence technologies, the so-called next generation sequencing (NGS), provide a faster and simpler alternative to previously established techniques for the comprehensive investigation of microbial community structures (Moorthie et al., 2011). Fairly recent works using NGS include the evaluation of microbial community compositions of soils polluted with organochlorine pesticides including Dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexane (HCH), and 2-chloro-4-ethylamino-6-isopropylamino-s-triazine (ATZ) (Fang et al., 2014). Based on the metagenomic survey of functional genes, these studies have suggested that diverse bacterial species complement one another to degrade these organochlorides (Fang et al., 2014).

In crop fields, biodegradation of the insecticides is one of the pivotal factors affecting the recovery and sustainability of soil ecosystems. Furthermore, it has been reported that one or more previous applications of the same insecticides and/or other structurally-analogous insecticides frequently result to less persistence of the insecticide in the environment (Felsot, 1989). In soil environments where the same insecticide has been continuously sprayed, it has been hypothesized that insecticide-degrading microbes grow by assimilating the insecticide and are enriched in the soil, which cause excessively enhanced biodegradation of the sprayed insecticides (Arbeli and Fuentes, 2007). However, it remains unclear how microbial communities transit under and adapt to insecticide-spraying and how the history of insecticide-spraying affects these succession and adaptation processes of soil microbial communities.

By combining the culture-independent approach using the NGS technique with culture-dependent isolation of MEP-degrading bacteria, we investigated the detailed process of microbial succession when MEP was sprayed on field-collected soils with or without a history of MEP-spraying prior to collection. Among the collected soils, one has been sprayed with MEP at least for 4 years and the other has never been sprayed with MEP nor with other organophosphorus compounds. The NGS analysis combined with culture-dependent approach clearly demonstrates previously unseen dynamics of soil microbiota during MEP-spraying, highlighting the different responses of microbiota against the experimental MEP-spraying in the different types of soil, and identifying the key players of MEP-degradation in soil environments.

MATERIALS AND METHODS

SOILS

We collected two andisol soils from agricultural fields located at the National Institute for Agro-Environmental Sciences (Tsukuba, Ibaraki, Japan; 36°0′N, 140°1′E): *soil S* (Sprayed soil) was collected from a crop field that had been sprayed with MEP for at least 4 years [pH(H_2O), 6.3; water content (w/w), 35.2%; total carbon, 5.0%; total nitrogen, <0.3%]; *soil N* (Naive soil) was collected from a field that had never been sprayed with MEP and other organophosphorus compounds [pH(H_2O), 6.6; water content (w/w), 33.7%; total carbon, 4.4%; total nitrogen, <0.3%]. Characterization of these soil samples is summarized in Table S1. Each soil was passed through a 2 mm sieve to remove large organic litters like leaves and roots.

INSECTICIDE-SPRAYING EXPERIMENTS

Experimental design of the insecticide-spraying test is shown in Figure S1. Approximately 150 g (dry weight) of each of the sieved soils were transferred into plastic pots (10 × 8 cm; opening diameter × depth). The potted soils were incubated at 25°C with weekly application of MEP solution (diluted by distilled water) at a specific loading of 50 mg kg^{-1}-dry soil (Tago et al., 2006). For both soils S and N, duplicate runs (pot 1 and pot 2) were prepared and individually subjected to experiments and further analyses. Control treatments were conducted under the same conditions, but the addition of MEP solution was replaced by equal amount of distilled water only once a week. These pots were covered with a sheet of aluminum foil during incubation to prevent water evaporation and photodegradation of MEP. Soil samples from the surface layer up to a depth of 1 cm were collected 1 week after the final spraying, e.g., soil samples treated three times with MEP were collected 1 week after the 3rd MEP-spraying (see Figure S1), and then subjected to the various analyses described below.

CFU COUNTING OF MEP-DEGRADING BACTERIA

The total CFU (colony forming units) counts of MEP-degrading bacteria was measured by plating serial dilutions of soil samples [1% (w/w) diluted in distilled water] on 1.5% agar plates of MEP medium [0.08% MEP, 20 mM potassium phosphate (pH 7.0), 0.1% $(NH_4)_2SO_4$, 0.02% NaCl, 0.01% $MgSO_4$•$7H_2O$, 0.05% $CaCl_2$•$2H_2O$, 0.0002% $FeSO_4$•$7H_2O$, 0.1% yeast extract] and incubated for 4 days at 25°C, as previously described (Tago et al., 2006). The CFU of MEP-degrading bacteria were determined by counting the colonies with a halo on the MEP plates. In the halo-forming assay, the bacteria that are capable of degrading MEP to 3-methyl-4-nitrophenol were detected.

ISOLATION AND IDENTIFICATION OF MEP-DEGRADING BACTERIA

Isolated colonies were identified based on the partial sequences (c.a. 600 bp) of their 16S rRNA genes. During isolation, each of the colonies was picked with a sterile toothpick and then suspended in 35 μl TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] in each well of a 96 well titer plate. The cells were subjected to heat shock treatment (98°C for 3 min) in order to extract the genomic DNA, which was used as template for PCR amplification. The PCR amplification of bacterial 16S rRNA gene was performed with the use of a KOD Fx

Neo polymerase (TOYOBO, Tokyo, Japan), a universal primer set, 16SA1 [5′-AGAGTTTGATCMTGGCTCAG-3′] and 16SB1 [5′-TACGGYTACCTTGTTACGACTT-3′] (Fukatsu and Nikoh, 1998), and the template DNA extracted as described above. The temperature program for PCR is as follows: 94°C for 2 min, followed by 20 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 90 s. The resulting 1.5 kb amplicons were used as templates of sequencing reaction conducted with a universal primer 357F [5′-CCTACGGGAGGCAGCAG-3′] (Muyzer et al., 1993). Taxonomic assignment of the resulting sequences was double checked by the RDP multiclassifier ver. 1.1 (Wang et al., 2007) and BLASTN-search (http://ncbi.nlm.nih.gov/blast/) against GreenGene database (DeSantis et al., 2006).

DEEP SEQUENCING

DNA was extracted from soil S and soil N samples before treatment (S0, N0), after the 2nd (S2) and 3rd (S3, N3) MEP-treatments, and after the 3rd control treatment (S3C, N3C) by using a slightly modified published protocol (Noll et al., 2005). Briefly, 0.5 g wet soil was combined with 0.08 g skim milk, 700 μl extraction buffer [50 mM Tris-HCl (pH 8.0), 1.7% Polyvinylpyrrolidone, 20 mM $MgCl_2$], and 0.5 ml zirconia/silica beads (0.1 mm diameter, Biospec, OK, USA), and blended in a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) at 2000 rpm for 60 s. Crude extracts were purified by phenol/chloroform/isoamyl alcohol (25:24:1) treatment followed by isopropanol precipitation. Contaminating RNA was digested with Ribonuclease (Nippon Gene, Toyama, Japan). The prepared DNA was subjected to PCR amplification of 16S rRNA gene for deep sequencing. The variable region (V4) of bacterial 16S rRNA gene was amplified using universal primers 515F [5′-GTGCCAGCMGCCGCGGTAA-3′] and 806R [5′-GGACTACHVGGGTWTCTAAT-3′] (Caporaso et al., 2012). The PCR reaction mixture comprised of 50 μM each dNTP, 0.4 μM 515F with Illumina P5 sequences, 0.4 μM 806R with 6-bases indexes and Illumina P7 sequences (Caporaso et al., 2012) (Illumina, San Diego, CA, USA), Q5 High-Fidelity DNA polymerase with Q5 reaction buffer (New England BioLabs, Ipswich, MA, USA) and the extracted soil DNA template. The PCR conditions were as follows: initial denaturation at 98°C for 90 s, followed by 25 cycles of 98°C for 10 s, 54°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 2 min. The PCR amplicons were purified as described previously (Itoh et al., 2014). DNA libraries containing all tagged-amplicons and internal control phiX were generated for paired-end sequencing by the MiSeq sequencer using MiSeq Reagent kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer's instruction.

QUANTITATIVE PCR

Quantitative PCR (qPCR) of bacterial 16S rRNA gene was performed to amplify bacterial 16S rRNA genes using a Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the LightCycler 96 System (Roche Applied Science, Indianapolis, IN, USA). The reaction mixture was comprised of 2× SYBR Green PCR Master Mix, 0.2 μM 515F and 806R primer pairs (Caporaso et al., 2012), 0.5 μg/μl BSA, and soil DNA as a template. The PCR conditions were as follows: initial

denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The amount of bacterial 16S rRNA gene copies was calculated on the basis of the standard curve constructed using a dilution series of the target PCR product of *Burkholderia* sp. SFA1 (DDBJ accession no. AB232333).

DATA ANALYSIS

Internal control phiX sequences and low-quality sequences were removed, and paired sequences were joined as described previously (Itoh et al., 2014). Chimeric sequences were removed using uchime algorithm on Mothur program ver. 1.29.2 (Schloss et al., 2009) through the alignment with Greengene database (DeSantis et al., 2006). The resulting sequences were subjected to taxonomic assignment by using RDP multiclassifier ver. 1.1 (Wang et al., 2007) with an 80% confidence threshold. Operational taxonomic units (OTU) based analyses, including estimation of diversity indices and Principal Coordinate Analysis (PCoA) based on OTUs with 3% differences, were performed by using the macqiime ver. 1.6.0 (Caporaso et al., 2010). Phylogenetic tree was constructed by the neighbor-joining method with the bootstrap test (1000 replicates) under MEGA ver. 4.0.2 (Tamura et al., 2007). Analysis of variance (ANOVA) was performed using R software ver. 3.0.1 (R Development Core Team, 2008) to analyze differences in qPCR data among samples.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The nucleotide sequences reported in this study were deposited in the DDBJ/Genbank/EBI databases under the accession numbers: AB904935–AB905196 (16S rRNA gene sequences of isolates, Table S2) and the MG-RAST database (http://metagenomics.anl.gov/, Meyer et al., 2008) as a "Microbial succession under MEP-spraying in 2012" project under the ID: 4562358.3–4562369.3 (deep sequencing, **Table 1**).

RESULTS

POPULATION DYNAMICS OF MEP-DEGRADING BACTERIA IN MEP-SPRAYED SOILS

Abundance changes of MEP-degrading strains by a culture-dependent method were investigated using two types of soils with different MEP-spraying histories: MEP-sprayed "soil S" and MEP-naive "soil N" (Table S1). Although the CFU of MEP-degrading bacteria were below the detectable level ($<10^3$ cfu g^{-1} dry soil) even 1 week after the insecticide application, they became detectable after the 2nd and 3rd treatments in soil S and soil N, respectively, (**Figure 1**). In the control, with only the addition of distilled water, no MEP-degraders were detected even after the 3rd treatment (**Figure 1**). These results indicate that the repeated spraying of MEP acts as a strong selective pressure on soil microbiota, confirming our findings from previous studies (Tago et al., 2006; Kikuchi et al., 2012). It has also been reported that some bacteria are capable of utilizing MEP as a sole carbon source, fostering their population increase (Hayatsu et al., 2000; Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009), which likely explains the above-observed population dynamics of MEP-degrading strains in soil environments.

Table 1 | Summary of deep sequencing and qPCR.

Soil	No. of spraying	Pot No.	Library ID	No. of sequences[b]	No. of OTU$^{c}_{0.03}$	Cx[d]	Diversity indices[e]			No. of copies[f]
							chao1	shannon	1/simpson	
S	0	1	S0p1	13,202	1,206	0.97	1,944 ± 107	7.98 ± 0.04	65 ± 2.7	$3.6 ± 0.1 × 10^8$
S	2	1	S2p1	13,346	796	0.99	1,188 ± 103	5.9 ± 0.03	15 ± 0.3	$4.5 ± 0.3 × 10^8$
S	3	1	S3p1	14,600	575	0.99	813 ± 79	3.86 ± 0.05	4.5 ± 0.1	$4.7 ± 0.2 × 10^8$
S	0	2	S0p2	6,218	1,062	0.93	2,446 ± 78	8.62 ± 0.01	109 ± 0.7	$4.6 ± 0.2 × 10^8$
S	2	2	S2p2	10,223	1,154	0.94	2,324 ± 116	7.72 ± 0.03	42 ± 0.8	$4.0 ± 0.6 × 10^8$
S	3	2	S3p2	8,213	897	0.96	1,731 ± 100	6.43 ± 0.03	13 ± 0.3	$4.4 ± 0.5 × 10^8$
S	0[a]	control	S3C	17,933	1,533	0.94	2,260 ± 123	8.6 ± 0.04	124 ± 2.8	$4.8 ± 1.2 × 10^8$
N	0	1	N0p1	13,540	1,500	0.85	3,268 ± 190	9.07 ± 0.04	138 ± 5.8	$5.5 ± 1.8 × 10^8$
N	3	1	N3p1	10,171	1,028	0.95	1,919 ± 114	7.3 ± 0.03	36 ± 0.8	$6.3 ± 0.9 × 10^8$
N	0	2	N0p2	15,054	1,747	0.87	3,655 ± 180	9.1 ± 0.02	151 ± 3.4	$6.3 ± 0.2 × 10^8$
N	3	2	N3p2	34,865	1,970	0.90	2,977 ± 187	7.78 ± 0.04	33 ± 1.2	$6.8 ± 0.5 × 10^8$
N	0[a]	control	N3C	72,066	2,459	0.90	3,470 ± 139	9.06 ± 0.04	149 ± 9.3	$8.6 ± 1.0 × 10^8$

[a] Control pots received distilled water (DW) once a week: soils were collected 1 week after 3rd DW-spraying.

[b] Number of sequences after removal of low-quality, chimeric, and archaeal sequences.

[c] Clustered at the 3% distance level.

[d] Calculated from the equation, $Cx = 1-(n/N)$, where "n" is the number of OTUs composed of only one sequence (singleton) and N is the total number of sequences.

[e] Each index was calculated based on the same amount of sequences (5174) sub-sampled from original libraries (Mean ± SDs of 10 time sub-samplings is shown).

[f] Copy numbers (copies g^{-1}-dry soil) of 16S rRNA genes estimated by qPCR (Mean ± SDs of three time measurements of the same DNA sample is shown). Analysis of variance (ANOVA) was performed using R software ver. 3.0.1 (R Development Core Team, 2008), showing the copy numbers were not significantly different between samples that were originated from the same field soil.
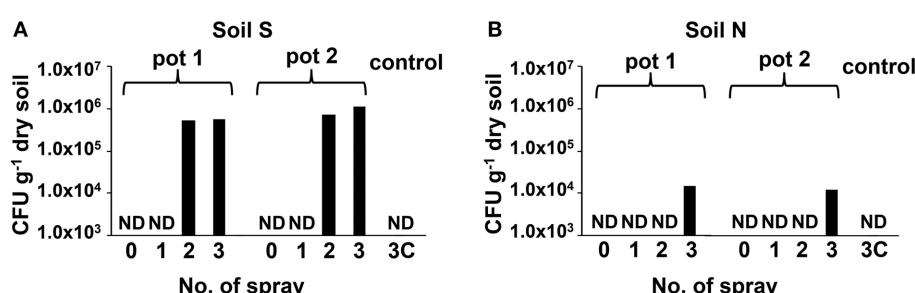
FIGURE 1 | Transition of CFU of MEP-degrading bacteria in MEP-sprayed soils. CFU of MEP-degrading bacteria in soil S **(A)** and soil N **(B)** were counted by using MEP medium plates. Results of replicated experiments (pot 1 and pot 2) are shown. ND, not detected (< $1.0 × 10^3$ cfu g^{-1}-dry soil).

ISOLATION AND IDENTIFICATION OF MEP-DEGRADING BACTERIA

From the soil S after the 2nd and 3rd MEP-treatments (S2 and S3), and soil N after the 3rd MEP-treatment (N3), colonies of the MEP-degrading bacteria were isolated and identified based on the partial sequences of 16S rRNA gene. The sequence analyses demonstrated that the majority belonged to the betaproteobacterial genus *Burkholderia*. Specifically, they occupied around 97.9, 96.9, and 61.1% of the total sequenced-bacteria isolated from the S2, S3, and N3 soils, respectively, (**Figure 2**; Table S2). Notably, in contrast to the predominance of *Burkholderia* strains in the S2 and S3 samples, MEP-degraders from the N3 sample were more phylogenetically diverse with the presence of strains from the genera *Dyella*, *Ralstonia*, *Pandoraea*, *Achromobacter*, and *Cupriavidus*.

SUCCESSION OF MICROBIOTA IN MEP-SPRAYED SOILS AS REVEALED BY DEEP SEQUENCING

For comprehensive understanding of the temporal dynamics of microbiota in the MEP-sprayed soils, we performed deep sequencing of bacterial 16S rRNA genes from both soils before treatment (S0 and N0), after the 2nd (S2) and 3rd (S3 and N3) MEP-treatments, and after the 3rd control-treatment (S3C and N3C). The sequencing of PCR amplicons of partial 16S rRNA genes produced > $2 × 10^5$ sequences in total (**Table 1**). The qPCR data showed that the amount of bacterial 16S rRNA genes was stable at around $4 × 10^8$ and $6 × 10^8$ g^{-1}-dry soil in soil S and soil N, respectively, during the treatments (**Table 1**). Meanwhile, in both soils S and N, diversity indices, chao1, Shannon, and reciprocal simpson decreased after MEP-spraying (**Table 1**), suggesting
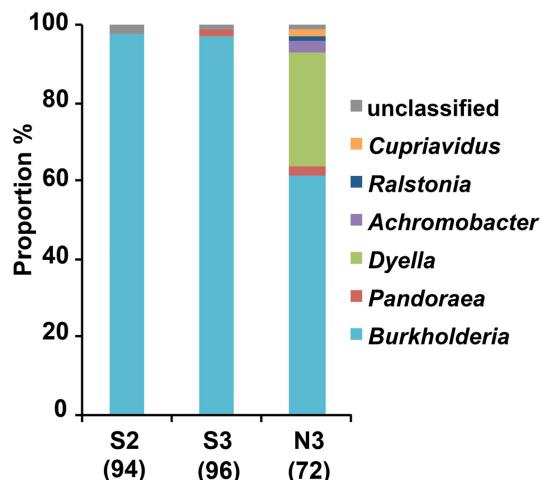
FIGURE 2 | Genus-level compositions of MEP-degrading bacteria isolated from MEP-sprayed soils. MEP-degrading isolates from soil S after two times and three times MEP spraying (S2 and S3, respectively) and from soil N after three times MEP spraying (N3) were identified by partial (c.a. 600 bp) sequencing of 16S rRNA gene. Numbers in parentheses indicate total numbers of inspected MEP-degrading strains that were randomly selected from 2 pots in each soil (i.e., results of pots 1 and 2 are merged here). Sequences were subjected to taxonomic assignment by using RDP multiclassifier with a threshold level of 80%.

that MEP treatment caused the decrease of soil microbial diversity.

Similarities among the sequence libraries of each soil sample were analyzed by PCoA based on weighted unifrac distance (Figure S2). The results suggest that the bacterial compositions of S2, S3, and N3 were distinct from those of S0 and N0 samples, although the shift direction was different between the soil S and soil N. The controls, S3C and N3C, were closely related to the soils before treatments, S0 and N0. These results also clearly demonstrate that MEP-spraying dramatically altered the community structure of soil microbiota.

BACTERIAL GROUPS RESPONDING TO MEP-SPRAYING

Figures 3A,B show the phylum-level (class-level in the *Proteobacteria*) distribution of sequences obtained from the deep sequence libraries. Among the diverse bacterial groups, *Betaproteobacteria* drastically increased after MEP-spraying in all samples from both soils S and N. Especially in soil S, the relative abundance of *Betaproteobacteria* was over 40% (80.4% in pot 1 and 49.8% in pot 2) after the 3rd MEP-treatment (**Figure 3A**). In the control samples (S3C and N3C), the bacterial composition appeared to be stable based from the initial states (S0 and N0), as also supported by the PCoA profile (Figure S2).

Within the increasing community of *Betaproteobacteria*, the genus *Burkholderia* was the most abundant, followed by *Ralstonia* and *Pandoraea* (**Figures 3C,D**). Before MEP treatment, few *Burkholderia* sequences were detected. However, after the 3rd treatment, the frequencies of *Burkholderia* drastically increased up to 53.8% in soil S pot 1, 41.1% in soil S pot 2, 55.7% in soil N pot 1, and 66.9% in soil N pot 2.

Figure 4 shows increase in the relative proportion of specific bacteria genera during the MEP-spraying experiments. It depicts the top 10 genera with the higher frequency after the 3rd treatment. From the list, the *Burkholderia*, *Cupriavidus*, *Pseudomonas*, and *Arthrobacter* are already well-known MEP-degrading strains (Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009). Furthermore, the genera *Pandoraea*, *Ralstonia*, and *Dyella*, which were additionally identified earlier as MEP-degrading group (**Figure 2**), were also included in the top 10 genera that highly responded to MEP (**Figure 4**). In both soils S and N, the frequencies of *Burkholderia* and *Ralstonia* drastically increased in response to MEP-spraying at two to three orders of magnitude, suggesting that these genera might possess a strong MEP-assimilation ability to highly adapt to the biological niche resulting from MEP-sprayed soils. Other bacterial groups, such as *Rhodanobacter*, *Nevskia*, and *Methylobacillus*, were also included in the top 10 (**Figure 4**), although they were not detected through the culture-dependent approach (**Figure 2**). These strains might assimilate the intermediates of MEP degradation such as 3-methl-4-nitrophenol and p-nitrophenol (Hayatsu et al., 2000; Arora et al., 2013), and thus proliferate under the MEP treatment. The frequencies of these bacterial groups with high increasing ratios after MEP treatment were almost stable in the control set-ups in both of soils S and N (Fold: 0.2~3.4), emphasizing that these bacteria respond to MEP-spraying.

BURKHOLDERIA STRAINS RESPONDING TO MEP-SPRAYING

To clarify the diversity of *Burkholderia* strains in the MEP-sprayed soils, 16S rRNA gene sequences of *Burkholderia* derived from the MEP-degrading isolates and the deep sequencing libraries of the soils treated with MEP three times (i.e., S3 and N3 soils) were classified into OTUs defined by 100% sequence identity. In the MEP-degrading isolates, 4 and 8 OTUs were identified from the S3 and N3 soils, respectively, in which a single and distinct OTU dominantly occupied at >70–90% frequency (**Figures 5A,B**). Although >100 OTUs were determined from all of the S3p1, S3p2, N3p1, and N3p2 libraries, a single and distinct OTU was dominant with >40% frequency in each soil sample (**Figures 5C–F**). In sequence libraries of both soil S and soil N, the dominant OTUs were not detected or a few sequences were detected before the MEP-treatment (Figure S3). These culture-dependent and -independent studies strikingly demonstrated that a particular phylotype of *Burkholderia* dominantly responds in soil environments in the presence of MEP.

We then estimated the phylogenetic relationship of these dominant *Burkholderia* strains based on 255 bp sequences of the V4 region of 16S rRNA gene (**Figure 6**). Regardless of whether the strain was identified by the culture-dependent or -independent method, each of the dominant *Burkholderia* OTUs derived from isolates and deep sequences was clustered together with the OTUs detected from the soil of the same origin (**Figure 6**), strongly indicating that our isolates are representative strains of MEP-degrading *Burkholderia* dominating the MEP-sprayed soils. This also indicates that the *Burkholderia* frequently detected from deep sequencing in MEP-sprayed soils are most likely to have MEP-degrading activity. The most dominant OTUs in soil S samples were related to pathogenic *Burkholderia* such as *B. cepacia*,
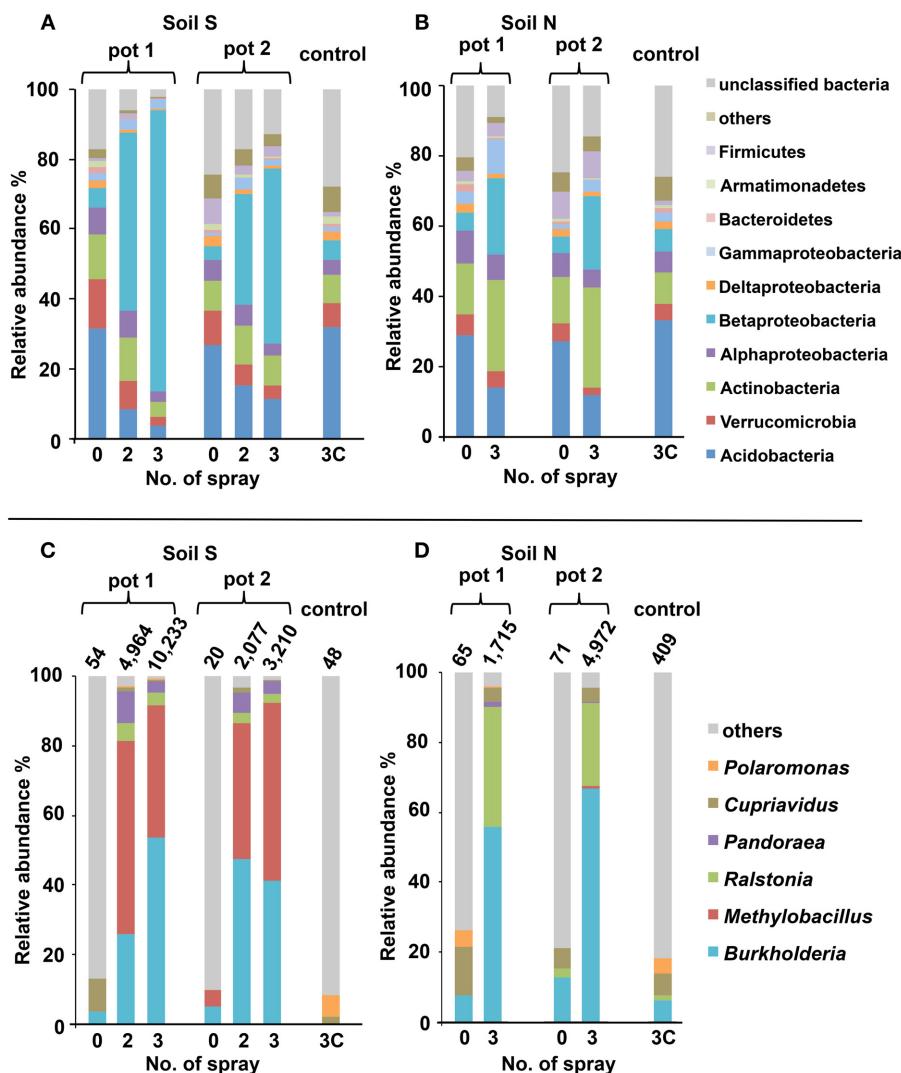
FIGURE 3 | Structural changes of microbial communities in MEP-sprayed soils. Soil S **(A,C)** and soil N **(B,D)** were analyzed by deep sequencing of 16S rRNA gene. For each soil, results of replicated experiments (pot 1 and pot 2) are shown. Relative distributions of the sequences at **(A,B)** phylum-level (class-level in the *Proteobacteria*) and **(C,D)** genus-level within *Betaproteobacteria* are shown. In **(C,D)**, total numbers of betaproteobacterial sequences excluding those of unclassified genus are depicted on the bars. Sequences were subjected to taxonomic assignment by using RDP multiclassifier with a threshold level of 80%.

B. glumae, and *B. pseudomallei* of the *Burkholderia cepacia* complex (Bcc) group (Coenye et al., 2001), while those in soil N samples were related to known MEP-degrading bacteria isolated from soils and symbiotic *Burkholderia* in stinkbugs (Tago et al., 2006; Kikuchi et al., 2011). These results suggest that the phylogenic lineage of *Burkholderia* responding to MEP-spraying appears likely to not be general, but specific for each soil environment.

DISCUSSION

EFFECTS OF FIELD-USE HISTORY ON SOIL MICROBIOTA RESPONDING TO MEP TREATMENT

Deep sequencing combined with culture-dependent approach clearly demonstrated that soil microbiota is strongly affected by the application of MEP, and furthermore, bacterial communities responding to MEP-spraying were remarkably different between the two andisol soils (soil S and soil N) (**Figures 3**, **4**, **6** and Figure S2) despite their almost similar origin and chemical properties (Table S1). Notably, the two soils have different histories of insecticide application: the agricultural field where soil S was obtained had experienced at least 4 years of MEP spraying; the field where soil N was collected was not subjected to such insecticide application (Table S1). Therefore, in soil S, MEP-degrading bacteria (i.e., *Burkholderia* species) might already exist at a certain level, thus allowing them to respond quickly to the presence of the chemical. Meanwhile, the greater variety of MEP-degrading bacteria in soil N may be due to the absence of selection pressure from MEP. Although the enhanced biodegradation caused by repeated treatment with the same insecticide were reported from the 1970s
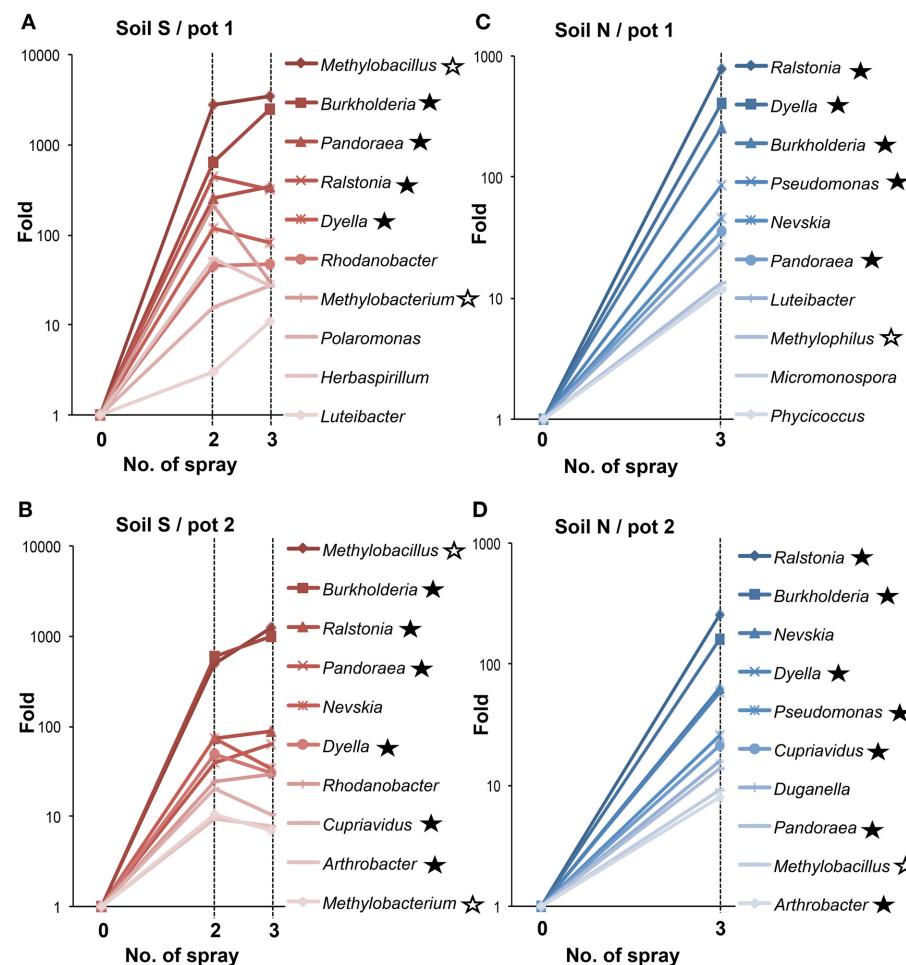
FIGURE 4 | Temporal changes of relative proportions of bacterial genera during MEP-spraying. (A,B) Soil S; **(C,D)** soil N. Relative proportions of bacterial genera were estimated based on the read numbers of deep sequencing of 16S rRNA sequences. Closed asterisks indicate the genera, which have been reported to degrade MEP (Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009), or are newly identified as MEP-degrading bacteria in this study (**Figure 2**). Open asterisks indicate methylotrophs. In addition to the relative increasing rates in MEP-sprayed soils, those in DW-sprayed soils (controls) are shown in text.

(Felsot, 1989), little is known about the relation between soil microbial succession/adaptation and insecticide-spraying history. This is the first report to show the different microbial-community responses against insecticide re-treatment in soils that previously experienced such chemical application vs. naive soil with no such treatment prior to the experiment.

It should be noted that the bacterial composition of the initial states in both soils S and N was similar to each other (Figure S2) despite their different MEP-spraying history. This result strongly suggests that, although speculative, microbial community-structure disturbed by insecticide-spraying could recover to an initial community composition once the insecticide application is terminated. To clarify the plasticity of microbial community in more detail, it would be of great interest to investigate succession process of insecticide-enriched microbiota after terminating insecticide spraying. Interestingly, our results demonstrated that soil diagnostics of biological activity should not be inferred only based on a snapshot of environmental

microbial community. To estimate potentials of a soil (microbiota) more precisely, it may be more important to trace the microbial succession under environmental stimuli.

BURKHOLDERIA IS A KEY PLAYER IN MEP-DEGRADATION

Among the bacterial groups that dominantly responded to this insecticide, the genus *Burkholderia* was the most abundant after MEP-treatment (**Figures 3C,D**) and included a number of MEP-degrading isolates (**Figure 2**). Previous studies from China, Japan, and South Korea that were based on culture-dependent methods have repeatedly isolated MEP-degrading *Burkholderia* strains from MEP-contaminated soils at a higher frequency relative to other genera (Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009). Taken together, our findings from both culture-dependent and -independent approaches strongly suggest that *Burkholderia* is a key bacterial group for MEP-degradation in soil environments. Remarkably, *Burkholderia* species were also found to degrade other organophosphorus insecticides, which includes
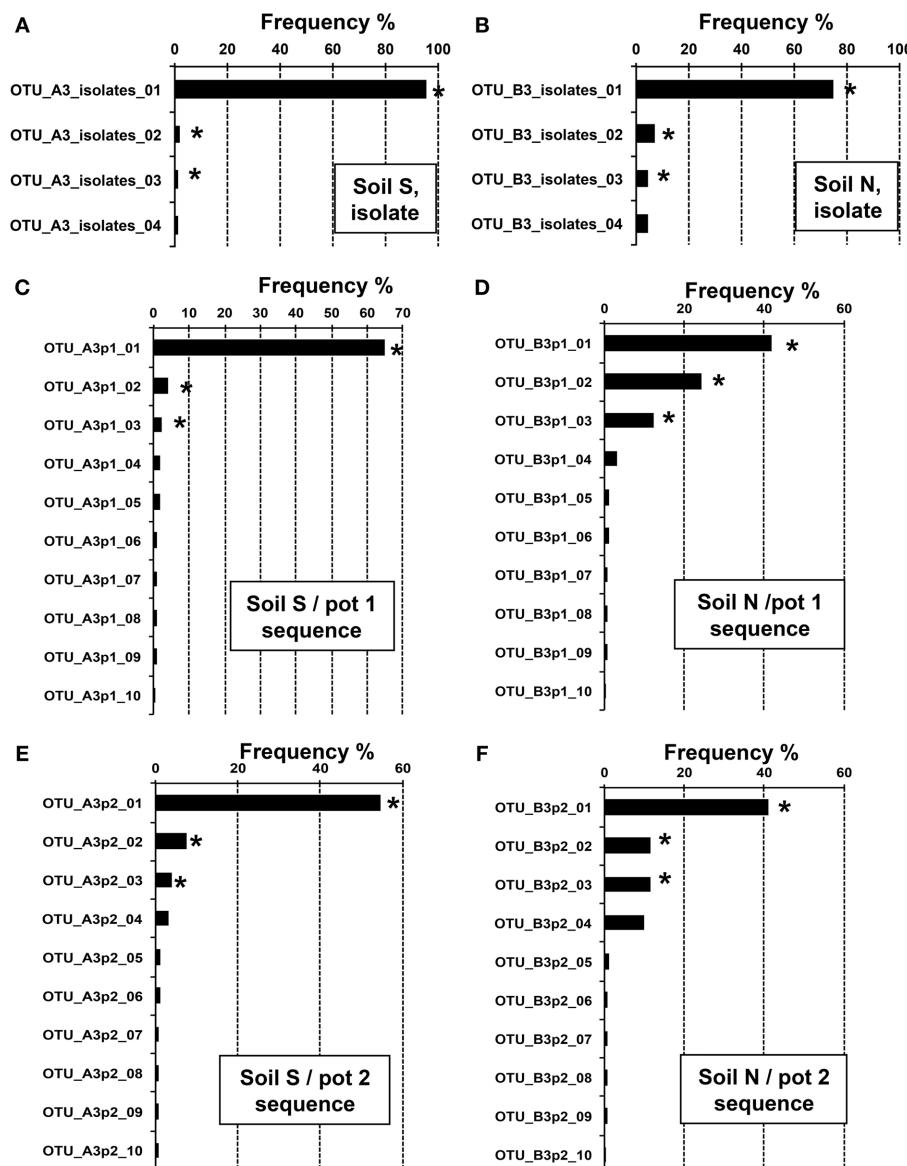
FIGURE 5 | Relative abundance of *Burkholderia* OTUs in MEP-sprayed soils. Relative abundance of 16S rRNA gene sequences assigned to different OTUs (100% sequence identity thresholds) of the genus *Burkholderia* in the MEP-degrading isolates **(A,B)** and the deep sequence libraries **(C–F)**. **(A,C,E)** Soil S; **(B,D,F)** soil N. Data showed the top 4 or 10 OTUs with high frequency in *Burkholderia* isolates or sequences, respectively. OTUs with asterisks were subjected to phylogenetic analysis (see **Figure 6**).

parathion, methyl parathion, glyphosate, and chlorpyrifos-methyl (Keprasertsupa et al., 2001; Kuklinsky-Sobral et al., 2005; Kim et al., 2007b, 2009). Considering that MEP-degrading strains of *Burkholderia* frequently show cross-acclimation against other organophosphorus compounds (Hayatsu et al., 2000; Kim and Ahn, 2009; Kim et al., 2009; Kikuchi et al., 2012), *Burkholderia* might have a pivotal role in the degradation of various insecticides. To clarify this point, it would be of great interest to also employ a deep sequencing survey of 16S rRNA genes for various types of organophosphorus insecticide-contaminated soils.

OTHER BACTERIAL GROUPS RESPONDING TO MEP-SPRAYING

Previous studies have isolated and characterized MEP-degrading bacteria from genera *Cupriavidus*, *Pseudomonas*, *Sphingomonas*, *Corynebacterium*, *Arthrobacter*, and *Burkholderia* (Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009). In addition to *Burkholderia*, members of genera *Dyella*, *Ralstonia*, *Pandoraea*, and *Achromobacter* were also isolated as MEP-degrading bacteria in our culture-dependent experiments (**Figure 2**). Hence, this is the first record of MEP-degrading strains from these four genera, suggesting that broader taxonomic groups could contribute to MEP-degradation in natural soil environments. Some
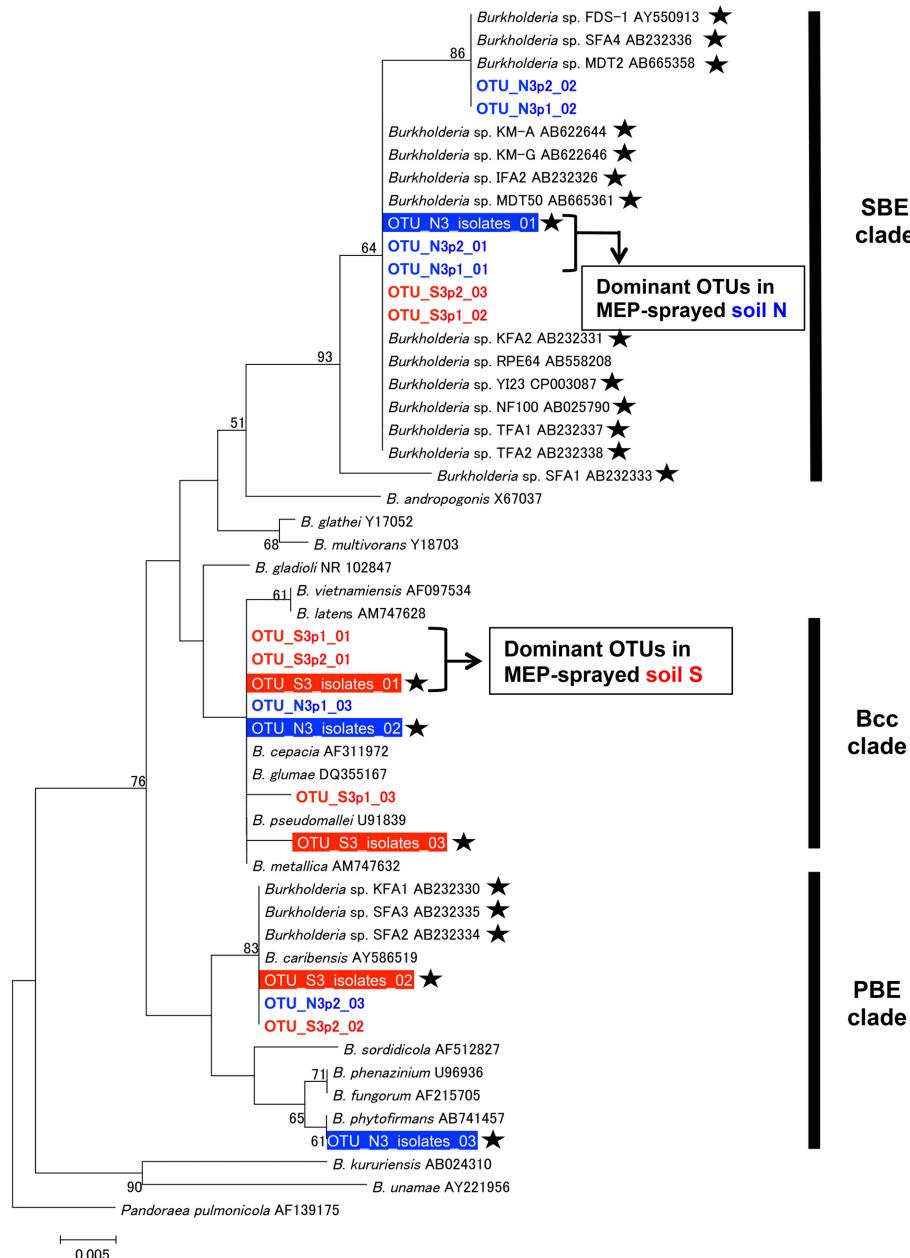
FIGURE 6 | Phylogenetic analysis of *Burkholderia* strains dominating in MEP-sprayed soils. A neighbor-joining tree inferred from aligned 255 bp sequences of 16S rRNA gene is shown. Phylogenetic placements of OTUs (OTUs at 100% identity thresholds) of *Burkholderia*, identified from the MEP-degrading isolates and deep sequences, were estimated. Among dominant OTUs in each soil samples, top 3 OTUs (see **Figure 5**) were included in the analysis. Red and blue colored OTUs are detected from soil S and soil N, respectively. Outline letters on a filled background indicate OTUs determined by the isolation. Colored letters on a white background indicate OTUs detected by the deep sequencing. Asterisks indicate MEP-degrading *Burkholderia* strains. Clade names of SBE (stinkbug-associated beneficial and environmental group), Bcc (*Burkholderia cepacia* complex), and PBE (plant-associated beneficial and environmental group), respectively, according to Itoh et al. (2014), Coenye et al. (2001), and Suárez-Moreno et al. (2012), are shown on the right side. Bootstrap values higher than 50% are depicted at the nodes. The 16S rRNA sequence of *Pandoraea pulmonicola* (AF139175) was used as an outgroup.

strains of *Ralstonia* and *Achromobacter* have already been reported to degrade organophosphorus compounds that are analogous to MEP, particularly fenamifos (organophosphorus nemati-cide) and methyl parathion (organophosphorus insecticide), respectively, (Zhang et al., 2005; Cabrera et al., 2010). Similar to *Burkholderia* strains, these bacteria probably have an ability for cross-acclimation toward other organophosphorus compounds.

In addition to the complete degradation of MEP by a single bacterium, the cooperative degradation of MEP has also been reported *in vitro*. In this case, MEP-hydrolyzing

and MEP-hydrolysate-degrading bacteria cooperate for complete consumption of the chemical compound (Katsuyama et al., 2009). Previous studies have shown that some *Burkholderia* strains, though ineffective against MEP, are able to degrade its hydrolysate, 3-methyl-4-nitrophenol (Kim et al., 2007a; Katsuyama et al., 2009). Hence, some of the increasing bacteria identified by deep sequencing (**Figure 4**) from the MEP-sprayed soils might be directly involved in the hydrolysis of MEP, while others might contribute to the degradation of the resulting hydrolysates. Such a synergistic interaction among bacterial species could promote efficient degradation of MEP in soils.

METHYLOTROPHS COULD PLAY A ROLE IN MEP-DEGRADATION

Notably, deep sequencing revealed that the frequency of *Methylobacillus*, *Methylobacterium*, and *Methylophilus*, which were never detected by the culture-dependent approach in this study and from previous researches (Tago et al., 2006; Zhang et al., 2006; Kim et al., 2009), also dramatically increased in soils after MEP-spraying (**Figure 4**). Most members of these genera are methylotrophic, that is, they actively use one-carbon compounds such as methanol as a sole carbon source (Kolb and Stacheter, 2013). Past studies have demonstrated that bacteria break down MEP into 3-methyl-4-nitrophenol and dimethyl phosphate during the initial hydrolysis step (Spillner et al., 1979; IPCS, 1992) (**Figure 7**). Though the succeeding stages for biodegrading the former aromatic compound have been described in detail (Spillner et al., 1979; IPCS, 1992; Hayatsu et al., 2000), the fate of dimethyl phosphate in soil still remains unclear. In the case of the degradation of paraoxon, an analog of MEP, the initial hydrolysis process produces diethyl phosphate, which is then further hydrolyzed into two molecules of ethanol by *Delftia acidovorans* and *Pseudomonas putida* through phosphodiesterase and phosphatase enzymes (Tehara and Keasling, 2003).

Although speculative, dimethyl phosphate may also be hydrolyzed into two molecules of methanol by a similar route during MEP-degradation. If so, the methylotrophs detected in this study may consume and utilize such generated methanol for growth (**Figure 7**).

It has been reported that accumulated methanol is harmful to organisms that are unable to metabolize it (Martin-Amat et al., 1978). In fact, growth of MEP-degrading *Burkholderia* strains isolated in this study was depressed by a tiny amount of methanol contamination (Figure S4). If methylotrophs rapidly consume any methanol generated during MEP-degradation, they might protect other bacteria involved in the overall degradation process from the toxicity of this by-product. This could probably lead to better growth of such relevant bacterial communities, thus contributing to more efficient and complete mineralization of MEP. This hypothesis could be verified through future evaluations on the (1) degradability of dimethyl phosphate by methylotrophs and (2) co-culture of MEP-degrading bacteria and methylotrophs.

CONCLUDING REMARKS

This study demonstrated the effect of MEP-spraying history to the microbial succession under MEP application and identified key bacteria for MEP-degradation by performing both culture experiments and high-throughput sequencing. Owing to the enormous information generated by the deep sequencing of bacterial 16S rRNA gene, we comprehensively revealed the community structures of bacterial species responding to MEP, and found previously unseen members, methylotrophs, that may play an important role in the complete degradation of MEP in soil environments. In contrast, it should be noted that the partial sequencing of 16S rRNA gene gave us only indirect information about the MEP-degrading strains. In fact, our previous study
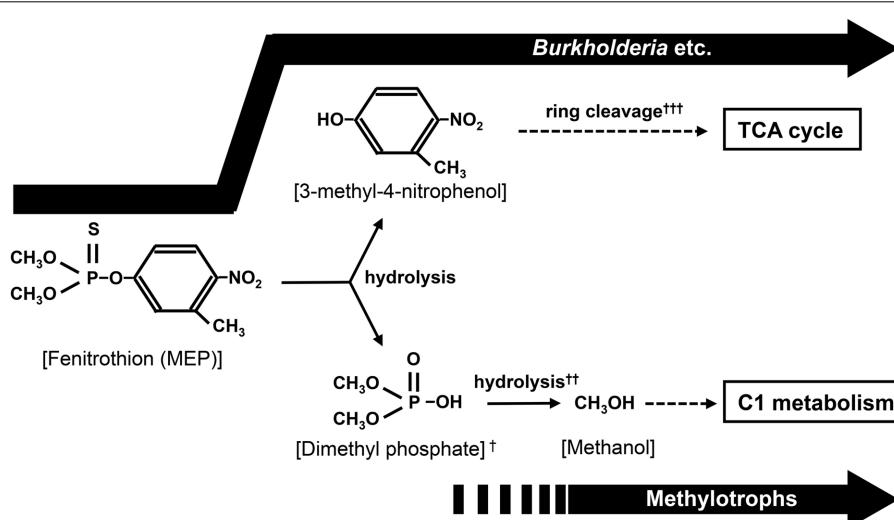
FIGURE 7 | Proposed biodegradation pathway of MEP. In addition to the utilization of 3-methyl-4-nitrophenole by *Burkholderia* and other bacteria, methanol generated from hydrolysis of dimethyl phosphate might be used as a carbon source by soil bacteria like methylotrophs. [†]P=S was rapidly substituted to P=O by oxidation in environment (Bajgar, 2004; Karpouzas and Singh, 2006). [††]$(CH_3O)_2POOH + 2H_2O \rightarrow 2CH_3OH + PO_4^{3-} + 3H^+$. [†††] The ring cleavage process has been reported in the biodegradation of MEP or MEP analog (Hayatsu et al., 2000; Zhang et al., 2006).

reported that even *Burkholderia* strains exhibiting high (>99.8%) sequence similarities in 16S rRNA gene possess quite different MEP-degrading activities (Kikuchi et al., 2012). Sequencing of functional genes involved in the MEP-degradation pathway might give more direct information for MEP-degradation in environmental soils, although MEP-degradation genes are not well understood (Singh, 2009). In addition, fungal biodegradation of MEP has been also reported (Baarschers and Heitland, 1986), although here we focused only on bacteria. Metagenomic approaches of functional genes and more comprehensive survey including fungal communities in soils should improve our knowledge of MEP-degradation by soil microorganisms.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb. 2014.00457/abstract

REFERENCES

Arbeli, Z., and Fuentes, L. C. (2007). Accelerated biodegradation of pesticides: an overview of the phenomenon, its basis and possible solutions; and a discussion on the tropical dimension. *Crop Protect.* 26, 1733–1746. doi: 10.1016/j.cropro. 2007.03.009

Arora, P. K., Srivastava, A., and Singh, V. P. (2013). Bacterial degradation of nitrophenols and their derivatives. *J. Hazard. Mater.* 266C, 42–59. doi: 10.1016/j.jhazmat.2013.12.011

Baarschers, H. W., and Heitland, S. H. (1986). Biodegradation of fenitrothion and fenitrooxon by the fungus *Trichoderma viride*. *J. Agric. Food Chem.* 34, 707–709. doi: 10.1021/jf00070a029

Bajgar, J. (2004). Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. *Adv. Clin. Chem.* 38, 151–216. doi: 10.1016/S0065-2423(04)38006-6

Cabrera, J. A., Kurtz, A., Sikora, R. A., and Schouten, A. (2010). Isolation and characterization of fenamiphos degrading bacteria. *Biodegradation* 21, 1017–1027. doi: 10.1007/s10532-010-9362-z

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Coenye, T., Vandamme, P., Govan, J. R., and LiPuma, J. J. (2001). Taxonomy and identification of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* 39, 3427–3436. doi: 10.1128/JCM.39.10.3427-3436.2001

da Silva, N. A., Birolli, W. G., Seleghim, M. H. R., and Porto, A. L. M. (2013). "Biodegradation of the organophosphate pesticide profenofos by Marine Fungi," in *Applied Bioremediation-Active and Passive Approaches*, eds Y. B. Patil and P. Rao (Rijeka: InTechWeb), 149–180. doi: 10.5772/56372

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., and Brodie, E. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Diez, M. C. (2010). Biological aspects involved in the degradation of organic pollutants. *J. Soil. Sci. Plant Nutr.* 10, 244–267. doi: 10.4067/S0718-95162010000 100004

Fang, H., Cai, L., Yang, Y., Ju, F., Li, X., Yu, Y., et al. (2014). Metagenomic analysis reveals potential biodegradation pathways of persistent pesticides in freshwater and marine sediments. *Sci. Total Environ.* 1, 983–992. doi: 10.1016/j.scitotenv.2013.10.076

Felsot, A. S. (1989). Enhanced biodegradation of insecticides in soil: implications for agroecosystems. *Ann. Rev. Entomol.* 34, 453–476. doi: 10.1146/annurev.en.34.010189.002321

Fukatsu, T., and Nikoh, N. (1998). Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). *Appl. Environ. Microbiol.* 64, 3599–3606.

Hayatsu, M., Hirano, M., and Tokuda, S. (2000). Involvement of two plasmids in MEP degradation by *Burkholderia* sp. strain NF100. *Appl. Environ. Microbiol.* 66, 1737–1740. doi: 10.1128/AEM.66.4.1737-1740.2000

International Programme on Chemical Safety (IPCS). (1992). "Environmental health criteria 133: fenitrothion," in *World Health Organization* (Accessed December 22, 2013). http://www.inchem.org/documents/ehc/ehc/ehc133.htm

Itoh, H., Aita, M., Nagayama, A., Meng, X. Y., Kamagata, Y., Navarro, R., et al. (2014). Evidence of environmental and vertical transmission of *Burkholderia* symbionts in the oriental chinch bug *Cavelerius saccharivorus* (Heteroptera: Blissidae). *Appl. Environ. Microbiol.* doi: 10.1128/AEM.01087-14. (in press).

Jacobsen, C. S., and Hjelmsø, M. H. (2014). Agricultural soils, pesticides and microbial diversity. *Curr. Opin. Biotech.* 27, 15–20. doi: 10.1016/j.copbio.2013.09.003

Karpouzas, D. G., and Singh, B. K. (2006). Microbial degradation of organophosphorus xenobiotics: metabolic pathways and molecular basis. *Adv. Microb. Physiol.* 51, 119–185. doi: 10.1016/S0065-2911(06)51003-3

Katsuyama, C., Nakaoka, S., Takeuchi, Y., Tago, K., Hayatsu, M., and Kato, K. (2009). Complementary cooperation between two syntrophic bacteria in pesticide degradation. *J. Theor. Biol.* 256, 644–654. doi: 10.1016/j.jtbi.2008. 10.024

Keprasertsupa, C., Upathamb, E. S., Sukhapanthb, N., and Prempreed, P. (2001). Degradation of methyl parathion in an aqueous medium by soil Bacteria. *Scienceasia* 27, 261–270. doi: 10.2306/scienceasia1513-1874.2001.27.261

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K., and Fukatsu, T. (2012). Symbiont-mediated insecticide resistance. *Proc. Natl. Acad. Sci. U.S.A.* 109, 8618–8622. doi: 10.1073/pnas.1200231109

Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2011). An ancient but promiscuous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. *ISME J.* 5, 446–460. doi: 10.1038/ismej.2010.150

Kim, J. R., and Ahn, Y. J. (2009). Identification and characterization of chlorpyrifos-methyl and 3,5,6-trichloro-2-pyridinol degrading *Burkholderia* sp. strain KR100. *Biodegradation* 20, 487–497. doi: 10.1007/s10532-008-9238-7

Kim, K. D., Ahn, J. H., Kim, T., Park, S. C., Seong, C. N., Song, H. G., et al. (2009). Genetic and phenotypic diversity of fenitrothion-degrading bacteria isolated from soils. *J. Microbiol. Biotechnol.* 19, 113–120. doi: 10.4014/jmb. 0808.467

Kim, S. H., Park, M. R., Han, S., Whang, K., Shim, J. H., and Kim, I. S. (2007a). Degradation of 3-Methyl-4-nitrophenol, a Main Product of the Insecticide Fenitrothion, by *Burkholderia* sp. SH-1 Isolated from Earthworm (*Eisenia fetida*) Intestine. *J. Appl. Biol. Chem.* 50, 281–287.

Kim, T., Ahn, J. H., Choi, M. K., Weon, H. Y., Kim, M. S., Seong, C. N., et al. (2007b). Cloning and expression of a parathion hydrolase gene from a soil bacterium, *Burkholderia* sp. JBA3. *J. Microbiol. Biotechnol.* 17, 1890–1893.

Kolb, S., and Stacheter, A. (2013). Prerequisites for amplicon pyrosequencing of microbial methanol utilizers in the environment. *Front. Microbiol.* 4:268. doi: 10.3389/fmicb.2013.00268

Kuklinsky-Sobral, J., Araújo, W. L., Mendes, R., Pizzirani-Kleiner, A., and Azevedo, J. (2005). Isolation and characterization of endophytic bacteria from soybean (*Glycine max*) grown in soil treated with glyphosate herbicide. *Plant Soil* 273, 91–99. doi: 10.1007/s11104-004-6894-1

Martin-Amat, G., McMartin, K. E., Hayreh, S. S., Hayreh, M. S., and Tephly, T. R. (1978). Methanol poisoning: ocular toxicity produced by formate. *Toxicol. Appl. Pharmacol.* 45, 201–208. doi: 10.1016/0041-008X(78)90040-6

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9:386. doi: 10.1186/1471-2105-9-386

Moorthie, S., Mattocks, C. J., and Wright, C. F. (2011). Review of massively parallel DNA sequencing technologies. *Hugo J.* 5, 1–12. doi: 10.1007/s11568-011-9156-3

Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* 7, 382–395. doi: 10.1111/j.1462-2920.2005.00700.x

O'Connell, J. L., and Nyman, J. A. (2011). Effects of marsh pond terracing on coastal wintering waterbirds before and after Hurricane Rita. *Environ. Manage.* 48, 975–984. doi: 10.1007/s00267-011-9741-1

Peñuelas, J., Sardans, J., Estiarte, M., Ogaya, R., Carnicer, J., Coll, M., et al. (2013). Evidence of current impact of climate change on life: a walk from genes to the biosphere. *Glob. Chang. Biol.* 19, 2303–2338. doi: 10.1111/gcb.12143

Phillips, O. L., Aragão, L. E., Lewis, S. L., Fisher, J. B., Lloyd, J., López-González, G., et al. (2009). Drought sensitivity of the Amazon rainforest. *Science* 323, 1344–1347. doi: 10.1126/science.1164033

R Development Core Team. (2008). *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing. http://www.R-project.org

Rota, C. T., Millspaugh, J. J., Rumble, M. A., Lehman, C. P., and Kesler, D. C. (2014). The role of wildfire, prescribed fire, and mountain pine beetle infestations on the population dynamics of black-backed woodpeckers in the black hills, South dakota. *PLoS ONE* 9:e94700. doi: 10.1371/journal.pone.0094700

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Singh, B. K. (2009). Organophosphorus-degrading bacteria: ecology and industrial applications. *Nat. Rev. Microbiol.* 7, 156–164. doi: 10.1038/nrmicro2050

Singh, B. K., and Walker, A. (2006). Microbial degradation of organophosphorus compounds. *FEMS Microbiol. Rev.* 30, 428–471. doi: 10.1111/j.1574-6976.2006.00018.x

Spillner, C. J., DeBaun, J. R., and Menn, J. J. (1979). Degradation of fenitrothion in forest soil and effects on forest soil microbes. *J. Agric. Food Chem.* 27, 1054–1060. doi: 10.1021/jf60225a009

Suárez-Moreno, Z. R., Caballero-Mellado, J., Coutinho, B. G., Mendonça-Previato, L., and James, E. K. (2012). Common features of environmental and potentially beneficial plant-associated *Burkholderia. Microb. Ecol.* 63, 249–266. doi: 10.1007/s00248-011-9929-1

Tago, K., Sekiya, E., Kiho, A., Katsuyama, C., Hoshito, Y., Yamada, N., et al. (2006). Diversity of fenitrothion-degrading bacteria in soils from distant geographical areas. *Microbes Environ.* 21, 58–64. doi: 10.1264/jsme2.21.58

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599. doi: 10.1093/molbev/msm092

Tehara, S. K., and Keasling, J. D. (2003). Gene cloning, purification, and characterization of a phosphodiesterase from *Delftia acidovorans. Appl. Environ. Microbiol.* 9, 504–508. doi: 10.1128/AEM.69.1.504-508.2003

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve bayesian classifier for rapid assignment of rRNA Sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Whalon, M. E., Monte-Sanchez, D., and Hollingworth, R. M. (2008). *Global Pesticide Resistance in Arthropods.* London: CAB International. doi: 10.1079/9781845933531.0000

Wilson, M. C., and Piel, J. (2013). Metagenomic approaches for exploiting uncultivated bacteria as a resource for novel biosynthetic enzymology. *Chem. Biol.* 20, 636–647. doi: 10.1016/j.chembiol.2013.04.011

Zhang, R., Cui, Z., Jiang, J., He, J., Gu, X., and Li, S. (2005). Diversity of organophosphorus pesticide-degrading bacteria in a polluted soil and conservation of their organophosphorus hydrolase genes. *Can. J. Microbiol.* 51, 337–343. doi: 10.1139/w05-010

Zhang, Z., Hong, Q., Xu, J., Zhang, X., and Li, S. (2006). Isolation of fenitrothion-degrading strain *Burkholderia* sp. FDS-1 and cloning of mpd gene. *Biodegradation* 17, 275–283. doi: 10.1007/s10532-005-7130-2

Survival of free-living *Acholeplasma* in aerated pig manure slurry revealed by ^{13}C-labeled bacterial biomass probing

Dai Hanajima[1]*, Tomo Aoyagi[2] and Tomoyuki Hori[2]

[1] Dairy Research Division, Hokkaido Agricultural Research Center, National Agricultural and Food Research Organization, Sapporo, Japan, [2] Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

Many studies have been performed on microbial community succession and/or predominant taxa during the composting process; however, the ecophysiological roles of microorganisms are not well understood because microbial community structures are highly diverse and dynamic. Bacteria are the most important contributors to the organic-waste decomposition process, while decayed bacterial cells can serve as readily digested substrates for other microbial populations. In this study, we investigated the active bacterial species responsible for the assimilation of dead bacterial cells and their components in aerated pig manure slurry by using ^{13}C-labeled bacterial biomass probing. After 3 days of forced aeration, ^{13}C-labeled and unlabeled dead *Escherichia coli* cell suspensions were added to the slurry. The suspensions contained ^{13}C-labeled and unlabeled bacterial cell components, possibly including the cell wall and membrane, as well as intracellular materials. RNA extracted from each slurry sample 2 h after addition of *E. coli* suspension was density-resolved by isopycnic centrifugation and analyzed by terminal restriction fragment length polymorphism, followed by cloning and sequencing of bacterial 16S rRNA genes. In the heavy isotopically labeled RNA fraction, the predominant ^{13}C-assimilating population was identified as belonging to the genus *Acholeplasma*, which was not detected in control heavy RNA. *Acholeplasma* spp. have limited biosynthetic capabilities and possess a wide variety of transporters, resulting in their metabolic dependence on external carbon and energy sources. The prevalence of *Acholeplasma* spp. was further confirmed in aerated pig manure slurry from four different pig farms by pyrosequencing of 16S rRNA genes; their relative abundance was ~4.4%. Free-living *Acholeplasma* spp. had a competitive advantage for utilizing dead bacterial cells and their components more rapidly relative to other microbial populations, thus allowing the survival and prevalence of *Acholeplasma* spp. in pig manure slurry.

Keywords: stable isotope probing, dead bacterial biomass, assimilation, heterotroph, aerated slurry, Acholeplasma

INTRODUCTION

Composting is a waste treatment process by which organic material is degraded and stabilized to produce fertilizer and amend soil. Aerobic bacteria are the most important decomposers during the composting process; they obtain energy by oxidizing organic material, especially the carbon fraction. Composting is typically applied to solid waste; however, aerobic treatment is also effective for liquid waste. Animal manure slurry consisting of a mixture of feces and urine is normally stored in a manure pit until land application. During storage, malodorous compounds such as volatile fatty acids and amines, indoles, phenols, ammonia, and sulfur-containing compounds accumulate due to the anaerobic degradation of organic matter (Zhu, 2000). When the stored slurry is then directly applied to agricultural fields, it often leads to complaints from residents of surrounding areas. In addition, the application of excessive amounts of easily digestible organic matter is phytotoxic to plants. Forced aeration of manure slurry has been employed to mitigate slurry odor and stabilize its quality.

In batch solid/liquid composting, the majority of organic carbon is derived from a single initial input present within the substrate itself. Labile substrates are consumed first by copiotrophic microbial taxa, which are later replaced by more oligotrophic taxa that metabolize the remaining more recalcitrant organic carbon pools (Fierer et al., 2010). It is hypothesized that microbial populations that arise due to slurry aeration are responsible for degrading the complex mixture of organic matter such as intestinal cells, microbial cells, undigested feed, or soluble organic carbons, including odorous substances. Within several hours of initiating aeration, a distinct shift in bacterial community structure occurs, accompanied by drastic changes in slurry conditions, including physicochemical parameters such as pH, oxidation-reduction potential (ORP), and reducing organic carbon (Hanajima et al., 2009, 2011). Bacteria act as important decomposers in an aerated slurry environment, while decayed bacterial cells can serve as readily digested substrates for other microbial populations. Biomass accumulation is often highest in the earlier stages of the composting process, tapering off as succession progresses (Fierer et al., 2010). Decay of the accumulated biomass releases ammonia during the decomposition process and may trigger the regrowth of unfavorable microbial populations (e.g., pathogens).

There have been many studies on microbial community succession and/or predominant taxa during the composting process (Ishii and Takii, 2003; Danon et al., 2008; Hanajima et al., 2009, 2011). However, the ecophysiological roles of microorganisms are not well understood because microbial community structures are highly diverse and dynamic. Stable isotope probing (SIP) has been used to investigate the link between the metabolic functions and phylogenetic identities of uncultured microorganisms in natural environments (Hori et al., 2007; Lee et al., 2011). For instance, SIP of rRNA has been used to trace the incorporation of ^{13}C-labeled substrates by microorganisms, which has advanced our understanding of metabolically active microbial species in a particular environment. Isotopically labeled rRNA is density-resolved by isopycnic centrifugation, and the density fraction of rRNA is analyzed by fingerprinting techniques and subsequently identified by cloning and sequencing. It was previously reported that carbon from bacterial biomass is incorporated into a soil microbial food web (Lueders et al., 2006; Murase et al., 2011); however, carbon flow mediated by microbial communities has been poorly studied in copiotrophic environments such as solid/liquid compost.

The objective of the present study was to identify active heterotrophic bacteria that are able to directly incorporate the dead *Escherichia coli* cells and their components present in aerated pig manure slurry. To achieve this objective, *E. coli* cells were cultivated with ^{13}C-labeled and unlabeled glucose to prepare ^{13}C-labeled and unlabeled bacterial biomass, respectively. These cells were heat-inactivated and dead cells and their components were added as substrates to aerated pig manure slurry. To clarify the fate of dead cells and their components, the incorporation of the substrate-derived ^{13}C into bacterial rRNA was investigated. After identifying the microorganisms that assimilated carbon from dead bacterial cells and their components by using SIP of rRNA, their widespread distribution in several pig manure slurry was examined by pyrosequencing of 16S rRNA genes.

MATERIALS AND METHODS

SIP with ^{13}C-labeled Bacterial Biomass in Aerated Pig Manure Slurry
Preparation of Pig Manure Slurry

Fresh pig feces were collected from a pig fattening house at the National Institute of Livestock and Grassland Science (Tsukuba, Japan). The feces were transported to Hokkaido Agricultural Research Center (Sapporo, Japan) in a cold box, and mixed with distilled water (dry weight of feces:weight of distilled water = 1:14). The mixture was filtered through a metallic sieve (0.5-mm mesh size) to remove large suspended solids that resist biological degradation. We simulated the actual content of pig manure slurry by adding 2 g urea instead of urine to 1 l of the filtered mixture.

Preparation of ^{13}C-labeled Bacterial Cells as a Substrate for SIP

To obtain ^{13}C-labeled bacterial biomass, *E. coli* strain NBRC 3301 was cultured on mineral medium [(6.97 g l^{-1} Na$_2$HPO$_4$, 3.42 g l^{-1} KH$_2$PO$_4$, 0.2 g l^{-1} MgCl$_2$·7H$_2$O, 1.0 g l^{-1} NH$_4$Cl, 0.1 g l^{-1} CaCl$_2$, 0.01 g l^{-1} FeSO$_4$, 0.2 mg l^{-1} ZnSO$_4$, and 0.2 mg l^{-1} MnSO$_4$ (pH 7.2)] (Kindler et al., 2006) containing 2 g l^{-1} ^{13}C-labeled glucose (D-glucose–^{13}C$_6$, min. 99 atom% ^{13}C; Sigma–Aldrich, St. Louis, MO, USA). Unlabeled cells for the control experiment were obtained by cultivation on the same medium except for containing unlabeled glucose in place of ^{13}C-labeled glucose. Cultures were incubated on a rotary shaker at 37°C and 200 rpm. *E. coli* cultures were incubated for 16 h and cells were harvested by centrifugation (4,000 × *g*, 10 min, 4°C) and washed three times with phosphate-buffered saline (PBS; pH 7.2). The labeling of *E. coli* cells was 97.5 ^{13}C atom%.

[13]C-labeled or unlabeled *E. coli* cell pellets were resuspended in 35-ml PBS and the number of viable cells in the suspension was counted by plating on LB agar (BD Biosciences, Franklin Lakes, NJ, USA). ^{13}C-labeled and unlabeled *E. coli* suspensions (2.2×10^{10} CFU/ml) were inactivated by heating at 90°C for 30 min and stored at −20°C until use. The suspensions contained ^{13}C-labeled and unlabeled bacterial cell components possibly including cell wall and membrane, as well as intracellular materials (e.g., amino acids and fatty acids).

Batch Treatment of Aerated Pig Manure Slurry Supplemented with ^{13}C-labeled Biomass

Two batch aerobic treatments were carried out in a 5-l jar fermenter (TJM-502WS; Takasaki Scientific Instruments Corp., Saitama, Japan) containing 1.5-l manure slurry and equipped with a foam cutter. The liquid was maintained at 40°C and stirred at 350 rpm. Constant airflow at 50 ml min^{-1} l^{-1} was provided from the bottom of the jar fermenter through a ceramic stone diffuser (1.2 cm inner diameter × 5.5 cm) for 5 days. This airflow rate corresponds to the average value used by farmers in Japan (1–5 m^3 air h^{-1} m^{-3} slurry) (Japan Livestock Industry Association, 1989). On day 3 after aeration was initiated, 30-ml suspensions of ^{13}C-labeled dead *E. coli* and unlabeled dead *E. coli* (control) were added to the aerated slurry. Every 30 min, the ORP and pH of the slurry were automatically measured and recorded (Thermodac EF, Model 5020A; Eto Electrics, Tokyo, Japan). The slurry in the reactor was periodically sampled using a syringe. Samples were stored at −80°C for subsequent molecular analyses.

Physicochemical Analyses of Aerated Slurry

Moisture content was determined by drying samples for 24 h at 105°C. Chemical oxygen demand (COD_{Cr}) was measured using the closed reflux colorimetric method in reaction tubes with prepared reagents provided by Hach Company (Loveland, CO, USA), according to the manufacturer's instructions. Analysis of $\delta^{13}C$ was carried out at the International Research Center for Agricultural Sciences (Tsukuba, Japan) using an infrared mass spectrometer (Finnigan Delta PLUS XP; Thermo Scientific, Hamburg, Germany) connected to an element analyzer (EA Flash 1112; Carlo Erba, Milan, Italy). To detect total carbon, freeze-dried slurry samples were incinerated in the element analyzer furnace and separated as pure CO_2 gas, a small quantity of which was used to measure the ratio of $^{13}CO_2/^{12}CO_2$ as different mass weights of 45/44 to obtain $\delta^{13}C$ (‰).

RNA Extraction and Density Gradient Centrifugation

RNA was extracted from 0.5 ml of slurry samples that were obtained after 2- and 6-h incubations with ^{13}C-labeled and unlabeled dead *E. coli* cells using a direct lysis protocol involving bead beating (Noll et al., 2005). Total RNA was quantified using the Ribogreen RNA quantification kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA extracts (500 ng RNA) from ^{13}C-labeled and unlabeled samples were mixed with cesium trifluoroacetate (CsTFA) (Wako Pure Chemical Industries Ltd., Osaka, Japan) solution. The mixture was subjected to equilibrium density gradient centrifugation as previously described (Lueders et al., 2004). The RNA density gradients were fractionated, and their CsTFA buoyant density (BD) was determined (Lueders et al., 2004).

Reverse Transcription (RT)-PCR and Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

Each density fraction of RNA from ^{13}C-labeled and unlabeled samples was subjected to RT-PCR using a PrimeScript RT-PCR kit (Takara Bio, Otsu, Japan) for T-RFLP fingerprinting. The PCR primer set 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′-6-carboxyfluorescein) and 926r (5′-CCG TCA ATT CCT TTR AGT TT-3′) was used to amplify bacterial 16S rRNA genes. RT was carried out at 65°C for 5 min for annealing, followed by 42°C for 30 min and 95°C for 5 min for transcription. The thermal profile for PCR amplification consisted of 20 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min under stringent conditions, followed by final extension at 72°C for 5 min. RT-PCR products were verified by electrophoresis on a 1.5% agarose gel and were purified using the MonoFas DNA purification kit (GL Sciences, Tokyo, Japan). Two hundred nanograms of the amplicon was digested with *Msp*I (Nippon Gene, Tokyo, Japan) and then desalted by ethanol precipitation. Prior to electrophoresis, 1 μl of the digests was resuspended in 12 μl Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and 0.5 μl of GeneScan 600 LIZ Size Standard (Applied Biosystems). The mixture was denatured at 95°C for 3 min and cooled immediately on ice. Size separation of T-RFs was performed using an ABI 310 genetic analyzer (Applied Biosystems).

Cloning and Sequencing of the RNA Density Fractions

Selected density fractions of RNA were amplified for cloning using the primer set 27f/926r under the thermal conditions described above. RT-PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and the ligated product was used to transform *ECOS* competent *E. coli* JM109 cells (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. We randomly selected 63 clones from ^{13}C-labeled samples and 92 clones from unlabeled samples. The 16S rRNA segments were sequenced on a 3130xl Genetic Analyzer (Applied Biosystems) using the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems). The 16S rRNA gene sequences obtained was compared with those in the nucleotide sequence database by using the BLAST program[1]. Chimeric structures were detected by separately analyzing the phylogenies of terminal stretches at the 5′ and 3′ ends, known as fractional treeing (Ludwig et al., 1998).

Analysis of Bacterial Communities in Pig Manure Slurry Samples using Pyrosequencing

Preparation of Aerated Pig Manure Slurry from Different Pig Farms

To analyze bacterial communities in aerated pig manure slurry, samples from four different locations (designated as R, N, O,

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[1]http://ddbj.nig.ac.jp/blast/

and K) were collected. N corresponded to a pig fattening house at the National Institute of Livestock and Grassland Science, and R, O, and K were pig farms located in various areas of Japan separated by a distance of at least 300 km. Pig feces were transported to Hokkaido Agricultural Research Center in a cold box and slurry samples were prepared as described above. Batch aerobic treatment of the slurry was also performed as described, except that a 3-l volume was used. To prevent bacterial cross-contamination between samples, the jar fermenter including sensors and air inlet were autoclaved prior to each batch aerobic treatment. Air was supplied through a filter with a pore size of 0.22 μm.

Barcoded Pyrosequencing of Bacterial 16S rRNA Genes

Total genomic DNA from pig manure slurry was extracted using the UltraClean Fecal DNA Isolation kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions. The V4–V5 regions of the bacterial 16S rRNA gene were amplified using primers T6m563F (5′-NNNNNN-AYTGGGYDTAAAGNG-3′) and T6m926R (5′-NNNNNN-CCGTCAATTCMTTTRAGT-3′), where NNNNNN denotes a unique 6-mer barcode. PCR amplification was performed on a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) in a 50-μl reaction volume containing the BIOTAQ HS DNA Polymerase mixture (Bioline, London, UK), 1 μl genomic DNA, and 20 pmol of each primer. The reaction conditions were as follows: 95°C for 10 min; 20 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. PCR products were resolved by electrophoresis on a 1.5% agarose gel and the DNA band of the correct size was excised and purified using the MonoFas DNA purification kit. Equal amounts of purified PCR product were pooled and ligated with adapters using the GS Titanium Rapid Library Preparation kit (Roche, Mannheim, Germany). Pyrosequencing was carried out on a 454 GS Junior instrument (Roche) following the Roche Amplicon Lib-L protocol, and the resulting sequencing reads were analyzed using the Ribosomal Database Project (RDP) pyrosequencing pipeline[2] (Wang et al., 2007) with default parameters (maximum number of Ns = 0 and minimum average quality score = 20). Pyrosequencing reads were assigned to specific samples based on their unique bar codes; these as well as primers were then trimmed off. DECIPHER was used to identify chimeric sequences[3] (Wright et al., 2012). Taxonomic assignment of bacterial high quality reads was performed using the RDP naive Bayesian rRNA Classifier with 80% confidence thresholds.

Phylogenetic Analyses and Deposition of Identified 16S rRNA Genes

Phylogenetic Analyses of 16S rRNA Gene Sequences

Phylogenetic analyses were carried out using MEGA software version 6 (Tamura et al., 2013). Phylogenetic trees with reference 16S rRNA sequences (>1,400 nucleotides) and representative partial 16S rRNA sequences (i.e., ~850 bp from clone analysis

[2]http://pyro.cme.msu.edu/
[3]http://decipher.cee.wisc.edu/

or 325 bp from pyrosequencing) obtained in this study were analyzed using the neighbor-joining method. Bootstrap values were obtained from 1,000 replicates.

Nucleotide Sequence Accession Numbers

Nucleotide sequence data obtained in this study have been deposited in the DNA Data Bank of Japan[4] under accession numbers LC055721–LC055728.

RESULTS

Aerated Slurry Conditions and Addition of Labeled Biomass

Organic matter in pig manure slurry decreased linearly from 35,000 to 15,000 mg l^{-1} over 5 days of aeration, as evaluated by COD_{Cr} (**Figure 1A**). ^{13}C-labeled or unlabeled dead *E. coli* cell suspension was added on day 3; at this point, the COD_{Cr} value was approximately 21,000 mg l^{-1}, while the value for ^{13}C-labeled or unlabeled dead *E. coli* cell suspension was 34,510 mg l^{-1}. Hence, the bacterial suspension (30 ml) corresponded to 3.3% of the total COD_{Cr} of the bulk slurry. Immediately after adding

[4]http://www.ddbj.nig.ac.jp
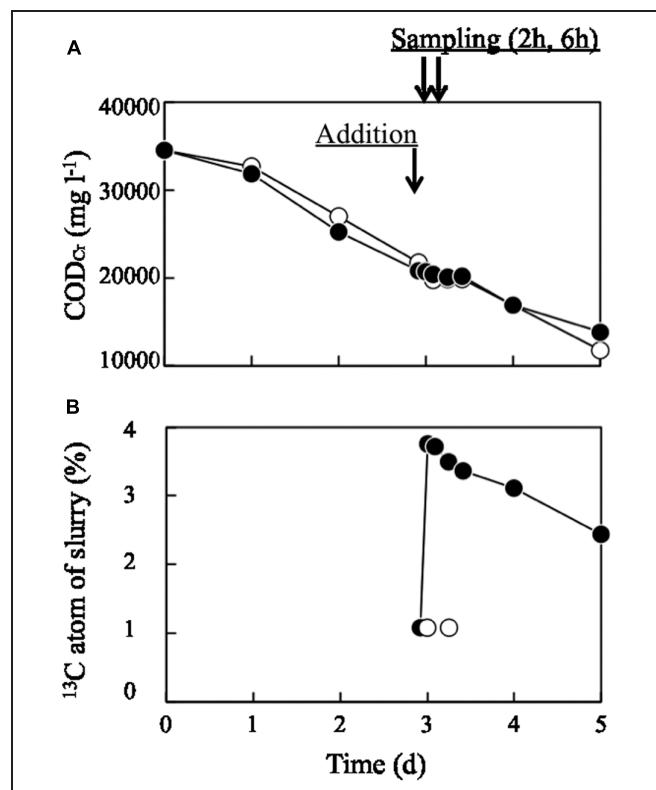
FIGURE 1 | Changes in (A) COD_{Cr} and (B) ^{13}C atom% in aerated pig manure slurry. Closed and open circles represent treatments with ^{13}C-labeled and unlabeled (control) dead *Escherichia coli* cells, respectively. Arrows represent the time at which dead *E. coli* cells were added and sampling times (2 and 6 h later).

[13]C-labeled bacterial biomass, the COD_{Cr} value of the slurry did not change significantly, while ^{13}C atom% increased from 1.07 to 3.76% (**Figure 1B**) and then decreased to 2.44% on day 5. The ORP decreased to less than −400 mV within 12 h of the operation (Supplementary Figure S1A). This low ORP value lasted through day 3. After day 3, ORP began to increase and finally reached −30 mV on day 5. The pH fluctuated between 8.1 and 8.6 during the process (Supplementary Figure S1B). The time courses of ORP and pH were similar between runs incubated with ^{13}C-labeled and unlabeled dead *E. coli* cells and their components.

Bacterial Community Structures in RNA Density Gradients

RNA-based SIP was performed to identify bacterial populations capable of assimilating bacterial biomass in aerated pig manure slurry 2 and 6 h after adding dead *E. coli* cell suspension. Bacteria-specific amplicons were obtained from density fractions with the highest BD (>1.811 g ml^{−1}) obtained for the ^{13}C-labeled sample, while no amplicons were obtained in this fraction (>1.811 g ml^{−1}) in unlabeled control samples. T-RFLP fingerprinting patterns were similar in light fractions of RNA (BD: 1.760–1.786 g ml^{−1}) after a 2-h incubation (**Figure 2**). The community profiles changed with increasing BD in ^{13}C-labeled samples, in which two major T-RFs (277 and 278 bp in length) predominated in heavy fractions (BD: >1.811 g ml^{−1}), accounting for ∼71% of total peak heights. However, this predominance was not observed either in light fractions (BD < 1.786 g ml^{−1}) of ^{13}C-labeled samples or in entire-range fractions of unlabeled samples. In addition, the two T-RF peaks differed from that of the 16S rRNA from ^{13}C-labeled dead *E. coli* cells (**Figure 2**). Although several minor T-RFs (94, 96, 151, 296, and 300 bp) were detected in heavy fractions, most of these were also present in light fractions. A high relative abundance of the two major peaks, reaching ∼62% of total peak heights, was still detected in heavy fractions after 6 h (Supplementary Figure S2).

Phylogenetic Identification of Bacteria Incorporating ^{13}C-labeled Bacterial Biomass

Major populations found in heavy fractions by T-RFLP were identified by cloning and sequencing their 16S rRNAs from ^{13}C-labeled and unlabeled samples. Phylogenetic affiliation and number of 16S rRNA clones are shown in **Table 1**. A high relative abundance of *Acholeplasma* sequences was present in heavy fractions of ^{13}C-labeled sample as compared to unlabeled sample (63% of total clones). T-RF sizes of the sequences were estimated to be 277 and 278 bp, which corresponded to the major peaks in the T-RFLP fingerprint (**Figure 2**). These clones were related to *Acholeplasma axanthum* (90–94% sequence identity, 850 bp in length); two representative sequences, AS1 and AS2, were obtained (**Figure 3**) that belonged to clades that were distinct from the only free-living *Acholeplasma*, *A. laidlawii* (∼88% sequence identity, 850 bp in length). Besides *Acholeplasma*, specific sequences of Clostridia or Bacteroidia were obtained from the heavy fraction in ^{13}C-labeled samples. However, some of these were also detected as minor T-RFs in the unlabeled T-RFLP fingerprint. A large fraction of clones in the unlabeled sample library was related to Bacilli (57% of total clones), Clostridia (12%), γ-Proteobacteria (11%), and *Corynebacterium* (11%); only one clone related to *Acholeplasma* was obtained.

Prevalence of *Acholeplasma* in Different Sources of Aerated Pig Manure Slurry

To evaluate the prevalence of *Acholeplasma* bacteria, pig manure slurry was obtained from four different locations (R, N, O, and K) in Japan. The pig manure slurry was sampled 3 days after the start of aeration; the sampling time was same as for SIP. Bacterial community composition was analyzed by pyrosequencing of 16S rRNA genes. The relative abundances of the *Acholeplasma* sequences (323 or 325 bp in length) in the four sample libraries were as follows: R, 4.5% (29 out of 649 sequences); N, 0.6% (10
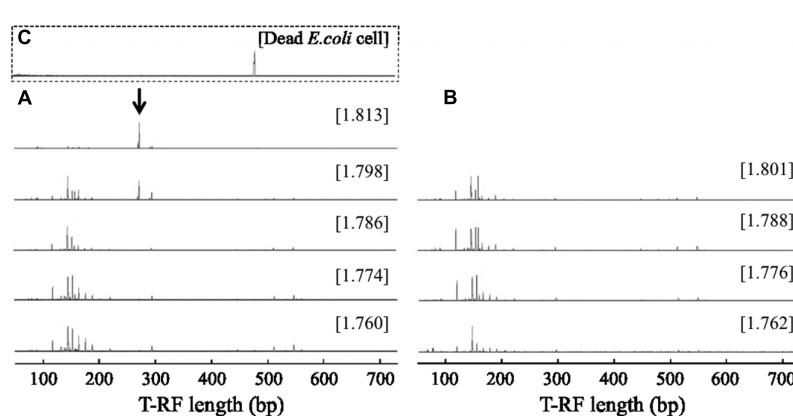
FIGURE 2 | Terminal restriction fragment length polymorphism (T-RFLP) fingerprints of bacterial 16S rRNA separated by isopycnic centrifugation from (A) ^{13}C-labeled **and (B) unlabeled samples 2 h after addition of dead *E. coli* cell suspension.** T-RFLP fingerprint of **(C)** the dead *E. coli* cell suspension is shown in the square with broken lines. The CsTFA BD (g ml^{−1}) of each fraction is shown in square brackets. The arrow indicates specific T-RFs observed in the heavy fraction of a ^{13}C-labeled sample.

TABLE 1 | Phylogenic affiliations, T-RF length, and number of 16S rRNA clones obtained from the highest-density fraction of bacterial RNA following addition of ^{13}C-labeled and unlabeled bacterial biomass.

Phylogenetic group	^{13}C-labeled		Unlabeled	
	Clones (n)	T-RF (bp)	Clones (*n*)	T-RF (bp)
Acholeplasma	39	**277, 278 (71%)**, 279	1	278
Bacilli				
Bacillus	3	153, 165, 168	11	155, 167, **169 (4%)**
Planococcaceae	1	151	25	145, 147, **151 (25%)**, 159, 558
Lactobacillus	–	–	10	29, **181 (2%)**
Streptococcus	–	–	6	**555 (3%)**
Clostridia				
Sporanaerobacter	4	296	–	–
Tissierella	2	300	–	–
Proteiniborus	1	167	–	–
Clostridium XI	–	–	6	**195 (5%)**
Clostridium sensu stricto	–	–	2	520
Cryptanaerobacter	–	–	2	508
Clostridium XIVa	–	–	1	287
Bacteroidia				
Petrimonas	6	**94 (3%)**	–	–
Proteiniphilum	2	96	1	96
α-Proteobacteria	2	150	2	439
β-Proteobacteria	–	–	4	129, 488, 496
γ-Proteobacteria	1	123	10	**123 (10%)**, 226
Erysipelothrix	1	173	—	—
Corynebacterium	–	–	10	**165 (25%)**
Flavobacterium	–	–	1	29
Total	62		92	

T-RF detected for more than five clones within a group is indicated in boldface. Relative abundance of corresponding peaks in the T-RFLP profile is presented in parentheses.

out of 1583 sequences); O, 0.2% (3 out of 1431 sequences); and K, 3.9% (44 out of 1128 sequences). All representative sequences obtained from these libraries were similar to the bacteria (i.e., AS1 or AS2) obtained in the ^{13}C-labeled sample (100% sequence identity, 323 or 325 bp in length) (**Figure 3**).

DISCUSSION

Forced aeration of pig manure slurry has been used to mitigate odor emission and stabilize slurry quality. During this process, the bacterial community—mainly comprising pig gastrointestinal bacteria in raw manure—immediately shifts to become predominantly composed of Bacilli (Hanajima et al., 2009). *Bacillus* species are ubiquitous and are typically but variably capable of growing aerobically on complex, low molecular mass substrates (Sneath, 1986). These bacteria are implicated in organic matter degradation under aeration, which converts a portion of decayed bacterial biomass to carbon dioxide, ammonia, and/or water; however, it was assumed that a significant fraction of decayed bacterial biomass is recycled by newly proliferating populations. If the slurry is mixed thoroughly under stirring and forced aeration within the reactor, most microorganisms have access to

bacterial cell components (e.g., cell wall and membrane, as well as intracellular materials) from decayed bacterial cells. However, with longer incubation times, carbon derived from labeled microbial products rapidly spread throughout the slurry microorganisms via trophic interactions. We therefore focused on identifying the first-to-arrive scavengers in the aerated slurry, and demonstrated by SIP of rRNA that dead cells and their components were incorporated specifically by *Acholeplasma*.

Dead cells and their components were added just prior to ORP elevation. ORP is closely linked to the level of dissolved oxygen (DO) in slurry, and a decline in ORP has been attributed to increased DO consumption by microorganisms (Hanajima et al., 2009). In contrast, a reduction in available carbon in slurry decreases the requirement for oxygen in the decomposition of carbon substrates. This fluctuation in ORP is generally observed during aerated slurry treatment, and drastic shifts in bacterial community composition occur along with changes in ORP (Hanajima et al., 2009, 2011). Under these conditions, a certain amount of dead bacterial cells is likely produced in response to drastic shifts in the bacterial community. To prevent the continued existence and/or proliferation of ^{13}C-labeled *E. coli* in aerated slurry, cell cultures were inactivated before their addition as substrate. Given that the bacterial suspension
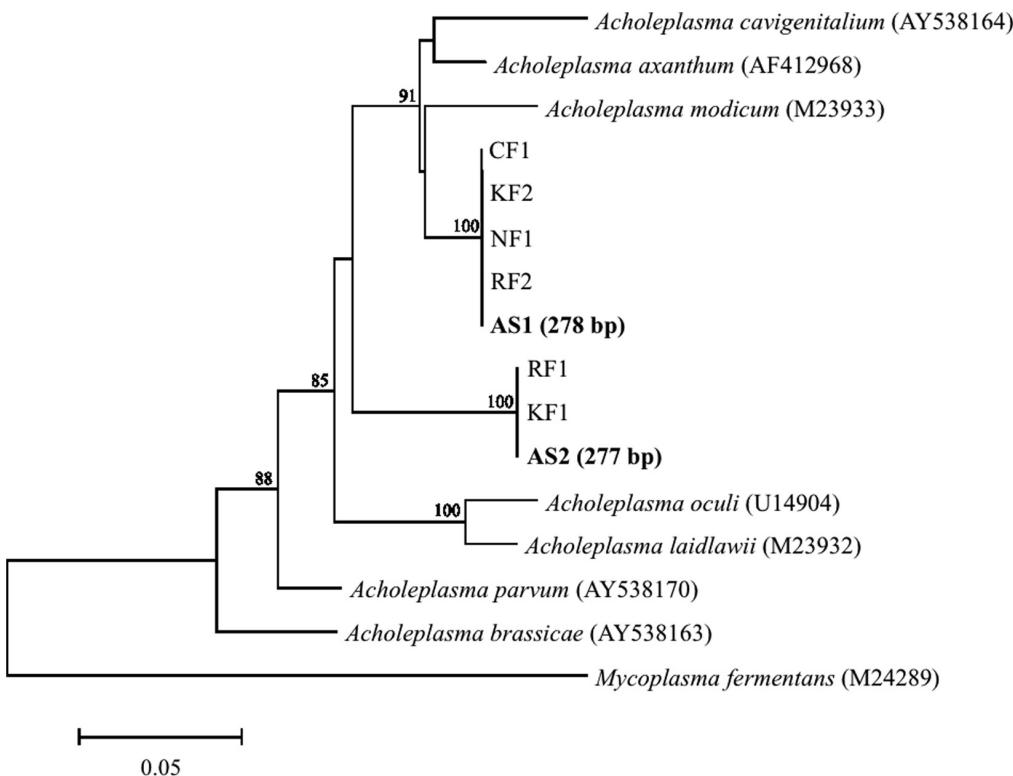
FIGURE 3 | Phylogenetic tree generated with the neighbor-joining method showing relationships among clones obtained from aerated pig manure slurry samples. Clones were designated AS (heavy fraction of ^{13}C-labeled sample, in boldface) and RF, NF, CF, or KF (sequences obtained from slurry from four different pig farms). Numbers in parentheses for AS1 and AS2 represent expected T-RF lengths of clones. Bootstrap values are shown at each node that had >70% support in a bootstrap analysis of 1,000 replicates. Scale bar represents 5% sequence divergence. DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank accession numbers of reference sequences are given.

was <3.3% of the total COD_{Cr} of bulk slurry, it had little influence on the aerobic treatment process. Moreover, *E. coli* suspensions were not contaminated with *Acholeplasma* and none of the T-RFs derived from ^{13}C-labeled *E. coli* were detected in the T-RFLP fingerprint. Therefore, sequences enriched in heavy fractions of RNA were derived from metabolically active bacteria that assimilated the ^{13}C-labeled dead cells and their components. It was noted that these heavy fractions contained *Acholeplasma* sequences. Because none of the *Acholeplasma*-related T-RFs were observed in the unlabeled control or light fractions of the ^{13}C-labeled sample, it was concluded that *Acholeplasma* was directly involved in the utilization of the dead cells and their components, albeit at a small population size.

Bacteria of the class *Mollicutes*, which includes the genus *Acholeplasma*, are widespread in nature and engage in a parasitic lifestyle; they are characterized by the absence of a cell wall and drastic reduction of genome size (Razin et al., 1998). *Acholeplasma* spp. possessing relatively large genomes of 1.5–1.8 Mbp (Lazarev et al., 2011), do not require sterols for cultivation, and are able to synthesize fatty acids from precursors, unlike other mycoplasma (Saito et al., 1977). *Acholeplasma* members have been detected as parasitic bacteria in nearly all organisms, including plants, crustaceans, mammals, and insects

(Atobe et al., 1983; Clark et al., 1986; Tully et al., 1994; Chen et al., 2011). One of the best-characterized species is *A. laidlawii*, which is regarded as a universalist that adapts to various environmental conditions. It was isolated from wastewater and is the only known *Acholeplasma* capable of surviving and reproducing in animals and plants through a parasitic lifestyle and in wastewaters through a free-living lifestyle (Lazarev et al., 2011). However, *Acholeplasma* sequences obtained in this study constituted a clade distinct from that of *A. laidlawii*, demonstrating for the first time that another free-living *Acholeplasma* species exists and is metabolically active in aerated slurry. *Acholeplasma* sequences have been previously detected in swine waste lagoons (Goh et al., 2009), aerated pig manure slurry (Hanajima et al., 2009), and pigpen pit slurry (Hwang et al., 2014). The latter study found a relative abundance of 3.5% from *Acholeplasma* in pig manure slurry, which is in accordance with results from our survey of four different farm samples (∼4.4%). These results strongly suggest that *Acholeplasma* is prevalent in the pig manure slurry environment. In addition to these environments, *Acholeplasma* were found in marine sediments (Masui et al., 2008; Imachi et al., 2011), suggesting that these organisms are concentrated in copiotrophic environments in which bacterial cell components released from decayed microbial biomass accumulate.

It is widely known that Mollicutes have limited biosynthetic capabilities (Fraser et al., 1995; Sasaki et al., 2002; Vasconcelos, 2005); these are primarily for energy acquisition, with synthetic pathways being considerably reduced or absent. As such, many nutrients must be obtained from external sources (e.g., the host organism). Metabolic and genomic analyses have been carried out to elucidate the principles of metabolic regulation and adaptation to environmental conditions by Mollicutes (Lazarev et al., 2011; Kube et al., 2014; Vanyushkina et al., 2014). The *Acholeplasma* genome encodes a wide variety of ATP-binding cassette (ABC) transporters, and most genetic modules are implicated in the conversion of intermediates and are linked to ABC transporter genes for the uptake of oligopeptides and amino acids (Kube et al., 2014). This demonstrates that *Acholeplasma* are capable of incorporating oligopeptides and amino acids released from dead cells and their components. However, this raises questions about the assimilation of labile carbon sources by heterotrophic bacteria in slurry, such as why only the rRNA recovered from *Acholeplasma* was ^{13}C-labeled despite most heterotrophic bacteria having access to dead cells and their components, and why ^{13}C-labeled rRNA was recovered after a very short (2-h) incubation period. *Acholeplasma* has the capacity to generate dNTPs from adenosine, guanosine, uracil, and thymine (Kube et al., 2014), and *A. laidlawii* was reported to incorporate nucleic acid precursors during the first 4 h of incubation in medium (McIvor and Kenny, 1978). The detection of ^{13}C-labeled rRNA within a 2-h incubation period leads us to speculate that *Acholeplasma* incorporated ^{13}C-labeled nucleic acid precursors released by dead *E. coli* cells. Although most heterotrophic bacteria may incorporate ^{13}C-labeled dead cells and their components, a longer incubation time would be required to obtain ^{13}C-labeled rRNA from these bacteria, with the exception of *Acholeplasma*.

Acholeplasma axanthum, the closest relative to metabolically active bacteria identified in our study, has been isolated from a variety of habitats and hosts, including the lung of swine (Stipkovits et al., 1974). The genotypic patterns of these strains differed markedly from one another, suggesting considerable heterogeneity within the same species (Razin et al., 1983). Growth in varied environments requires regulatory switches and cross-talk between metabolic pathways to allow rapid adaptation to environmental changes in nutritional flux. The *A. laidlawii* genome contains genes encoding components of signal transduction pathways and the SOS response system, which are closely related to the regulation of gene expression and mutagenic response to stress (Lazarev et al., 2011). These functions allow *A. laidlawii* to adapt to various environmental conditions. It is unclear whether the *Acholeplasma* spp. detected in this study originated from the body of pigs or from the manure slurry itself; nonetheless, the results suggest that *Acholeplasma* has a competitive advantage for utilizing dead bacterial biomass more rapidly relative to other microbial populations.

Linking the phylogeny and function of microorganisms is one of the most fundamental challenges in microbial ecology. Generating defined mixed cultures of isolated microorganisms is one promising approach to achieve this end (Kato et al., 2005, 2014). However, in natural and engineered environments, unidentified microbial components may nonetheless play critical ecophysiological roles. In this study, we identified the first-to-arrive scavengers in aerated pig manure slurry by ^{13}C-labeled bacterial biomass probing of rRNA. *Acholeplasma* spp. were identified for the first time as a major heterotroph that assimilates the dead cells and their components in pig manure slurry. Substrate competition has been found in many types of biological processes, in which physiologically similar microorganisms co-exist and fill ecological niches owing to the difference in the substrate availability and affinity (Hori et al., 2006; Aoyagi et al., 2015). Due to the limited biosynthetic capabilities, free-living *Acholeplasma* spp. most likely require biomolecules released from dead bacterial cells as substrates. Actually, they were capable of incorporating the dead cells and their components more rapidly than the other microbial populations, which allowed the survival and prevalence of *Acholeplasma* spp. in pig manure slurry. These findings provide deeper understanding of carbon flow mediated by microorganisms in copiotrophic environments and unveil one of the mechanisms underlying the microbial ecosystem development.

AUTHOR CONTRIBUTIONS

DH designed the research. DH and TH wrote the manuscript. DH, TA, and TH carried out the experiments and analyzed the data.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01206

REFERENCES

Aoyagi, T., Kimura, M., Yamada, N., Navarro, R. R., Itoh, H., Ogata, A., et al. (2015). Dynamic transition of chemolithotrophic sulfur-oxidizing bacteria inresponse to amendment with nitrate in deposited marine sediments. *Front. Microbiol.* 6:426. doi: 10.3389/fmicb.2015.00426

Atobe, H., Watabe, J., and Ogata, M. (1983). *Acholeplasma parvum*, a new species from horses. *Int. J. Syst. Bacteriol.* 33, 344–349. doi: 10.1099/00207713-33-2-344

Chen, J. G., Lou, D., and Yang, J. F. (2011). Isolation and identification of *Acholeplasma* sp. from the mud crab. Scylla serrata. *Evid. Based Complement. Alternat. Med.* 2011, 209406. doi: 10.1155/2011/209406

Clark, T. B., Tully, J. G., Rose, D. L., Henegar, R., and Whitcomb, R. F. (1986). Acholeplasmas and similar nonsterol-requiring mollicutes from insects—missing link in microbial ecology. *Curr. Microbiol.* 13, 11–16. doi: 10.1007/BF01568152

Danon, M., Franke-Whittle, I. H., Insam, H., Chen, Y., and Hadar, Y. (2008). Molecular analysis of bacterial community succession during prolonged compost curing. *FEMS Microbiol. Ecol.* 65, 133–144. doi: 10.1111/j.1574-6941.2008.00506.x

Fierer, N., Nemergut, D., Knight, R., and Craine, J. M. (2010). Changes through time: integrating microorganisms into the study of succession. *Res. Microbiol.* 161, 635–642. doi: 10.1016/j.resmic.2010.06.002

Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., et al. (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* 270, 397–403. doi: 10.1126/science.270.5235.397

Goh, S. H., Mabbett, A. N., Welch, J. P., Hall, S. J., and McEwan, A. G. (2009). Molecular ecology of a facultative swine waste lagoon. *Lett. Appl. Microbiol.* 48, 486–492. doi: 10.1111/j.1472-765X.2009.02560.x

Hanajima, D., Fukumoto, Y., Yasuda, T., Suzuki, K., Maeda, K., and Morioka, R. (2011). Bacterial community dynamics in aerated cow manure slurry at different aeration intensities. *J. Appl. Microbiol.* 111, 1416–1425. doi: 10.1111/j.1365-2672.2011.05151.x

Hanajima, D., Haruta, S., Hori, T., Ishii, M., Haga, K., and Igarashi, Y. (2009). Bacterial community dynamics during reduction of odorous compounds in aerated pig manure slurry. *J. Appl. Microbiol.* 106, 118–129. doi: 10.1111/j.1365-2672.2008.03984.x

Hori, T., Haruta, S., Ueno, Y., Ishii, M., and Igarashi, Y. (2006). Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. *Appl. Environ. Microbiol.* 72, 1623–1630. doi: 10.1128/AEM.72.2.1623-1630.2006

Hori, T., Noll, M., Igarashi, Y., Friedrich, M. W., and Conrad, R. (2007). Identification of acetate-assimilating microorganisms under methanogenic conditions in anoxic rice field soil by comparative stable isotope probing of RNA. *Appl. Environ. Microbiol.* 73, 101–109. doi: 10.1128/AEM.01676-06

Hwang, O. H., Raveendar, S., Kim, Y. J., Kim, J. H., Kim, T. H., Choi, D. Y., et al. (2014). Deodorization of pig slurry and characterization of bacterial diversity using 16S rDNA sequence analysis. *J. Microbiol.* 52, 1056–1056. doi: 10.1007/s12275-014-4251-5

Imachi, H., Aoi, K., Tasumi, E., Saito, Y., Yamanaka, Y., Saito, Y., et al. (2011). Cultivation of methanogenic community from subseafloor sediments using a continuous-flow bioreactor. *ISME J.* 5, 1913–1925. doi: 10.1038/ismej.2011.64

Ishii, K., and Takii, S. (2003). Comparison of microbial communities in four different composting processes as evaluated by denaturing gradient gel electrophoresis analysis. *J. Appl. Microbiol.* 95, 109–119. doi: 10.1046/j.1365-2672.2003.01949.x

Japan Livestock Industry Association (1989). *Facility of Liquid Composting.* Tokyo: Japan Livestock Industry Association.

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., and Igarashi, Y. (2005). Stable coexistence of five bacterial strains as a cellulose-degrading community. *Appl. Environ. Microbiol.* 71, 7099–7106. doi: 10.1128/AEM.71.11.7099-7106.2005

Kato, S., Yoshida, R., Yamaguchi, T., Sato, T., Yumoto, I., and Kamagata, Y. (2014). The effects of elevated CO_2 concentration on competitive interaction between aceticlastic and syntrophic methanogenesis in a model microbial consortium. *Front. Microbiol.* 5:575. doi: 10.3389/fmicb.2014.00575

Kindler, R., Miltner, A., Richnow, H. H., and Kastner, M. (2006). Fate of gram-negative bacterial biomass in soil—mineralization and contribution to SOM. *Soil Biol. Biochem.* 38, 2860–2870. doi: 10.1016/j.soilbio.2006.04.047

Kube, M., Siewert, C., Migdoll, A. M., Duduk, B., Holz, S., Rabus, R., et al. (2014). Analysis of the complete genomes of *Acholeplasma brassicae*. *A. palmae* and *A. laidlawii* and their comparison to the obligate parasites from 'Candidatus *Phytoplasma*'. *J. Mol. Microbiol. Biotechnol.* 24, 19–36. doi: 10.1159/000354322

Lazarev, V. N., Levitskii, S. A., Basovskii, Y. I., Chukin, M. M., Akopian, T. A., Vereshchagin, V. V., et al. (2011). Complete genome and proteome of *Acholeplasma laidlawii*. *J. Bacteriol.* 193, 4943–4953. doi: 10.1128/JB.05059-11

Lee, C. G., Watanabe, T., Sato, Y., Murase, J., Asakawa, S., and Kimura, M. (2011). Bacterial populations assimilating carbon from ^{13}C-labeled plant residue in soil: analysis by a DNA-SIP approach. *Soil Biol. Biochem.* 43, 814–822. doi: 10.1016/j.soilbio.2010.12.016

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., et al. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* 19, 554–568. doi: 10.1002/elps.1150190416

Lueders, T., Kindler, R., Miltner, A., Friedrich, M. W., and Kaestner, M. (2006). Identification of bacterial micropredators distinctively active in a soil microbial food web. *Appl. Environ. Microbiol.* 72, 5342–5348. doi: 10.1128/AEM.00400-06

Lueders, T., Manefield, M., and Friedrich, M. W. (2004). Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ. Microbiol.* 6, 73–78. doi: 10.1046/j.1462-2920.2003.00536.x

Masui, N., Morono, Y., and Inagaki, F. (2008). Microbiological assessment of circulation mud fluids during the first operation of riser drilling by the deep-earth research vessel Chikyu. *Geomicrobiol. J.* 25, 274–282. doi: 10.1080/01490450802258154

McIvor, R. S., and Kenny, G. E. (1978). Differences in incorporation of nucleic acid bases and nucleosides by various *Mycoplasma* and *Acholeplasma* species. *J. Bacteriol.* 135, 483–489.

Murase, J., Hordijk, K., Tayasu, I., and Bodelier, P. L. (2011). Strain-specific incorporation of methanotrophic biomass into eukaryotic grazers in a rice field soil revealed by PLFA-SIP. *FEMS Microbiol. Ecol.* 75, 284–290. doi: 10.1111/j.1574-6941.2010.01007.x

Noll, M., Matthies, D., Frenzel, P., Derakshani, M., and Liesack, W. (2005). Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ. Microbiol.* 7, 382–395. doi: 10.1111/j.1462-2920.2005.00700.x

Razin, S., Tully, J. G., Rose, D. L., and Barile, M. F. (1983). DNA cleavage patterns as indicators of genotypic heterogeneity among strains of *Acholeplasma* and *Mycoplasma* species. *J. Gen. Microbiol.* 129, 1935–1944.

Razin, S., Yogev, D., and Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev.* 62, 1094–1156.

Saito, Y., Silvius, J. R., and McElhaney, N. (1977). Membrane lipid biosynthesis in *Acholeplasma laidlawii* B: de novo biosynthesis of saturated fatty acids by growing cells. *J. Bacteriol.* 132, 497–504.

Sasaki, Y., Ishikawa, J., Yamashita, A., Oshima, K., Kenri, T., Furuya, K., et al. (2002). The complete genomic sequence of *Mycoplasma penetrans*, an intracellular bacterial pathogen in humans. *Nucl. Acids Res.* 30, 5293–5300. doi: 10.1093/nar/gkf667

Sneath, P. H. A. (1986). "Endospore-forming Gram-Positive Rods and Cocci," in *Bergey's Manual of Systematic Bacteriology*, Vol.2, eds P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (Baltimore: Williams & Wilkins Co), 1104–1207.

Stipkovits, L., Romvary, J., Nagy, Z., Bodon, L., and Varga, L. (1974). Studies on the pathogenicity of *Acholeplasma axanthum* in swine. *J. Hyg.* 72, 289–296. doi: 10.1017/S0022172400023500

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Tully, J. G., Whitcomb, R. F., Rose, D. L., Bove, J. M., Carle, P., Somerson, N. L., et al. (1994). *Acholeplasma brassicae* sp. nov. and *Acholeplasma palmae* sp. nov., two non-sterol-requiring mollicutes from plant surfaces. *Int. J. System. Bacteriol.* 44, 680–684. doi: 10.1099/00207713-44-4-680

Vanyushkina, A. A., Fisunov, G. Y., Gorbachev, A. Y., Kamashev, D. E., and Govorun, V. M. (2014). Metabolomic analysis of three mollicute species. *PLoS ONE* 9:e89312. doi: 10.1371/journal.pone.0089312

Vasconcelos, A. T. R. (2005). Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*. *J. Bacteriol.* 187, 7548–7548. doi: 10.1128/JB.187.16.5568-5577.2005

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wright, E. S., Yilmaz, L. S., and Noguera, D. R. (2012). DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl. Environ. Microbiol.* 78, 717–725. doi: 10.1128/AEM.06516-11

Zhu, J. (2000). A review of microbiology in swine manure odor control. *Agric. Ecosyst. Environ.* 78, 93–106. doi: 10.1016/S0167-8809(99)00116-4

The effects of elevated CO_2 concentration on competitive interaction between aceticlastic and syntrophic methanogenesis in a model microbial consortium

Souichiro Kato[1,2,3] *, **Rina Yoshida**[4], **Takashi Yamaguchi**[4], **Tomoyuki Sato**[1], **Isao Yumoto**[1] **and Yoichi Kamagata**[1,2]

[1] Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Sapporo, Japan
[2] Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan
[3] Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan
[4] Department of Civil and Environmental Engineering, Nagaoka University of Technology, Nagaoka, Japan

Investigation of microbial interspecies interactions is essential for elucidating the function and stability of microbial ecosystems. However, community-based analyses including molecular-fingerprinting methods have limitations for precise understanding of interspecies interactions. Construction of model microbial consortia consisting of defined mixed cultures of isolated microorganisms is an excellent method for research on interspecies interactions. In this study, a model microbial consortium consisting of microorganisms that convert acetate into methane directly (*Methanosaeta thermophila*) and syntrophically (*Thermacetogenium phaeum* and *Methanothermobacter thermautotrophicus*) was constructed and the effects of elevated CO_2 concentrations on intermicrobial competition were investigated. Analyses on the community dynamics by quantitative RT-PCR and fluorescent *in situ* hybridization targeting their 16S rRNAs revealed that high concentrations of CO_2 have suppressive effects on the syntrophic microorganisms, but not on the aceticlastic methanogen. The pathways were further characterized by determining the Gibbs free energy changes (ΔG) of the metabolic reactions conducted by each microorganism under different CO_2 concentrations. The ΔG value of the acetate oxidation reaction (*T. phaeum*) under high CO_2 conditions became significantly higher than -20 kJ per mol of acetate, which is the borderline level for sustaining microbial growth. These results suggest that high concentrations of CO_2 undermine energy acquisition of *T. phaeum*, resulting in dominance of the aceticlastic methanogen. This study demonstrates that investigation on model microbial consortia is useful for untangling microbial interspecies interactions, including competition among microorganisms occupying the same trophic niche in complex microbial ecosystems.

Keywords: model consortia, methanogenesis, acetate, thermodynamics, CO_2 concentration

INTRODUCTION

In natural and engineered environments, many species of microorganisms coexist by interacting with each other. Comprehension of interspecies interactions is essential for describing the features of complex microbial ecosystems, and competition among microorganisms occupying similar trophic niches is a conventional and significant aspect of such interspecies interaction. Coexistence of multiple microorganisms with similar trophic niches is regarded as one of the major factors to confer functional stability and resiliency on microbial ecosystems (Loreau et al., 2001; Deng, 2012). It is important to grasp how the population of each microorganism changes depending on a specific environmental disturbance. Most microbial ecological research has assessed the effects of specific environmental factors on competitive interactions among multiple microbial species by observing the transition of abundances of each microorganism responding to environmental disturbances. Although this approach has produced

many excellent outcomes, existence of non-target microorganisms and uncontrollable environmental factors in the systems often hamper precise understanding of the effects of specific environmental factors on the competitive interactions among target microorganisms.

Construction of microbial model consortia, in which interspecies interactions in ecosystems are reproduced by defined co-culture of isolated microorganisms, is appreciated as a worthwhile method to investigate microbial interactions (Haruta et al., 2009; De Roy et al., 2014; Großkopf and Soyer, 2014). For instance, the complex phenomenon of bacterial competition as being similar to rock-paper-scissors among colisin-producing, colisin-resistant, and colisin-sensitive strains was untangled by constructing model co-culture systems (Kerr et al., 2002; Nahum et al., 2011). Kato et al. (2005, 2008) constructed model microbial consortia composed of 4–5 bacterial strains, in which all members stably coexisted for long period of time, and demonstrated

that existence of both positive and negative interspecies inter-actions among the members make these consortia stable. The construction of model consortia is a specific and beneficial feature of microbiological research fields, which will also be effective for proof-of-concept studies for theories in the field of macro-ecology (Haruta et al., 2009, 2013).

Methanogenesis from organic compounds is a complex microbial process accomplished by catabolic interactions among different trophic levels of microorganisms (Schink, 1997; Jones et al., 2008; Kato and Watanabe, 2010). Among the sequential biodegradation processes, acetate is the most important intermediary metabolite (Schink, 1997). Methanogenic acetate degradation proceeds by either aceticlastic methanogenesis or syntrophic acetate oxidation. The aceticlastic pathway is solely mediated by aceticlastic methanogens (Jetten et al., 1992). On the contrary, syntrophic acetate oxidation pathway requires cooperative interactions of two different types of microorganisms: acetate is first oxidized to H_2 and CO_2 by syntrophic acetate-oxidizing bacteria (SAOB), and then hydrogenotrophic methanogens convert the products to CH_4 (Zinder and Koch, 1984). As the acetate oxidation reaction is endoergic under the standard conditions and is feasible only under extremely low H_2 partial pressure, acetate oxidation by SAOB requires H_2 elimination by hydrogenotrophic methanogens (Karakashev et al., 2006; Hattori, 2008). These two different acetate-degrading methane-producing pathways and organisms involved can co-exist, but diverse environmental factors, such as temperature, pH, salinity, toxic compounds, and concentrations of substrates determine one pathway and organisms to dominate over the other (Nüsslein et al., 2001; Shigematsu et al., 2004; Karakashev et al., 2006; Hao et al., 2013; Kato et al., 2014).

In our previous studies, we demonstrated that the syntrophic pathway is the dominant methanogenic acetate degradation pathway in underground, thermophilic petroleum reservoirs (Mayumi et al., 2011). We further demonstrated that aceticlastic pathway becomes dominant under high CO_2 concentrations, which mimicked carbon capture and storage field conditions (Mayumi et al., 2013), whereas syntrophic acetate oxidation dominated over aceticlastic reactions under low CO_2 concentrations. Since CO_2 is either substrate or product of aceticlastic methanogenesis, acetate oxidation, and hydrogenotrophic methanogenesis, high CO_2 concentration alters the thermodynamics of each methanogenic reaction, which may cause the observed transition between syntrophic and aceticlastic methanogenic pathways. However, all the data were based on the analyses of complex microbial communities in field samples thus many other factors that affect the community shift could not be ruled out.

In the present study, the effect of CO_2 concentrations on methanogenic microorganisms were assessed by using a defined inorganic medium and a defined methanogenic consortium which is comprised of three organisms, i.e., SAOB, hydrogenotrophic methanogen and aceticlastic methanogen, namely, which contains two different acetate-degrading methanogenic pathways. The experiments allowed to precisely show the CO_2 concentrations to be a crucial factor affecting the dominance of respective pathways and organisms.

MATERIALS AND METHODS

MICROORGANISMS AND CULTURE CONDITIONS

Methanosaeta thermophila DSM6194[T] (Kamagata and Mikami, 1991) and *Thermacetogenium phaeum* DSM12270[T] (Hattori et al., 2000) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkukuren GmbH (Braunschweig, Germany). *Methanothermobacter thermautotrophicus* strain TM was isolated from a thermophilic anaerobic methanogenic reactor in Japan (Hattori et al., 2000). Routine cultivations were conducted at 55°C with 68-ml capacity serum vials containing 20 ml of a bicarbonate-buffered inorganic medium (pH 7.0; Kato et al., 2014) under an atmosphere of N_2-CO_2 [80/20 (v/v)] without shaking. Pyruvate (40 mM) or 200 kPa H_2-CO_2 [80/20 (v/v)] was supplemented as energy and carbon sources for the pure cultures of *T. phaeum* and *Methanothermobacter thermautotrophicus*, respectively. Sodium acetate (40 mM) was utilized as an energy and carbon source for the pure culture of *Methanosaeta thermophila*, the defined co-culture of *T. phaeum* and *Methanothermobacter thermautotrophicus*, and the tri-culture of the three strains. The tri-culture was constructed by simultaneously inoculating 1 and 2 ml of the early-stationary phases of pure culture of *Methanosaeta thermophila* and the defined co-culture of *T. phaeum* and *Methanothermobacter thermautotrophicus*, respectively, into the 20 ml of the medium. Although the long term stability of the tri-culture was not been tested, coexistence of the three microorganisms in the batch culture was confirmed.

CULTURES WITH DIFFERENT CO_2 CONCENTRATIONS

Three culture conditions were prepared to examine the effects of CO_2 concentrations on the microorganisms. For each condition, the media were supplemented with different concentrations of sodium bicarbonate and the gas phases were replaced with N_2/CO_2 mixed gas with different volume ratios, as described in **Table 1**. The medium was bubbled with the respective deoxygenated gas with 100 ml min^{-1} for 5 min and immediately capped with a butyl rubber stopper and an aluminum cap. The medium pH was adjusted to 7.0 by adding 1N NaOH solution before the cultivation, and the fluctuation of pH value throughout the cultivation was less than 0.2. For pH measurement, 100 μl of the medium was sampled with syringes and the pH value was determined using a compact pH meter B-212 (Horiba). The concentration of CO_2 in the aqueous phase [c_{aq} (M)] was calculated according to Henry's law ($c_{aq} = kp$), where k is the Henry's low constant (0.019 for CO_2 at 55°C) and p is the partial pressure of CO_2 in the gas phase (atm). Then the bicarbonate concentrations were calculated based on the equilibrium formula ($H_2CO_3 = H^+ + HCO_3^-$) with the

Table 1 | Media with different initial [ΣCO_2] used in this study.

[ΣCO_2]$_{initial}$ (mmol l^{-1})	NaHCO$_3$ added (mM)	Partial pressure of the gas phase CO_2 (atm)	Calculated [HCO_3^-]$_{initial}$ (mM)
5.0	5	0	0.8
50.7	35	0.2	8.1
113.4	35	1	18.1

equilibrium constant of 4.47×10^7. The culture experiments were conducted in triplicate and the student's t-test was used for the statistical analyses.

Growth of *Methanosaeta thermophila* and *Methanothermobacter thermautotrophicus* in pure and mixed cultures was determined by measuring methane production. Growth of *T. phaeum* pure culture was determined by measuring acetate production from pyruvate. The partial pressure of CH_4 was determined using a gas chromatograph GC-2014 (Shimadzu) as described previously (Kato et al., 2014). The partial pressure of H_2 was determined using a trace reduction gas analyzer TRA-1000 (ACE Inc.) according to the manufacturer's instruction. The concentrations of organic acids were determined using a high performance liquid chromatography (D-2000 LaChrom Elite HPLC system, HITACHI) equipped with Aminex HPX-87H Ion Exclusion column (BIO-RAD) and L2400 UV detector (HITACHI).

FLUORESCENT *IN SITU* HYBRIDIZATION (FISH)

Microbial cells of the tri-cultures in the early stationary phases were collected by centrifugation, fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) and left for 6 h at 4°C. The samples were washed three times with PBS, immobilized on glass slides, and dehydrated by successive passages through 50, 70, 80, 90, and 100% ethanol (3 min each). The following oligonucleotide probes complementary to specific regions of 16S rRNA were utilized for hybridizations: (i) Alexa488-labeled EUB338, specific for the domain *Bacteria* (Amann et al., 1990) and (ii) TexRed-labeled ARCH917, specific for the domain *Archaea* (Loy et al., 2002), (iii) Alexa594-labeled MSMX860, specific for the order *Methanosarcinales* (Raskin et al., 1994), and (iv) Alexa488-labeled MB311, specific for the order *Methanobacteriales* (Crocetti et al., 2006). Hybridizations were performed at 46°C for 3 h with hybridization buffer (0.9 M NaCl, 0.1 M Tris-HCl, pH 7.5) containing 5 ng μl^{-1} of each labeled probe. The specificity of each probe was confirmed by FISH observations using pure cultures of the three microorganisms used in this study even with the hybridization buffer not containing formamide. The washing step was done at 48°C for 30 min with washing buffer (0.2 M NaCl, 0.1 M Tris-HCl, pH 7.5). The samples hybridized with the probes were observed with a fluorescent microscope Provis AX70 (Olympus).

QUANTITATIVE RT-PCR (qRT-PCR)

Microbial cells were harvested from the mid-logarithmic phases by centrifugation at 10,000 X g and 4°C. Total RNA was isolated using ISOGEN II reagent (Nippon Gene, Japan) combined with a bead-beating method, as described previously (Kato et al., 2014). Total RNA was purified using an RNeasy Mini kit (Qiagen) with DNase treatment (RNase-free DNase set, Qiagen) as described in the manufacturer's instructions. The purified RNA was spectroscopically quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The PCR primers used for quantitative RT-PCR (qRT-PCR) were designed with Primer3 software (http://simgene.com/Primer3) and are listed in **Table 2**. Quantification of 16S rRNA copy numbers in the defined mixed culture

Table 2 | Quantitative RT-PCR (qRT-PCR) primers designed and used in this study.

Primer name	Sequence (5′–3′)	Target
PT387f	GATAAGGGGACCTCGAGTGCT	*Methanosaeta thermophila*
PT573r	GGCCGGCTACAGACCCT	*Methanosaeta thermophila*
PB486f	ACGGGACGAAGGGAGTGACGG	*Thermacetogenium phaeum*
PB646r	CTCCTCCCCTCAAGTCATCCAGT	*Thermacetogenium phaeum*
TM1139f	TTACCAGCGGAACCCTTATGG	*Methanothermobacter thermautotrophicus*
TM1275r	ACCTGGTTTAGGGGATTACCTCC	*Methanothermobacter thermautotrophicus*

were performed by one-step real-time RT-PCR using a Mx3000P QPCR System (Stratagene) and RNA-direct SYBR Green Realtime PCR Master Mix (Toyobo) as described previously (Kato et al., 2014). At least three biological replicates were subjected to qRT-PCR analysis, and at least two separate trials were conducted for each sample. Standard curves were generated with serially diluted PCR products (10^3–10^8 copies ml^{-1}) amplified using the respective primer sets and were used to calculate the copy number of rRNA in the total RNA samples.

RESULTS AND DISCUSSION

EFFECTS OF CO_2 CONCENTRATIONS ON THE MODEL METHANOGENIC CONSORTIUM
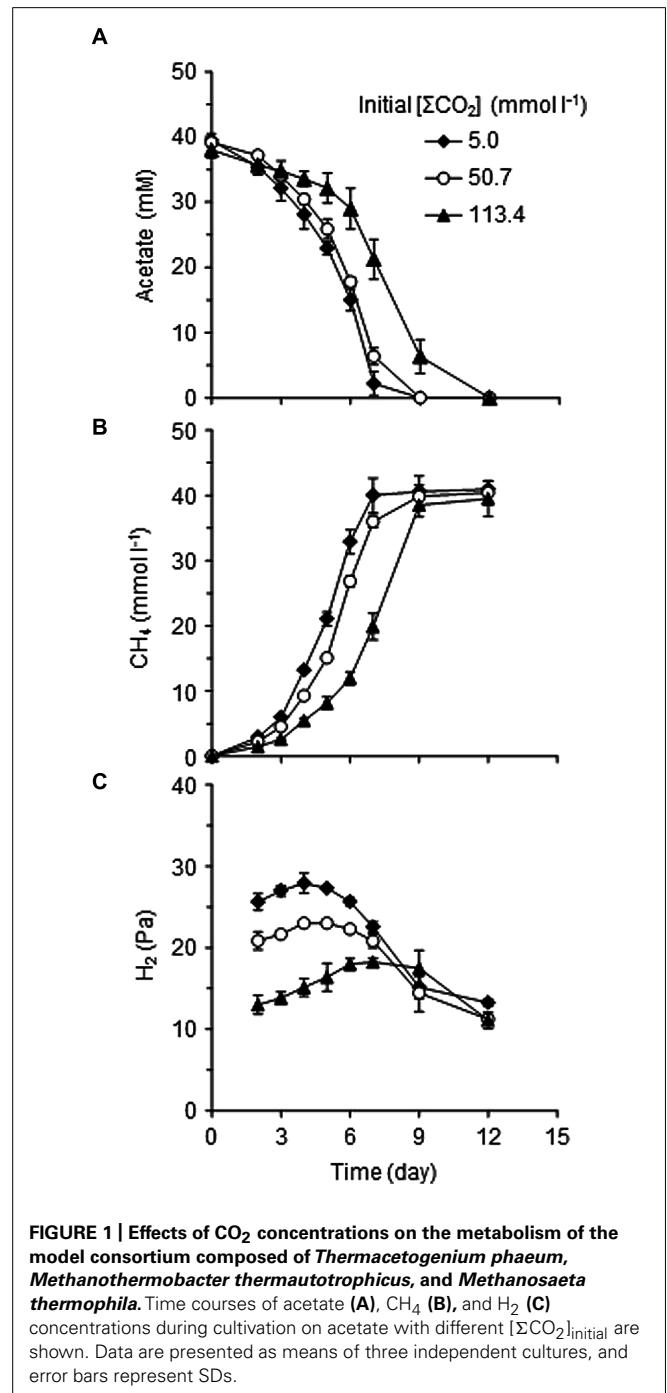
As the model consortium performing methanogenic acetate degradation, we utilized a defined mixed culture of an aceticlastic methanogen (*Methanosaeta thermophila*), a hydrogenotrophic methanogen (*Methanothermobacter thermautotrophicus*), and a SAOB (*T. phaeum*; **Table 3**). These microbial species were originally isolated from a thermophilic methanogenic digester (Kamagata and Mikami, 1991; Hattori et al., 2000) and are regarded as representative species for the methanogenic acetate degradation reactions that occur in various natural environments such as high-temperature petroleum reservoirs (Pham et al., 2009; Mayumi et al., 2011, 2013) and thermophilic methanogenic digesters (Sekiguchi et al., 1998; McHugh et al., 2003; Hori et al., 2011).

To adequately assess the effects of CO_2 concentration itself, media with different supplementation of CO_2/HCO_3^- were prepared (**Table 1**). The initial concentrations of total CO_2/HCO_3^- in the cultures, designated as $[\Sigma CO_2]_{initial}$, were 5.0, 50.7, or 113.4 mmol l^{-1}. The model consortium composed of *Methanosaeta thermophila*, *Methanothermobacter thermautotrophicus*, and *T. phaeum* was cultivated under the three different $[\Sigma CO_2]_{initial}$ conditions to evaluate their methanogenic acetate degradation abilities (**Figure 1**). A stoichiometric production of CH_4 from acetate in a 1:1 molar ratio was observed in all culture conditions tested. Both acetate consumption and CH_4 production rates slightly decreased with increasing the $[\Sigma CO_2]_{initial}$ (**Figures 1A,B**). Interestingly, the partial pressure of H_2, which is an important intermediate of syntrophic

Table 3 | The metabolic reactions and the respective standard Gibbs free energy changes ($\Delta G^{\circ\prime}$) of the microorganisms utilized in this study.
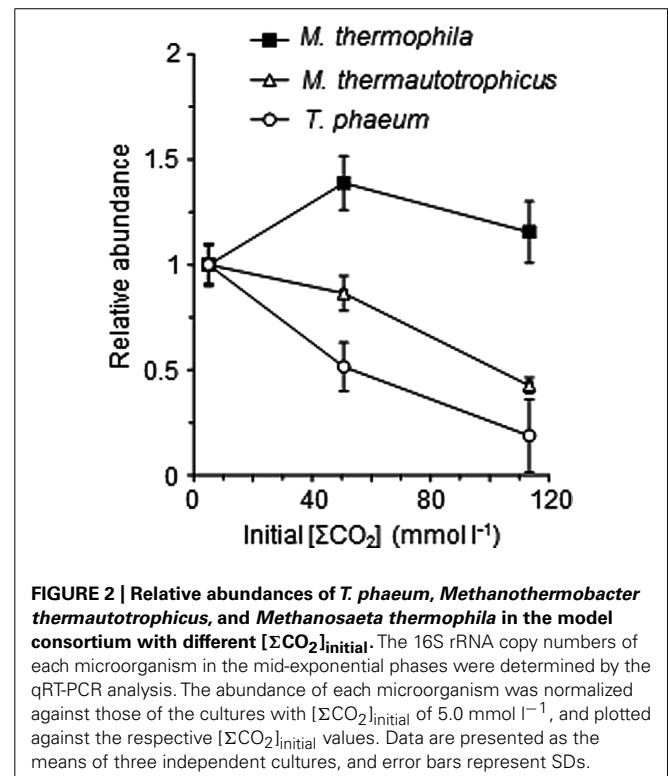
Microbial species	Metabolic reactions	$\Delta G^{\circ\prime}$ (kJ mol^{-1})[a]
Methanosaeta thermophila	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
Thermacetogenium phaeum	$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$	$+104.6$
Methanothermobacter thermautotrophicus	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6

[a]The $\Delta G^{\circ\prime}$ values were calculated according to the reference (*Thauer et al., 1977*).

FIGURE 1 | Effects of CO$_2$ concentrations on the metabolism of the model consortium composed of *Thermacetogenium phaeum*, *Methanothermobacter thermautotrophicus*, and *Methanosaeta thermophila*. Time courses of acetate **(A)**, CH$_4$ **(B)**, and H$_2$ **(C)** concentrations during cultivation on acetate with different [ΣCO_2]$_{initial}$ are shown. Data are presented as means of three independent cultures, and error bars represent SDs.

acetate degradation, significantly decreased with increasing the [ΣCO_2]$_{initial}$ (**Figure 1C**). This observation suggests that syntrophic methanogenic microorganisms are influenced by elevated CO$_2$ concentrations.

To assess the influence of the elevated CO$_2$ concentrations on each methanogenic pathway, the relative abundances of each microorganism in the exponentially growing cultures of the model consortium with the different [ΣCO_2]$_{initial}$ were evaluated by FISH and qRT-PCR analyses. The qRT-PCR analysis clearly demonstrated the decrease of the abundances of *Methanothermobacter thermautotrophicus* and *T. phaeum* in the higher [ΣCO_2]$_{initial}$ cultures (**Figure 2**). The FISH analysis also demonstrated that the relative abundances of *Methanothermobacter thermautotrophicus* and *T. phaeum* in the cultures with higher CO$_2$ concentrations are significantly lower than those in the low CO$_2$ cultures (**Figure 3**). These results indicate that the syntrophic methanogenic pathway is more strongly influenced by the elevation of CO$_2$ concentrations compared to the aceticlastic pathway.

FIGURE 2 | Relative abundances of *T. phaeum*, *Methanothermobacter thermautotrophicus*, and *Methanosaeta thermophila* in the model consortium with different [ΣCO_2]$_{initial}$. The 16S rRNA copy numbers of each microorganism in the mid-exponential phases were determined by the qRT-PCR analysis. The abundance of each microorganism was normalized against those of the cultures with [ΣCO_2]$_{initial}$ of 5.0 mmol l^{-1}, and plotted against the respective [ΣCO_2]$_{initial}$ values. Data are presented as the means of three independent cultures, and error bars represent SDs.

FIGURE 3 | Fluorescent microscopic images of the three-strains consortium cultivated with the $[\Sigma CO_2]_{initial}$ of 5.0 (A) or 113.4 mmol l^{-1} (B). The exponential phase cultures were subjected to *in situ* hybridization with the probes described in the Materials and Methods section. Red, *Methanosaeta thermophila* (hybridized with Archaea917-TexRed and MSMX860-Alexa594); Yellow, *Methanothermobacter thermautotrophicus* (hybridized with Archaea917-TexRed and MB311-Alexa488); Green, *T. phaeum* (hybridized with EUB338-Alexa488). Bars = 10 μm.

EFFECTS OF CO_2 CONCENTRATIONS ON THE ACETICLASTIC AND SYNTROPHIC PATHWAYS

To confirm the differences in the suppressive effects of elevated CO_2 concentrations on the two methanogenic pathways, the pure culture of *Methanosaeta thermophila* and the defined co-culture of *Methanothermobacter thermautotrophicus* and *T. phaeum* were separately cultivated in the media with the different $[\Sigma CO_2]_{initial}$ (**Figure 4**). The growth of *Methanosaeta thermophila* was barely affected by the elevated CO_2 concentration: the methanogenic rate in the $[\Sigma CO_2]_{initial}$ of 113.4 mmol l^{-1} cultures decreased only about 10% compared to the cultures with $[\Sigma CO_2]_{initial}$ of 5.0 mmol l^{-1} (**Figures 4A,C**). On the contrary, the methanogenic rate of the syntrophic co-culture in the $[\Sigma CO_2]_{initial}$ of 113.4 mmol l^{-1} dropped to less than half of that in the cultures with $[\Sigma CO_2]_{initial}$ of 5.0 mmol l^{-1} (**Figures 4B,C**). These observations confirm the assumption that the syntrophic acetate degradation pathway is more susceptible to elevated CO_2 concentrations than the aceticlastic pathway.

EFFECTS OF CO_2 CONCENTRATIONS ON THE PURE CULTURES OF *Methanothermobacter thermautotrophicus* AND *T. phaeum*

One possible explanation for the suppressive effects of CO_2 on the syntrophic methanogenesis is the susceptibility of
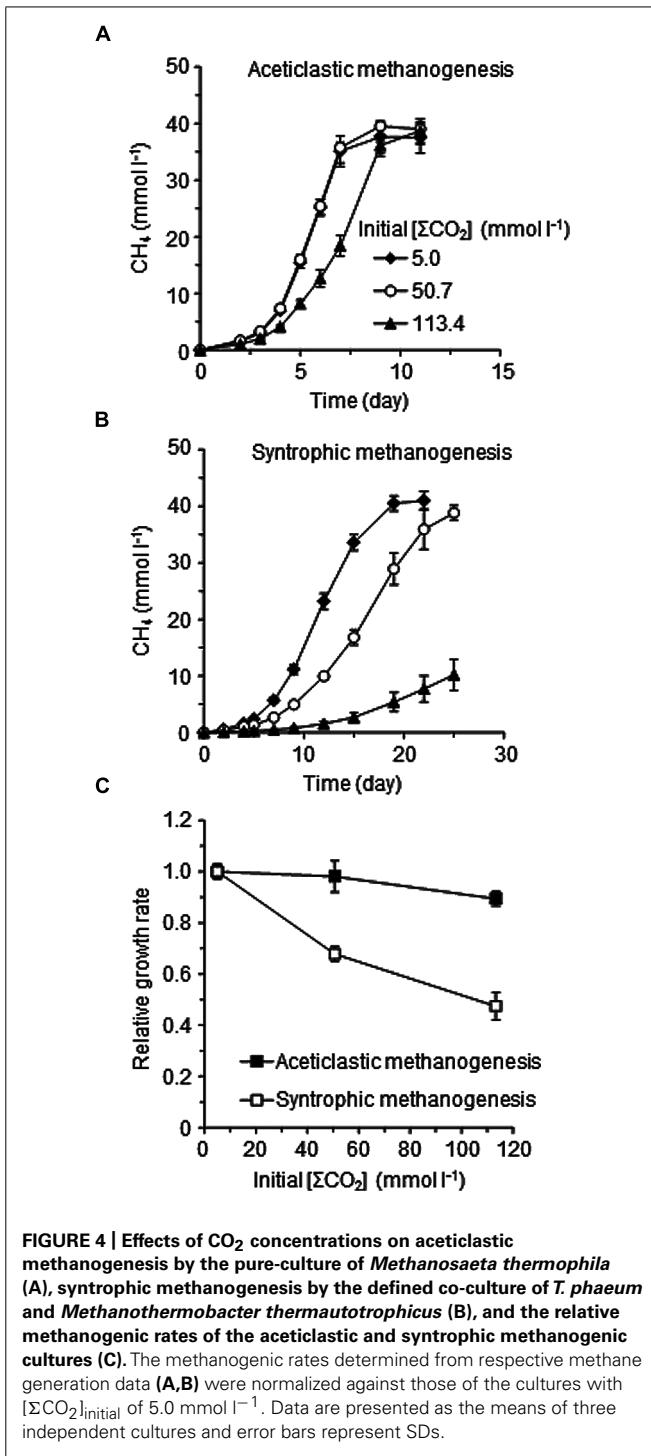
FIGURE 4 | Effects of CO_2 concentrations on aceticlastic methanogenesis by the pure-culture of *Methanosaeta thermophila* (A), syntrophic methanogenesis by the defined co-culture of *T. phaeum* and *Methanothermobacter thermautotrophicus* (B), and the relative methanogenic rates of the aceticlastic and syntrophic methanogenic cultures (C). The methanogenic rates determined from respective methane generation data **(A,B)** were normalized against those of the cultures with $[\Sigma CO_2]_{initial}$ of 5.0 mmol l^{-1}. Data are presented as the means of three independent cultures and error bars represent SDs.

Methanothermobacter thermautotrophicus and/or *T. phaeum* to some environmental alterations induced by increased CO_2 or to CO_2 itself. To evaluate this possibility, pure cultures of *Methanothermobacter thermautotrophicus* and *T. phaeum* were cultivated in media with different $[\Sigma CO_2]_{initial}$ (**Figure 5**). No significant differences were observed for the growth of both *Methanothermobacter thermautotrophicus* and *T. phaeum* under the different CO_2 conditions tested. These results suggest that elevated CO_2
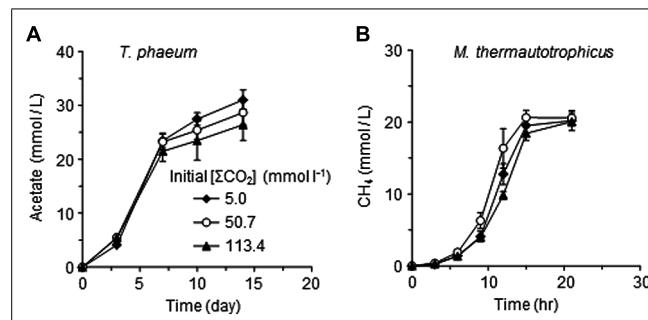
FIGURE 5 | Effects of CO$_2$ concentrations on the pure cultures of *T. phaeum* (A) and *Methanothermobacter thermautotrophicus* (B). Data are presented as the means of three independent cultures, and error bars represent SDs.

concentrations negatively affect the microbial activity only when *Methanothermobacter thermautotrophicus* and *T. phaeum* are in a syntrophic relationship.

EFFECTS OF CO$_2$ CONCENTRATIONS ON THE THERMODYNAMICS OF EACH REACTION

The other possible explanation for the suppression of syntrophic methanogenesis by elevated CO$_2$ concentration is alterations of thermodynamic conditions of each microbial reaction. A minimum energy required for biochemical energy conversion is estimated at around -20 kJ mol^{-1} (Schink, 1997), while some anaerobic microorganisms have been reported to thrive under more thermodynamically restricted conditions (Jackson and McInerney, 2002; Nauhaus et al., 2002). The value was estimated from the energetics of ATP formation (around -70 kJ mol^{-1} under the physiological conditions; Jetten et al., 1991; Tran and Unden, 1998) and the number of protons transported to ATP formation (between 3 and 4; Maloney, 1983; Stock et al., 1999). Since syntrophic methanogenesis from acetate

is one of the least exergonic microbial metabolisms (Schink, 1997), it is no wonder that only slight perturbations on the thermodynamics induce deteriorations of the syntrophic methanogenesis.

To evaluate the influences of elevated CO$_2$ concentrations on the thermodynamic properties, ΔG values of metabolic reactions conducted by each microorganism in the model consortium were determined using the data-set of metabolite concentrations shown in **Figure 1**. The ΔG values of the aceticlastic methanogenesis conducted by *Methanosaeta thermophila* were not significantly influenced by the elevated CO$_2$ concentrations (**Figure 6**). The average ΔG values during the logarithmic growth phase (day 2–5) with the $[\Sigma CO_2]_{initial}$ of 5.0, 50.7 and 113.4 mmol l^{-1} were -47.7 ± 3.5, -44.9 ± 2.6, and -44.6 ± 2.0 kJ mol^{-1}, respectively, which are substantially lower than the ΔG value required for microbial energy acquisition.

The ΔG values of the hydrogenotrophic methanogenesis catalyzed by *Methanothermobacter thermautotrophicus* were also largely not altered with different CO$_2$ settings and were constantly lower than -20 kJ mol^{-1} (**Figure 6**). The average ΔG values during the logarithmic growth phases with the $[\Sigma CO_2]_{initial}$ of 5.0, 50.7, and 113.4 mmol l^{-1} were -24.6 ± 1.0, -27.2 ± 1.0, and -26.0 ± 1.2 kJ mol^{-1}, respectively. Since CO$_2$ is the substrate for hydrogenotrophic methanogenesis, lower ΔG values under the higher CO$_2$ conditions are expected. However, the decrease in H$_2$ partial pressures under the higher CO$_2$ conditions (**Figure 1C**) compensates for the positive effects of increase in CO$_2$ concentration.

On the contrary, elevation of CO$_2$ concentrations significantly influenced the ΔG values of the acetate oxidation reaction performed by *T. phaeum* (**Figure 6**). While the average ΔG value during the logarithmic growth phases with the $[\Sigma CO_2]_{initial}$ of 5.0 mmol l^{-1} (-23.1 ± 2.7 kJ mol^{-1}) was less than the borderline ΔG value of -20 kJ mol^{-1}, those with the $[\Sigma CO_2]_{initial}$ of 50.7 and 113.4 mmol l^{-1} (-17.8 ± 1.3 and -18.7 ± 1.4 kJ
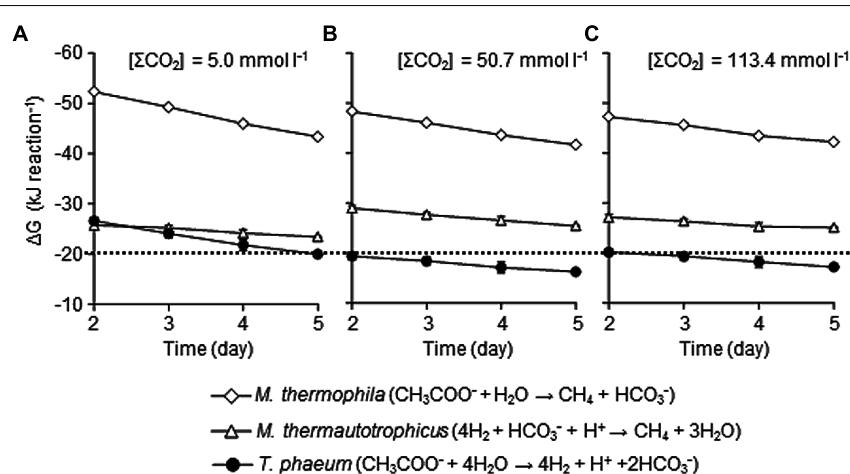
FIGURE 6 | Effects of CO$_2$ concentrations on the Gibbs free energy change (ΔG) of metabolisms of each microorganism under the conditions with the $[\Sigma CO_2]_{initial}$ of 5.0 (A), 50.7 (B), or 113.4 mmol l^{-1} (C). The ΔG values were calculated using metabolite concentration data presented in **Figure 1**. The dotted line represents ΔG value of -20 kJ/reaction. Data are presented as the means of three independent cultures and error bars represent SDs.

mol^{-1}, respectively) exceeded the borderline. As acetate oxidation reaction produces 2 mol of CO_2 from 1 mol of acetate, it is rational that this reaction is strongly influenced by the elevation of CO_2 concentration. The decrease in the partial pressure of H_2, the other metabolic product of acetate oxidation, is expected to compensate for the negative effects of increase in CO_2. However, the decrease in H_2 partial pressure would be limited by the minimum threshold for H_2 consumption by *Methanothermobacter thermautotrophicus*. The minimum thresholds for H_2 utilization by hydrogenotrophic methanogens have been reported as around 5–10 Pa (Lovley, 1985; Thauer et al., 2008). However, considering the energy required for active growth, H_2 partial pressure of around 10–15 Pa observed in the increased CO_2 conditions in this study may be the minimum H_2 threshold for the syntrophic interaction. Actually, if the H_2 partial pressure in the cultures with $[\Sigma CO_2]_{initial}$ of 113.4 mmol l^{-1} at the logarithmic growth phase (day 5) becomes 10 Pa, the ΔG value becomes > -20 kJ mol^{-1} (-19.7 ± 0.3 kJ mol^{-1}) from the actual value of -25.1 ± 1.4 kJ mol^{-1} (with H_2 partial pressure of 16.4 ± 1.7 Pa). These results clearly demonstrated that high concentrations of CO_2 thermodynamically constrain the acetate oxidizing reaction, which results in the deterioration of syntrophic methanogenesis from acetate.

CONCLUSION

This is the first paper to evaluate the influence of elevated CO_2 concentration on the two different methanogenic acetate degradation pathways, namely aceticlastic and syntrophic pathways, using a model microbial consortium. As expected from the observations based on *in situ* environments with complex microbial communities, high concentrations of CO_2 suppressed the syntrophic pathway rather than the aceticlastic pathway. Thermodynamic calculations revealed that the acetate oxidation reaction is more intensely constrained by elevated CO_2 concentrations. This study exemplified the importance of even slight changes in the ΔG values of microbial metabolisms in anaerobic biota. Furthermore, this study demonstrated that the construction of model microbial consortia is useful for assessing competitive interspecies interactions even in anaerobic, methanogenic environments.

AUTHOR CONTRIBUTIONS

Souichiro Kato, Tomoyuki Sato, and Yoichi Kamagata designed the research. Souichiro Kato, Rina Yoshida, Takashi Yamaguchi, Tomoyuki Sato, and Isao Yumoto carried out the experiments and analyzed the data. Souichiro Kato and Yoichi Kamagata wrote the manuscript.

ACKNOWLEDGMENTS

REFERENCES

Amann, R. I., Krumholz, L., and Stahl, D. A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172, 762–770.

Crocetti, G., Murto, M., and Björnsson, L. (2006). An update and optimization of oligonucleotide probes targeting methanogenic Archaea for use in fluorescence *in situ* hybridisation (FISH). *J. Microbiol. Methods* 65, 194–201. doi: 10.1016/j.mimet.2005.07.007

Deng, H. (2012). A review of diversity-stability relationship of soil microbial community: what do we not know? *J. Environ. Sci.* 24, 1027–1035. doi: 10.1016/S1001-0742(11)60846-2

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., and Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ. Microbiol.* 16, 1472–1481. doi: 10.1111/1462-2920.12343

Großkopf, T., and Soyer, O. S. (2014). Synthetic microbial communities. *Curr. Opin. Microbiol.* 18, 72–77. doi: 10.1016/j.mib.2014.02.002

Hao, L., Lü, F., Li, L., Wu, Q., Shao, L., and He, P. (2013). Self-adaption of methane-producing communities to pH disturbance at different acetate concentrations by shifting pathways and population interaction. *Bioresour. Technol.* 140, 319–327. doi: 10.1016/j.biortech.2013.04.113

Haruta, S., Kato, S., Yamamoto, K., and Igarashi, Y. (2009). Intertwined interspecies relationships: approaches to untangle the microbial network. *Environ. Microbiol.* 11, 2963–2969. doi: 10.1111/j.1462-2920.2009.01956.x

Haruta, S., Yoshida, T., Aoi, Y., Kaneko, K., and Futamata, H. (2013). Challenges for complex microbial ecosystems: combination of experimental approaches with mathematical modeling. *Microbes Environ.* 28, 285–294. doi: 10.1264/jsme2.ME13034

Hattori, S. (2008). Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ.* 23, 118–127. doi: 10.1264/jsme2.23.118

Hattori, S., Kamagata, Y., Hanada, S., and Shoun, H. (2000). *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. *Int. J. Syst. Evol. Microbiol.* 50, 1601–1609. doi: 10.1099/00207713-50-4-1601

Hori, T., Sasaki, D., Haruta, S., Shigematsu, T., Ueno, Y., Ishii, M., et al. (2011). Detection of active, potentially acetate-oxidizing syntrophs in an anaerobic digester by flux measurement and formyltetrahydrofolate synthetase (FTHFS) expression profiling. *Microbiology* 157, 1980–1989. doi: 10.1099/mic.0.049189-0

Jackson, B. E., and McInerney, M. J. (2002). Anaerobic microbial metabolism can proceed close to thermodynamic limits. *Nature* 415, 454–456. doi: 10.1038/415454a

Jetten, M. S. M., Stams, A. J. M., and Zehnder, A. J. B. (1991). Adenine nucleotide content and energy charge of *Methanothrix soehngenii* during acetate degradation. *FEMS Microbiol. Lett.* 84, 313–318. doi: 10.1111/j.1574-6968.1991.tb04616.x

Jetten, M. S. M., Stams, A. J. M., and Zehnder, A. J. B. (1992). Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. *FEMS Microbiol. Lett.* 88, 181–197. doi: 10.1111/j.1574-6968.1992.tb04987.x

Jones, D. M., Head, I. M., Gray, N. D., Adams, J. J., Rowan, A. K., Aitken, C. M., et al. (2008). Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* 451, 176–180. doi: 10.1038/nature06484

Kamagata, Y., and Mikami, E. (1991). Isolation and characterization of a novel thermophilic *Methanosaeta* strain. *Int. J. Syst. Bacteriol.* 41, 191–196. doi: 10.1099/00207713-41-2-191

Karakashev, D., Batstone, D. J., Trably, E., and Angelidaki, I. (2006). Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae. *Appl. Environ. Microbiol.* 72, 5138–5141. doi: 10.1128/AEM.00489-06

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., and Igarashi, Y. (2005). Stable coexistence of five bacterial strains as a cellulose-degrading community. *Appl. Environ. Microbiol.* 71, 7099–7106. doi: 10.1128/AEM.71.11.7099-7106.2005

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., and Igarashi, Y. (2008). Network relationships of bacteria in a stable mixed culture. *Microb. Ecol.* 56, 403–411. doi: 10.1007/s00248-007-9357-4

Kato, S., Sasaki, K., Watanabe, K., Yumoto, I., and Kamagata, Y. (2014). Physiological and transcriptomic analyses of a thermophilic, aceticlastic methanogen *Methanosaeta thermophila* responding to ammonia stress. *Microbes Environ.* 29, 162–167. doi: 10.1264/jsme2.ME14021

Kato, S., and Watanabe, K. (2010). Ecological and evolutionary interactions in syntrophic methanogenic consortia. *Microbes Environ.* 25, 145–151. doi: 10.1264/jsme2.ME10122

Kerr, B., Riley, M. A., Feldman, M. W., and Bohannan, B. J. (2002). Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. *Nature* 418, 171–174. doi: 10.1038/nature00823

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J. P., Hector, A., et al. (2001). Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science* 294, 804–808. doi: 10.1126/science.1064088

Lovley, D. R. (1985). Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl. Environ. Microbiol.* 49, 1530–1531.

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., et al. (2002). Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl. Environ. Microbiol.* 68, 5064–5081. doi: 10.1128/AEM.68.10.5064-5081.2002

Maloney, P. (1983). Relationship between phosphorylation potential and electrochemical H+ gradient during glycolysis in Streptococcus lactis. *J. Bacteriol.* 153, 1461–1470.

Mayumi, D., Dolfing, J., Sakata, S., Maeda, H., Miyagawa, Y., Ikarashi, M., et al. (2013). Carbon dioxide concentration dictates alternative methanogenic pathways in oil reservoirs. *Nat. Commun.* 13:1998. doi: 10.1038/ncomms2998

Mayumi, D., Mochimaru, H., Yoshioka, H., Sakata, S., Maeda, H., Miyagawa, Y., et al. (2011). Evidence for syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan). *Environ. Microbiol.* 13, 1995–2006. doi: 10.1111/j.1462-2920.2010.02338.x

McHugh, S., Carton, M., Mahony, T., and O'Flaherty, V. (2003). Methanogenic population structure in a variety of anaerobic bioreactors. *FEMS Microbiol. Lett.* 219, 297–304. doi: 10.1016/S0378-1097(03)00055-7

Nahum, J. R., Harding, B. N., and Kerr, B. (2011). Evolution of restraint in a structured rock-paper-scissors community. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10831–10838. doi: 10.1073/pnas.1100296108

Nauhaus, K., Boetius, A., Krüger, M., and Widdel, F. (2002). In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. *Environ. Microbiol.* 4, 296–305. doi: 10.1046/j.1462-2920.2002.00299.x

Nüsslein, B., Chin, K. J., Eckert, W., and Conrad, R. (2001). Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical Lake Kinneret (Israel). *Environ. Microbiol.* 3, 460–470. doi: 10.1046/j.1462-2920.2001.00215.x

Pham, V. D., Hnatow, L. L., Zhang, S., Fallon, R. D., Jackson, S. C., Tomb, J. F., et al. (2009). Characterizing microbial diversity in production water from an Alaskan mesothermic petroleum reservoir with two independent molecular methods. *Environ. Microbiol.* 11, 176–187. doi: 10.1111/j.1462-2920.2008.01751.x

Raskin, L., Poulsen, L. K., Noguera, D. R., Rittmann, B. E., and Stahl, D. A. (1994). Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* 60, 1241–1248.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol. Mol. Biol. Rev.* 61, 262–280.

Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H., and Nakamura, K. (1998). Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. *Microbiology* 144, 2655–2665. doi: 10.1099/00221287-144-9-2655

Shigematsu, T., Tang, Y., Kobayashi, T., Kawaguchi, H., Morimura, S., and Kida, K. (2004). Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation. *Appl. Environ. Microbiol.* 70, 4048–4052. doi: 10.1128/AEM.70.7.4048-4052.2004

Stock, D., Leslie, A. G. W., and Walker, J. E. (1999). Molecular architecture of the rotary motor in ATP synthase. *Science* 286, 1700–1705. doi: 10.1126/science.286.5445.1700

Thauer, R. K., Jungermann, K., and Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* 41, 100–180.

Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W., and Hedderich, R. (2008). Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* 6, 579–591. doi: 10.1038/nrmicro1931

Tran, Q. H., and Unden, G. (1998). Changes in the proton potential and the cellular energetics of *Escherichia coli* during growth by aerobic and anaerobic respiration or by fermentation. *Eur. J. Biochem.* 251, 538–543. doi: 10.1046/j.1432-1327.1998.2510538.x

Zinder, S. H., and Koch, M. (1984). Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Arch. Microbiol.* 138, 263–272. doi: 10.1007/BF00402133

Recombination Does Not Hinder Formation or Detection of Ecological Species of *Synechococcus* Inhabiting a Hot Spring Cyanobacterial Mat

Melanie C. Melendrez[1]*, Eric D. Becraft[1], Jason M. Wood[1], Millie T. Olsen[1], Donald A. Bryant[2], John F. Heidelberg[3], Douglas B. Rusch[4], Frederick M. Cohan[5] and David M. Ward[1]

[1] Department of Land Resources and Environmental Science, Montana State University, Bozeman, MT, USA, [2] Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA, USA, [3] Department of Biological Sciences, College of Letters, Arts and Sciences, University of Southern California, Los Angeles, CA, USA, [4] Informatics Group, J. Craig Venter Institute, Rockville, MD, USA, [5] Department of Biology, Wesleyan University, Middletown, CT, USA

Recent studies of bacterial speciation have claimed to support the biological species concept—that reduced recombination is required for bacterial populations to diverge into species. This conclusion has been reached from the discovery that ecologically distinct clades show lower rates of recombination than that which occurs among closest relatives. However, these previous studies did not attempt to determine whether the more-rapidly recombining close relatives within the clades studied may also have diversified ecologically, without benefit of sexual isolation. Here we have measured the impact of recombination on ecological diversification within and between two ecologically distinct clades (A and B') of *Synechococcus* in a hot spring microbial mat in Yellowstone National Park, using a cultivation-free, multi-locus approach. Bacterial artificial chromosome (BAC) libraries were constructed from mat samples collected at 60°C and 65°C. Analysis of multiple linked loci near *Synechococcus* 16S rRNA genes showed little evidence of recombination between the A and B' lineages, but a record of recombination was apparent within each lineage. Recombination and mutation rates within each lineage were of similar magnitude, but recombination had a somewhat greater impact on sequence diversity than mutation, as also seen in many other bacteria and archaea. Despite recombination within the A and B' lineages, there was evidence of ecological diversification within each lineage. The algorithm Ecotype Simulation identified sequence clusters consistent with ecologically distinct populations (ecotypes), and several hypothesized ecotypes were distinct in their habitat associations and in their adaptations to different microenvironments. We conclude that sexual isolation is more likely to follow ecological divergence than to precede it. Thus, an ecology-based model of speciation appears more appropriate than the biological species concept for bacterial and archaeal diversification.

Keywords: population genetics, speciation, *Synechococcus*, cyanobacteria, multi-locus sequence typing, Ecotype Simulation, ecotype, recombination

INTRODUCTION

For much of the last century, theories of the origin of species have revolved around genetic exchange (Mayr, 1942, 1963; Dobzhansky, 1951; Coyne and Orr, 2004). In highly sexual organisms such as animals and plants, genetic exchange is frequently seen as a cohesive force holding together the individuals within a species population, thus preventing divergence into irreversibly separate lineages (Cohan, 2013). The role of genetic exchange in bacteria and archaea, where recombination is not connected to reproduction (Levin and Bergstrom, 2000), has been vigorously debated. Recombination has been viewed by some as a rampant process that can erode the distinctness of nascent species (Doolittle and Zhaxybayeva, 2009); moreover, recombination is seen as a cohesive force binding a species together, as in the case of sexual species. Speciation theories based on Mayr's Biological Species Concept see the breaking of recombination between populations as a critical step in population divergence; in this view, populations can diverge ecologically and irreversibly only after their genetic exchange is blocked (Coyne and Orr, 2004).

On the other hand, theories of ecological speciation hold that ecological divergence is the critical step in the origin of diversity, and that recombination plays only a minor role in the subsequent fate of newly split, ecologically distinct populations (Van Valen, 1976; Mallet, 2008; Schluter, 2009). According to a theory developed long ago by Haldane, rare introduction of genes from one population to another cannot reverse the adaptive divergence between populations (Haldane, 1932). This is because natural selection against niche-specifying genes from other populations can limit these foreign genes to negligible frequencies, especially if the rate of recombination between populations is very low. The equilibrium frequency of a recombinant niche-specifying gene is equal to the rate of recombination between populations (c_b) divided by the intensity of selection (s) against the gene (Mallet, 2008; Wiedenbeck and Cohan, 2011). The equilibrium frequency of recombinant genes is thus extremely low, as recombination rates are not known to exceed the mutation rate by more than a factor of 10 (Vos and Didelot, 2009), and mutation rates are known to be very low in nearly all bacteria and archaea studied (about 10^{-6} per gene per generation; Drake, 2009). It can therefore be inferred that recombination rates are much too low to reverse adaptive divergence of microbial populations, and that recombination is unlikely to prevent ecological divergence among the most closely related bacteria and archaea (Wiedenbeck and Cohan, 2011).

The conflict between recombination-based and ecology-based theories of speciation has motivated several recent studies on the role of recombination in the evolution of bacterial and archaeal species. In a study of coexisting strains of *Sulfolobus islandicus* isolated from the same hot spring, Cadillo-Quiroz and colleagues found depressed rates of recombination between two major clades formed by these organisms compared to recombination within either clade (Cadillo-Quiroz et al., 2012). Inter-clade recombination was taken to be insufficient to prevent the divergence of the clades into easily distinguishable clusters, and the authors claimed this as support for the Biological

Species Concept in archaeal speciation. Ellegaard et al. came to a similar conclusion based on comparison of genomes derived from closely related *Wolbachia* strains (97–98% 16S rRNA identity) isolated from the same host fly (Ellegaard et al., 2013). Shapiro et al. obtained similar results for closely related clades of marine *Vibrio cyclitrophicus* strains that may be ecologically distinct, based on their isolation from particles of different sizes (Shapiro et al., 2012). They proposed a model in which ecological specialization, based on horizontal genetic transfer, leads to occupancy of different habitats; this causes a decrease in recombination between the ecologically distinct populations, allowing the ecological populations to further diverge (Shapiro et al., 2012; see also Retchless and Lawrence, 2010; Polz et al., 2013). In all these studies the reduced recombination between the clades under investigation was taken as evidence that recombination must be lowered to allow irreversible divergence among close relatives into stably coexisting, ecologically distinct populations. However, these studies did not address whether ecological divergence may have also occurred *within* each clade, where recombination rates are higher.

Here we test whether ecological divergence can occur among organisms so closely related that recombination between them is not reduced. Our model system is the set of unicellular cyanobacteria (*Synechococcus*) inhabiting the microbial mats of Mushroom Spring, Yellowstone National Park. This hypothesis emerged from a series of studies based on variation found in single genetic loci (Ferris and Ward, 1997; Ramsing et al., 2000; Ferris et al., 2003; Melendrez et al., 2011). First, closely related 16S rRNA genotypes were distributed differently along the effluent channel (genotypes A," A', A, B', and B along a temperature gradient from ∼72–74°C to ∼50°C Ferris and Ward, 1997) and with depth in the photic zone (Ramsing et al., 2000), suggesting differential adaptations to temperature and light that were later confirmed by studying isolates with genotypes representative of *in situ* populations (Allewalt et al., 2006). Second, analyses based on the internal transcribed spacer separating 16S and 23S rRNA genes revealed that 16S rRNA sequence variation was insufficient to detect all ecologically distinct populations (Ferris et al., 2003; Ward et al., 2006), limiting the utility of 16S rRNA-based methods for detecting closely related ecotypes (e.g., Eren et al., 2013). Third, analyses of several more rapidly evolving protein-encoding genes revealed numerous closely related and ecologically distinct populations, the number of which depended on the depth of coverage and on the degree of sequence divergence of the gene used (Becraft et al., 2011; Melendrez et al., 2011). It is clearly important to appreciate that the amount of molecular divergence between these closely related species is very small (see Discussion).

Our aim in these studies has been to discover the fundamental "ecotypes" within the A and B' clades (originally demarcated as 16S rRNA clusters), where we define an *ecotype* as a group of organisms that is ecologically distinct from other ecotypes and whose members are ecologically interchangeable (Kopac and Cohan, 2011). More specifically, different ecotypes are expected to coexist indefinitely owing to their ecological distinctness, while lineages within an ecotype are too similar to be able to coexist indefinitely (Kopac et al., 2014). In our recent work

we hypothesized the demarcations of putative ecotypes (PEs) from sequence clusters using a theory-based algorithm (Ecotype Simulation, based on the Stable Ecotype Model of species and speciation, Kopac et al., 2014; Cohan, 2016), which simulates the evolutionary dynamics of ecotype formation and the forces that purge sequence diversity within ecotypes (Koeppel et al., 2008). Unlike other studies that have used traditional or intuitive sequence cutoffs to define sequence clusters of interest (e.g., "main cloud" roughly equivalent to 3% 16S rRNA cutoff, see Rosen et al., 2015), we have used this dynamic approach to let the natural variation, itself, reveal the clusters that most likely fit the dynamic properties of species, given the resolution allowed by the sequence data. In recent pyrosequencing analyses of a gene encoding an essential photosynthesis protein (PsaA), Ecotype Simulation demarcated 18 B'-like, 14 A-like, and 5 A'-like *Synechococcus* PEs (Becraft et al., 2015). Importantly, these analyses demonstrated the ecological distinctness of the PEs: (i) the most abundant PEs had unique environmental distributions, (ii) the PEs showed unique gene expression patterns, (iii) cultivated strains with identical 16S rRNA sequences (see Nowack et al., 2015) from PEs with different depth distributions showed different light adaptations and acclimation responses (Nowack et al., 2015), and (iv) comparative genomic analyses of these strains are beginning to reveal the genetic mechanisms underlying these adaptations (Olsen et al., 2015). Moreover, the membership within PEs was shown to be ecologically interchangeable: (v) individuals predicted to belong to the same PE were distributed similarly along ecological gradients and (vi) the membership within a PE showed coordinated responses to environmental change (Becraft et al., 2015; Olsen et al., 2015).

Recently, Rosen et al. (2015) presented a view of the same Mushroom Spring *Synechococcus* populations that suggests recombination prevents the formation of such PEs. These authors pyrosequenced numerous PCR-amplified genes to study the genetic diversity within the A and B' lineages of the present model system (that is, the A and B' lineages of *Synechococcus* in Mushroom Spring) Although, Rosen et al. described their study as a multi-locus sequence analysis, any given organism was sampled for only one locus (i.e., multiple loci were unlinked in these analyses). Rosen et al. (2015) inferred from patterning of single-nucleotide polymorphisms (SNPs) in individual amplicons that recombination and mutation occur at similar rates within the sampled population. Viewing recombination to be "ubiquitous" and, based on comparison of neutral drift and sexual recombination models, they inferred that their "results were inconsistent with the presence of multiple ecotypes" within the A and B' lineages and conjectured that the A and B' lineages of *Synechococcus* were each behaving "as effectively [a] quasisexual species… occupying a broad environmental niche." However, as noted above we previously showed these lineages to each contain multiple, ecologically distinct populations (Becraft et al., 2015; Nowack et al., 2015; Olsen et al., 2015). Clearly, these *Synechococcus* populations provide a test case for understanding the roles of recombination and ecological specialization in speciation within these lineages. Here we will demonstrate in a true multilocus analysis that each

lineage has diversified into ecologically distinct populations in spite of recombination between populations.

Single-locus analyses may suffer from the effects of recombination, such that the evolutionary history of the gene may deviate from that of the organisms (Feil et al., 2001, 2004). Thus, we developed a novel multi-locus sequence analysis (MLSA) approach for cultivation-free sampling and analysis of population structure. MLSA provides a buffer against the effects of recombination in any single gene, increases molecular resolution, and allows detection of recombination events that involve both whole genes and parts of genes because the genes investigated are known to be linked on the same genome. MLSA was developed for population genetics studies of cultivated pathogenic isolates (Maiden et al., 1998; Feil et al., 2000a; Salerno et al., 2007; Vitorino et al., 2008; Cesarini et al., 2009) and has also been applied to nonpathogenic isolates (Papke et al., 2004, 2007; Whitaker et al., 2005; Koeppel et al., 2008; Mazard et al., 2012). Furthermore, the development of methods that permit access to large segments of genomes has extended its application to uncultivated organisms (Kashtan et al., 2014). Our cultivation-independent MLSA approach was based on the use of bacterial artificial chromosome (BAC) libraries (Shizuya et al., 1992; Tao et al., 2002; Liles et al., 2008) to sample multiple genes from individual genomes of *Synechococcus* inhabiting the microbial mat of Mushroom Spring.

BACs were derived from libraries constructed from DNA that was extracted from samples collected at either a 60°C site or a 65°C site. Consequently, like the other studies mentioned above, our focus populations have diverged into two major lineages (i.e., the A and B' lineages of *Synechococcus*, defined by 16S rRNA sequence variants A and B', see above) inferred to rarely recombine between lineages and known to be ecologically distinct (Ward et al., 1998, 2006; Klatt et al., 2011). BACs were screened to identify clones containing a *Synechococcus* A/B lineage-specific 16S rRNA region. Multiple loci were selected and PCR-amplified from these BAC clones and the sequence data obtained were analyzed using Ecotype Simulation of both concatenated MLSA and single-gene phylogenies, and alternatively, using eBURST analysis, which has routinely been used in MLSA studies to demarcate variants into clusters (see Methods; Feil et al., 2004).

Ecotype Simulation and eBURST use very different approaches to infer populations from sequence variation. Ecotype Simulation is a theory-based algorithm demarcating clades that are consistent with the dynamics of ecotype formation and purging of diversity within ecotypes; eBURST clusters variants on the basis of a pre-determined cutoff—that members of a cluster must be identical at all or all but one of the loci studied; however, concatenated MLSA sequences have been used in phylogenetic analyses (independent from eBURST analyses) to assist in demarcating species clusters (Maiden et al., 1998; Hanage et al., 2005). In previous analyses of this type, suspected recombinant sequences have simply been removed from Ecotype Simulation analyses, on the assumption that these recombinant sequences do not reflect the history of the organism at the locus undergoing recombination and, if not removed, might lead to overestimation of PEs (Koeppel et al., 2008). In this

study, however, we address the ability of Ecotype Simulation to predict PEs when recombined sequences are included.

The extent of recombination was assessed in single-gene and concatenated MLSA alignments using programs designed to detect recombination events and phylogenetic incongruency (also assessed by visual comparisons), and by SNP pattern analyses. The ecological distinctness of PEs, predicted either by MLSA or single-locus analysis, was determined through association with the temperature (60° or 65°) from which the BACs were obtained. We also tested for finer-scale habitat associations by tracking the distributions of genetic variants of an MLSA locus using pyrosequencing of DNA from samples collected over a range of vertical microenvironments in the 63–65°C mat (Becraft et al., 2015). In some cases genomic sequences were available for isolates representative of PEs demarcated in MLSA analyses (Olsen et al., 2015). This permitted comparison of distribution of the MLSA locus with *psaA* distributions that had previously been determined on the same samples (Becraft et al., 2011).

We will demonstrate not only that recombination has occurred more frequently within than between the A and B' lineages, but that it has neither prevented ecological diversification within each of these lineages nor our ability to detect ecotypes. We suggest that an ecology-based model of speciation is more appropriate than the biological species concept for bacterial speciation in hot spring *Synechococcus*, and possibly many other bacteria and archaea in diverse environments.

RESULTS

BAC Clone Libraries

Metagenomic libraries constructed from the 60° and 65° mat samples from the Mushroom Spring effluent channel contained 304,128 and 64,512 BAC clones with average insert sizes of 90 and 120 kb, respectively (see Supplemental Data Sheet Section I). BLAST analyses of paired-end sequences of 9216 randomly selected clones from each BAC library showed that the BAC libraries contained the same predominant taxonomic composition as small-insert metagenomes previously produced from the same DNA (Klatt et al., 2011; also see Supplemental Data Sheet Section II, Supplemental Data Tables 3, 4, and Supplemental Data Presentation Figure 2). While the BACs yielded poorer recovery of *Synechococcus* genes than the small-insert metagenomes, they provided broad coverage of *Synechococcus* strain JA-3-3Ab [accession: CP000239.1] and JA-2-3B'a(2-13) [accession: CP000240.1] reference genomes, which are representative of A-like and B'-like *Synechococcus* 16S rRNA lineages (Bhaya et al., 2007; **Figure 1A**, Supplemental Data Presentation Figure 3). BAC clones containing a *Synechococcus* strain A-like or B'-like 16S rRNA sequence were identified by oligonucleotide probe screening and sequence analysis (Supplemental Data Sheet Section I). Mapping the distribution of the paired-end sequences of each BAC clone relative to reference genomes suggested nearly equal and random coverage of the two unlinked 16S rRNA loci of these genomes (**Figure 1B**, Supplemental Data Presentation Figure
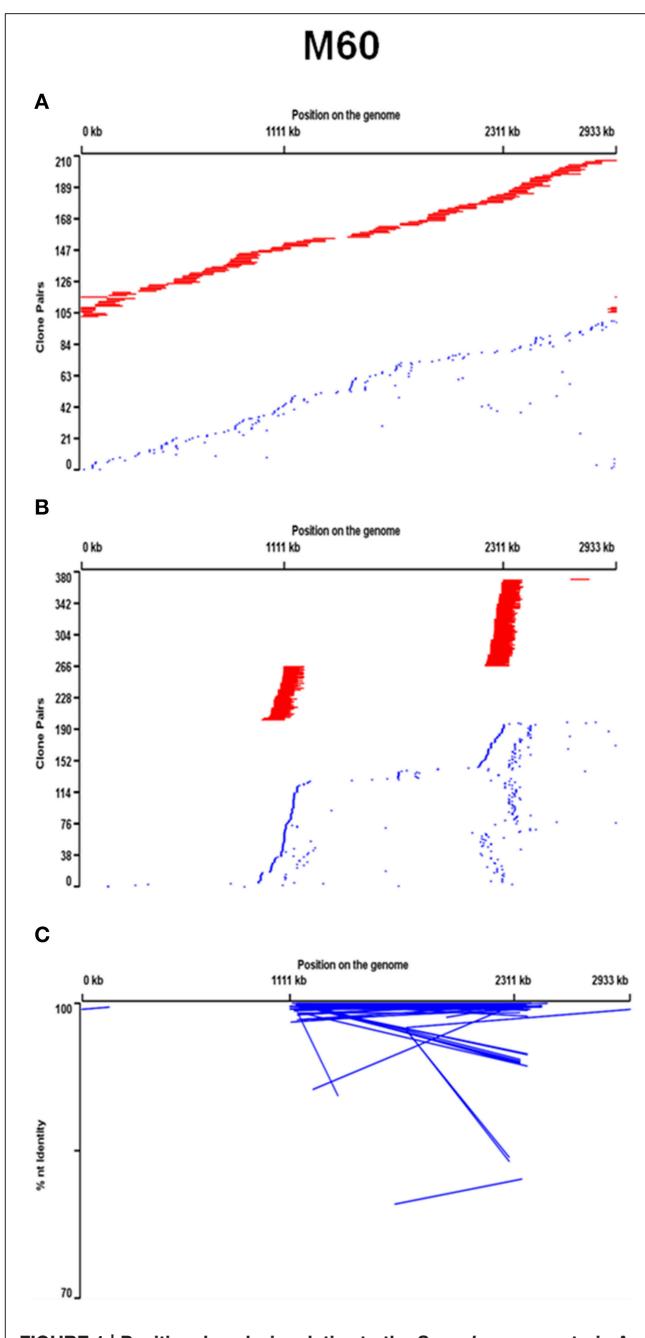
FIGURE 1 | Positional analysis relative to the *Synechococcus* strain A genome of jointly recruited syntenous (red line joins end sequences) and jointly recruited non-syntenous (blue) end sequences of 60°C BAC clones that are (A) recruited by this genome, and (B) contain an A-like *Synechococcus* 16S rRNA region, and (C) have anti-normal long end-sequence mate pairs. In (C) percent nucleotide identity of recruited sequences with genomic homologs is also plotted and lines connect mate-pairs.

3). Interestingly, many *Synechococcus* A-like or B'-like BACs containing a 16S rRNA sequence were non-syntenous when compared with the respective genome sequence. Syntenous sequences are considered here to be those whose mate pairs were of the same orientation as the reference genome and were

separated by a distance that was similar to the size of the DNA fragments used to construct the metagenomic library. Non-syntenous sequences are considered here to be those whose mate pairs deviated from the insert length and/or orientation of the reference genome (See Supplemental Data Sheet Section II and reference, Klatt et al., 2011). Such cases were revealed by the paired-end sequences of the same clone matching regions near both 16S rRNA loci, as opposed to one. The percentage of normal- and anti-normal long orientations of paired-end sequences in these non-syntenous clones was greater than in randomly sampled BACs (**Figure 1C**, Supplemental Data Sheet Section II, Supplemental Data Table 5, and Supplemental Data Presentation Figure 3). This suggested that non-syntenous BACs may have been associated with genomic inversions (Rusch et al., 2007).

MLSA Databases

BACs containing A-like and B'-like *Synechococcus* 16S rRNA genes were screened by PCR amplification of loci targeted for MLSA analysis (**Tables 1**, **2**; Supplemental Data Tables 1, 2). MLSA loci were selected on the basis of their being (i) single-copy genes, (ii) under neutral or purifying selection, that (iii) represented a range of degrees of divergences and distances from

the 16S rRNA locus and (iv) were not adjacent to potential mobile elements (see Supplemental Data Sheet Sections I, III).

The distributions of MLSA loci among the 267 A-like and 237 B'-like BACs containing the targeted 16S rRNA locus and at least one of the other MLSA loci are shown in Supplemental Data Sheet Section III and Supplemental Data Tables 6, 7. The majority of BACs (94 and 66% of *Synechococcus* A- and B'-like BACs, respectively) exhibited locus combinations consistent with the gene order of the reference genomes. The number of BACs positive for various loci decreased as the distance separating them from the 16S rRNA locus increased, providing additional evidence of genome rearrangements in these lineages (Supplemental Data Sheet Section III and Supplemental Data Presentation Figure 4). There was no obvious pattern of decaying synteny with increasing phylogenetic divergence (see end of legends in **Figure 2** and Supplemental Data Presentation Figure 10 identifying syntenous and non-syntenous clones in the dataset).There were 71 *Synechococcus* A-like BACs that contained the total set of 7 selected loci (*rbsK*, *PK* [locus tag CYA_2262], *hisF*, *lepB*, *CHP* [locus tag CYA_2291], *aroA* and *dnaG*) in addition to the 16S rRNA/16S-23S rRNA internal transcribed spacer (ITS) region (Supplemental Data Table 6). However, only two *Synechococcus* B'-like BACs contained 7 of

TABLE 1 | Results from recombination and mutation rate and ratio analyses for A-like *Synechococcus* BACs.

Locus (No. of Loci)	No. of STs in Analysis	No. of nts[b]	No. of Segregating Sites	LDHat						ClonalFrame			
				θ^c	R[d] |R[k] |R[l]	Ave PWD[i]	Var PWD[j]	R/θ[e]		θ^c(CI)	R[d] (CI)	ρ/θ[e](CI)	r/m[f](CI)
7[m]	50	4008	183	40.86	41|13|1.6	33.14	580.03	0.04–1.0		33.94	20.365 (12.92–30.29)	0.600 (0.38–0.892)	2.596 (1.57–4.01)
5[a,m]	69	2760	222	45.67	44|16|2.8	39.19	706.5	0.06–0.96		42.59	24.76 (16.75–34.65)	0.581 (0.39–0.81)	3.396 (2.21–4.92)
rbsK	h	520	119	24.48	5|9|0	23.24	509.7	0–0.368		h	h	h	h
PK[m]	h	584	30	6.17	11|1|0	3.63	18.85	0–1.7		h	h	h	h
hisF	h	429	0	g	g			g		g	g	g	g
lepB[m]	h	420	30	6.17	0|2|0	2.11	18.58	0–0.324		h	h	h	h
CHP[m]	h	629	28	5.76	7|1|0	9.13	93.59	0–1.2		h	h	h	h
aroA	h	607	15	3.09	0|0|0	1.10	2.67	0.0		h	h	h	h
dnaG[m]	h	547	26	5.80	0|0|0	1.49	20.48	0.0		h	h	h	h

CI: 95% credibility interval.

[a] *rbsK, CHP, PK, lepB, aroA.*

[b] Sum of all single gene lengths (number of nucleotides) and analysis pertains to a 73-sequence (145 sequence de-duplicated) 5-locus alignment except for hisF and dnaG which pertain to 7-locus alignment.

[c] Watterson's theta.

[d] Maximum at 4Ner(region); composite likelihood method (LDHat; pairwise) finite sites model (McVean et al., 2002, 2004).

[e] R/θ from LDHat is equivalent to the ρ/θ measure from Clonal Frame and define the ratio of the rates of recombination (R or ρ) to mutation (θ) illustrating how often recombination occurs relative to mutation (McVean et al., 2002, 2004; Didelot and Falush, 2007; Didelot and Maiden, 2010). For LDHat-pairwise analysis, R/θ range includes calculations from composite-likelihood, Rmin and Wakeleys moment method (McVean et al., 2002, 2004).

[f] Ratio of the probabilities that a given site is altered through recombination (r) and mutation (m) illustrating how important the effect of recombination was in the diversification of the samples relative to mutation (Didelot and Falush, 2007; Didelot and Maiden, 2010).

[g] There were no segregating sites in the sequence dataset.

[h] Clonal Frame analysis is designed for concatenated multi-locus alignments (Didelot and Falush, 2007; Didelot and Maiden, 2010).

[i] avePWD: average pairwise distance (LDHat pairwise).

[j] varPWD: variance in pairwise distance (LDHat pairwise).

[k] Rmin value (LDHat pairwise); minimum number of recombination events describing evidence for recombination in region (McVean et al., 2002, 2004) infinite sites model.

[l] Population scaled 4Ner (recombination estimate) by Wakeley Moment method (McVean et al., 2002, 2004).

[m] The look up table for LDHat pairwise not generated from data but from existing lkgen table provided in LDHat distribution (lk_n100_t0.01; https://github.com/auton1/LDHat).

TABLE 2 | Results from recombination and mutation rate and ratio analyses for B'-like *Synechococcus* BACs.

Locus (No. of Loci)–PE	No. of STs in Analysis	No. of Nts	No. of Segregating Sites	LDHat						ClonalFrame			
				θ^a	R^b $|R^h$ $|R^i$	Ave PWD^f	Var PWD^g	R/θ^c	θ^a	R^b (CI)	ρ/θ^d (CI)	r/m^e (CI)
4^j	51	2448	361	80.24	85|0|22.3	66.14	905.36	0–1.06	63.34	23.53 (15.18–33.89)	0.371 (0.24–0.535)	2.42 (1.58–3.44)
aroA^j	e	550	101	22.45	0|13|0	14.42	219.48	0–0.579	e	e	e	e
rbsk	e	535	122	27.12	15|15|2.9	34.05	539.83	0.44–0.55	e	e	e	e
pcrA^j	e	628	62	13.78	38|11|0.5	11.06	75.06	0.36–2.8	e	e	e	e
16S rRNA/ITS^j	e	733	76	16.89	15|0|0	6.725	43.46	0–0.889	e	e	e	e

CI: 95% credibility interval.

[a] *Watterson's theta.*

[b] *Maximum at 4Ner(region); composite likelihood method (LDHat; pairwise) finite sites model (McVean et al., 2002, 2004).*

[c] *R/θ from LDHat is equivalent to the ρ/θ measure from Clonal Frame and define the ratio of the rates of recombination (R or ρ) to mutation (θ) illustrating how often recombination occurs relative to mutation (McVean et al., 2002, 2004; Didelot and Falush, 2007; Didelot and Maiden, 2010). For LDHat-pairwise analysis, R/θ range includes calculations from composite-likelihood, Rmin and Wakeleys moment method (McVean et al., 2002, 2004).*

[d] *Ratio of the probabilities that a given site is altered through recombination (r) and mutation (m) illustrating how important the effect of recombination was in the diversification of the samples relative to mutation (Didelot and Falush, 2007; Didelot and Maiden, 2010).*

[e] *Clonal Frame analysis is designed for concatenated multi-locus alignments (Didelot and Falush, 2007; Didelot and Maiden, 2010).*

[f] *avePWD: average pairwise distance (LDHat pairwise).*

[g] *varPWD: variance in pairwise distance (LDHat pairwise).*

[h] *Rmin value (LDHat pairwise); minimum number of recombination events describing evidence for recombination in region (McVean et al., 2002, 2004) infinite sites model.*

[i] *Population scaled 4Ner (recombination estimate) by Wakeley Moment method (McVean et al., 2002, 2004).*

[j] *The look up table for LDHat pairwise **not** generated from data but from existing lkgen table provided in LDHat distribution (lk_n100_t0.01; https://github.com/auton1/LDHat).*

the targeted MSLA loci (Supplemental Data Table 7). In order to obtain a comparable sampling of *Synechococcus* B'-like and A-like BAC clones, the number of loci for the *Synechococcus* B'-like population was reduced to three protein-encoding loci (*rbsK, aroA* and *pcrA*) plus the 16S rRNA/16S-23S rRNA ITS sequence. The amount of nucleotide divergence at the 16S rRNA locus within A-like and B'-like BACs averaged 0.31% (±0.036% SE) and 0.71% (±0.064% SE), respectively, much less than that between BACS of the different lineages, which averaged 2.73% (±0.024% SE), which was somewhat lower than the 3.6% divergence reported for the *Synechococcus* strain A and B' genomes (Bhaya et al., 2007). We compared the *rbsK* sequences of A-like and B'-like BACs to *rbsK* sequences recovered by direct PCR amplification from the same mat samples and found that the BAC library sequences sampled broadly across B'-like and A-like lineages (Supplemental Data Sheet Section IV and Supplemental Data Presentation Figures 6, 7). All multi-locus sequence datasets were assembled into sequence types (STs) comprised of individual BACs with identical sequences at all loci (Supplemental Data Sheet Section V and Supplemental Data Tables 8, 9).

Recombination and Mutation within and Between A and B' *Synechococcus* Lineages

Evidence of recombination within the A- and B'-lineages was readily detected by various analyses of both single genes and multiple loci. The ability to detect recombination events was influenced by the number and length of sequences analyzed and by the number of genes included in the analysis. Furthermore, different methods detected different events. For instance, as shown in **Table 3**, RDP4 analysis of 7 loci in 71 A-like BACs detected 11 unique events. Clonal Frame detected 7 of the same events, and manual inspection of SNPs (using the definition from (Feil et al., 2000a), where >1 SNP within any single locus is indicative of recombination) suggested 24 additional putative events. Hence, 11–35 unique events were detected. However, in an analysis in which we more than doubled the number of sequences by removing 2 of the 7 genes (see below), RDP4 detected only 5 events; Clonal Frame detected 1 additional event and SNP analysis suggested 2 more events (range 6–8 events). Similarly, in an analysis of 4 genes in 72 B'-like BACs RDP4 detected 4 events, Clonal Frame detected an additional 9 events and SNP inspection suggested 11 additional unique events (range 13–24 events).

The majority of recombination events were detected in the *rbsK* locus in both lineages; to a lesser extent recombination events were also detected in *PK, CHP*, and *lepB* for A-like *Synechococcus* and in the 16S rRNA/ITS region and *pcrA* gene for B'-like *Synechococcus* (Supplemental Data Tables 10–13). Phylogenies for *rbsK*, constructed using RDP4 with sequence data from either side of the predicted breakpoints, were shown to be incongruent providing additional evidence of recombination at this locus in both lineages (Supplemental Data Presentation Figures 8, 9).

In contrast, evidence of recombination between the A-like and B'-like lineages was rare. Blast analyses of 7 MLSA loci on 71 A-like BACs and 4 MLSA loci on 72 B'-like BACs revealed only three examples, all involving *rbsK* loci on A-like (*n* =

TABLE 3 | The number of unique (u) and overlapping (ovl; supported by multiple methods) recombination events recorded by RDP4, Clonal Frame (CF), and single nucleotide polymorphism (SNP) analysis of MLSA datasets.

Organism	Datastet	No. Sequences	RDP4u*[a,b]	CFu	CFovl	SNPu	SNPovl	All Unique[e]
Synechoccocus A-like BACs	MLSA7[d]	71	11	0[a]	7[a]	24[a]	7[a]	35
	MLSA5	49	5	0[a]	0[a]	0[a]	0[a]	5
	MLSA5	145	5	1[a]	8[a]	2[a]	6[a]	8
Synechococcus B'-like BACs	MLSA4	72	4	9[b,c]	19[b]	11[b,c]	14[b,c]	24

All loci were considered in analysis.

*Overlapping RDP4 events with CF or SNP analyses were not recorded in CFu or SNPu columns, only the RDP4u column.

[a]See Supplemental Data Table 10.

[b]See Supplemental Data Table 11.

[c]See Supplemental Data Presentation Figure 10.

*[d]See **Figure 2**.*

[e]Sum of "u" columns only.

1) or B'-like ($n = 2$) BACs that were associated with the genome of the other lineage (Supplemental Data Sheet Section VI). Similarly, of the loci used to study both A-like and B'-like *Synechococcus* lineages, *rbsK* was the only gene for which RDP4 analysis detected evidence of recombination. Only 3 unique recombination events were identified among 123 combined A-like and B'-like *rbsK* sequences in the 511 nt overlapping region (all within B'-like BACs; Supplemental Data Table 14).

The importance of recombination relative to mutation for the evolution of A-like and B'-like *Synechococcus* lineages was estimated independently in two ways: (i) R/θ, which is the ratio at which recombination and mutation occur (McVean et al., 2002, 2004; Didelot and Falush, 2007) using LDHat analysis (reported as a range depending on LDHat method annotated in footnotes of **Tables 1, 2** and equivalent to ρ/θ in Clonal Frame analysis), and (ii) r/m, which is a ratio of the probabilities that a given site was altered through recombination or mutation (i.e., how important the effect of recombination was in the diversification of the sample relative to mutation in terms of the number of resulting nucleotide substitutions) as defined by Clonal Frame analysis (Didelot and Falush, 2007; Didelot and Maiden, 2010). For MLSA concatenated datasets, the ratios (R/θ, ρ/θ, and r/m) differed somewhat in the two lineages. Recombination events were generally less frequent than mutation events (ρ/θ ranging from 0.38 to 0.89 for A-like BACs and 0.24 to 0.54 for B'-like BACs, in Clonal frame analysis; R/θ ranging from 0.4 to 1.0 for A-like BACs and 0.44 to 1.06 for B'-like BACs in LDHat analysis; see **Tables 1, 2**). It was also clear from Clonal frame analysis that recombination had a greater impact than mutation on diversification of the lineage (r/m ratio ranging from 2.60 to 3.40 depending on number of loci for *Synechococcus* A-like BACs, and 2.42 for *Synechococcus* B'-like BACs; **Tables 1, 2**).
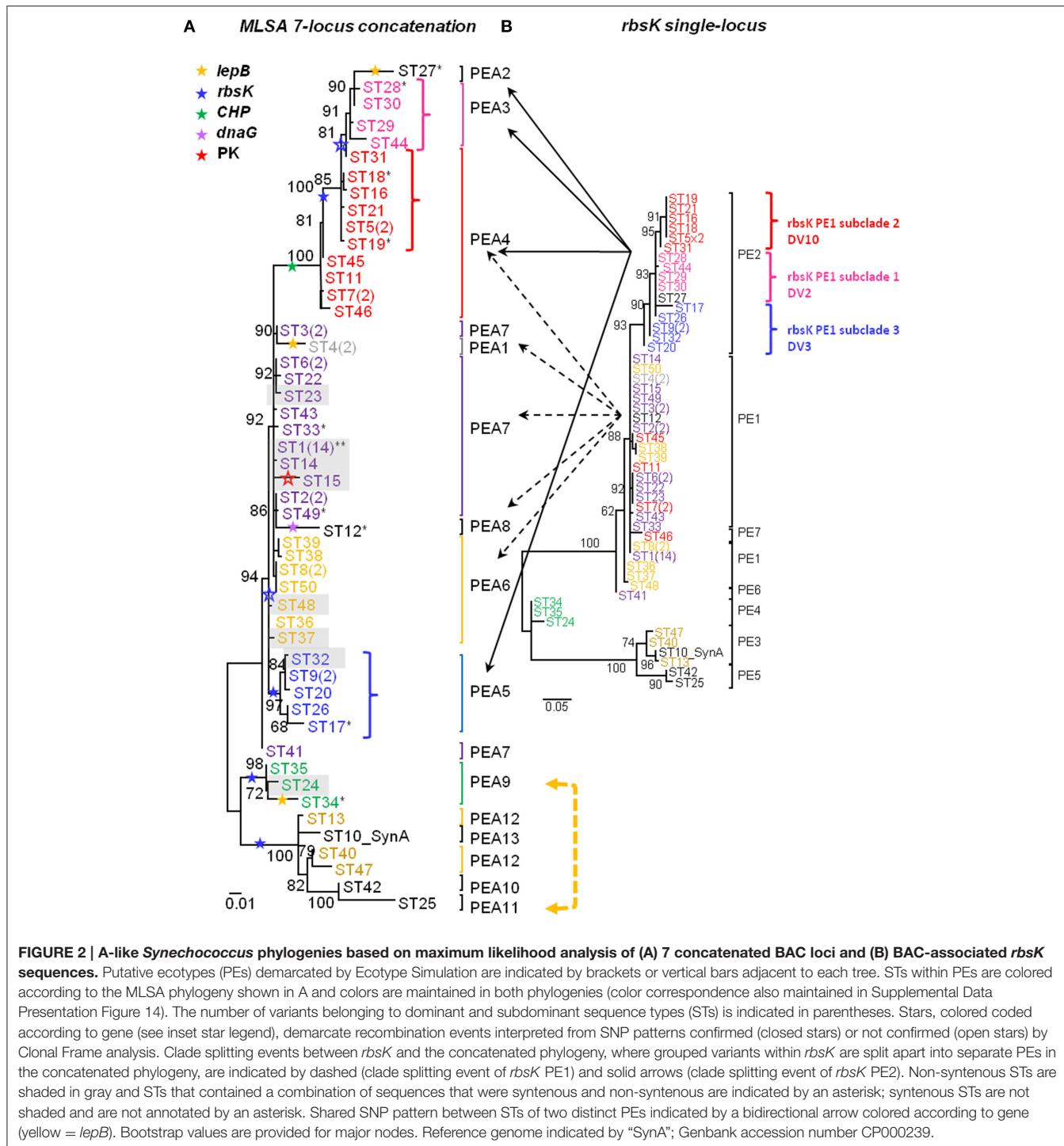
Ecotype Simulation Analyses of Concatenated A-like *Synechococcus* MLSA Datasets

We focus in the main text on the A-like *Synechococcus* lineage, which offered a greater number of genes for MLSA analyses (7 loci, hence termed MLSA7). Also, A-like *Synechococcus* BACs were recovered from both mat samples, thus enabling

analysis of habitat association. Comparable results for a 4-locus MLSA analysis (MLSA4) of B'-like lineage variants, which were recovered only from the 60°C sample, are presented in Supplemental Data Sheet Section VII. For clarity, PEs demarcated by Ecotype Simulation are named according to whether they are based on MLSA data (MLSA7 or MLSA4 followed by the PE designation; e.g., MLSA7 PEA1; MLSA4 PEB'4) or single-locus data (specified by the gene analyzed followed by the PE designation; e.g., *rbsK* PEA2).

All individual-locus sequences sampled by BACs and multi-locus concatenations constructed from them (STs) were analyzed using Ecotype Simulation (**Figure 2A**, **Table 4**). STs predicted by Ecotype Simulation to be members of the same putative ecotype in the MLSA7 analysis are given the same color, but members of different PEs are colored differently, and colors are maintained throughout all MLSA7 Figures. The MLSA7 concatenated phylogeny and the single-locus phylogeny for *rbsK* sequences sampled by the same BACs are compared in **Figures 2A,B** respectively, which also show PEs predicted by Ecotype Simulation. Phylogenetic comparisons with other loci (*CHP, lepB, dnaG PK, aroA,* and *hisF*) are discussed in Supplemental Data Sheet Section VIII and are shown in Supplemental Data Presentation Figure 14. Ecotype Simulation predicted 2–7 A-like PEs from individual BAC loci, depending on the locus analyzed. Among individual loci, the greatest number of PEs was predicted from the *rbsK* gene (**Figure 2B**), which had the greatest average evolutionary divergence among the loci studied (**Table 4**). Ecotype Simulation predicted 13 A-like MLSA7 PEs from concatenated MLSA sequence data (**Figure 2A**), though five of these PEs were based on a single ST sequence (i.e., singletons) and one was based on two occurrences of the same ST (i.e., a doubleton). MLSA7 PE clades contained from 1 to 28 BACs. Three of the 13 MLSA7 PE clades for *Synechococcus* A-like BACs contained a dominant variant (i.e., 2–14 clones that were identical at all MLSA7 loci) and singleton variants. In two cases, MLSA7 PEs A4 and A7, subdominant variants were also observed, along with singletons. Supplemental Data Table 23 shows how STs, dominant variants and subdominant variants were distributed among MLSA7 PEs.

Only one case of complete correspondence between *rbsK* PEs and MLSA7 PEs was observed (i.e., MLSA7 PE A9 = *rbsK*

FIGURE 2 | A-like *Synechococcus* phylogenies based on maximum likelihood analysis of (A) 7 concatenated BAC loci and (B) BAC-associated *rbsK* sequences. Putative ecotypes (PEs) demarcated by Ecotype Simulation are indicated by brackets or vertical bars adjacent to each tree. STs within PEs are colored according to the MLSA phylogeny shown in A and colors are maintained in both phylogenies (color correspondence also maintained in Supplemental Data Presentation Figure 14). The number of variants belonging to dominant and subdominant sequence types (STs) is indicated in parentheses. Stars, colored coded according to gene (see inset star legend), demarcate recombination events interpreted from SNP patterns confirmed (closed stars) or not confirmed (open stars) by Clonal Frame analysis. Clade splitting events between *rbsK* and the concatenated phylogeny, where grouped variants within *rbsK* are split apart into separate PEs in the concatenated phylogeny, are indicated by dashed (clade splitting event of *rbsK* PE1) and solid arrows (clade splitting event of *rbsK* PE2). Non-syntenous STs are shaded in gray and STs that contained a combination of sequences that were syntenous and non-syntenous are indicated by an asterisk; syntenous STs are not shaded and are not annotated by an asterisk. Shared SNP pattern between STs of two distinct PEs indicated by a bidirectional arrow colored according to gene (yellow = *lepB*). Bootstrap values are provided for major nodes. Reference genome indicated by "SynA"; Genbank accession number CP000239.

PEA 4) (green colored STs in **Figure 2**). Otherwise, the *rbsK* and MLSA7 phylogenies were mainly incongruent, providing further evidence that recombination complicates single-locus analyses of PEs (compare **Figures 2A,B**). For instance, some members of MLSA7 PE A4 were classified into three *rbsK* PEs (A1, A2, and A7; red colored sequences in **Figure 2**). Ecotype Simulation analysis of concatenated MLSA7 loci mainly split individual PEs

demarcated by *rbsK* analysis into multiple MLSA7 PEs, as shown by dashed and solid black arrows in **Figure 2** (e.g., *rbsK* PE2 was split into MLSA PEs 3 (pink), 4 (red), and 5 (blue).

Evidence of Habitat Association

In order to obtain a greater number of A-like BACs from samples collected at both temperatures sufficient to observe

TABLE 4 | Ecotype Simulation and eBURST output for 71 A-like *Synechococcus* BACs that contained all 7 loci assayed for the study.

Locus	Average evolutionary divergence (BACs)	Ecotype Simulation (ES)				eBURST[b]	
		PEs Demarcated-ES (95% CI)	Omega (95% CI)	Sigma (95% CI)	Sample-specific PEs	No. of alleles or sequence types	No. of clonal complexes
rbsK	0.03	7 (4–16)	0.05 (0.01–0.14)	3.5 (0.33–42)	Yes	24	[d]
PK	0.002	2 (2–60)	0.052 (0.04–66)	13 (1.2–12.2)	No	11	[d]
hisF[a]	< 0.0001	2 (2–71)	3113 ($<2e^{-7}$->100)	48,000 (0.7->100)	No	2	[d]
lepB	0.005	2 (2–6)	0.09 (0.003–0.6)	26.9 (1.7–99.8)	No	6	[d]
CHP	0.01	2 (2–5)	0.03 (0.001–0.23)	564 (5.4->100)	No	4	[d]
aroA	0.001	3 (2–71)	1.86 (0.03–99.6)	22.7 (0.07–100)	No	7	[d]
dnaG	0.002	4 (3–22)	0.3 (0.07–2.5)	180 (3.8->100)	No	7	[d]
Concatenation[b]	0.01	13 (9–44)	0.05 (0.03–0.12)	1.11 (0.07–100)	[c]	50	3

[a]*Molecular resolution of hisF was not significant enough for Ecotype Simulation to effectively predict ecotypes.*
[b]*Calculated from concatenated sequence datasets only.*
[c]*Not enough 65° sequences to determine sample specificity of demarcated PEs.*
[d]*Clonal complexes only apply to concatenated sequence datasets.*

habitat associations, we performed two separate MLSA analyses of 5 of the 7 loci (*rbsK, lepB, aroA, CHP*, and *PK*; MLSA5, see Supplemental Data Sheet Section VIII). One included 49 concatenated sequences with nearly equal representation of sequences from 60 and 65°C (Supplemental Data Presentation Figure 15). The other included the full dataset of 145 concatenated sequences that could be compared at these 5 loci (Supplemental Data Presentation Figure 16). Fisher's exact tests of contingency on both sets of BACs from 60 and 65°C yielded significant evidence of heterogeneity in habitat associations (i.e., collection temperatures; *p*-value = 0.001). Additionally, several MLSA5 PEs were comprised of multiple BACs obtained only from one of the two temperatures.

In order to investigate vertical distributions of alleles we pyrosequenced the *rbsK* locus in DNAs extracted from 80 μm-thick cryotome sections from different depths in the top green layer of the 63–65°C mat, where A-like PEs predominate. Pyrosequencing analysis of the *rbsK* locus resulted in a phylogeny (Supplemental Data Sheet Section IX; Supplemental Data Presentation Figure 19) that closely resembled the *rbsK* phylogeny based on sequences sampled by BAC clones (compare **Figure 2B** and Supplemental Data Presentation Figure 19). In cases where pyrosequences matched only STs of the same PE or subclade, we could test the hypothesis that these PEs represent ecologically distinct populations. As shown in **Figure 3A**, the subclade representing MLSAs PE A4 and A5 (red and blue STs in **Figure 2A**), which were grouped together into *rbsK* PE A2 (**Figure 2B**), were shown to exhibit different vertical distributions (Fisher's exact test, ANOVA and *G*-tests *p* < 0.001). *rbsK* sequences representative of MLSA PE A4 declined with depth, whereas those representative of MLSA PE A5 increased with depth. This provides an example of how a single-locus analysis can lump populations that are ecologically distinct, giving the impression of a clade containing ecologically heterogeneous members. Additionally, the availability of genome sequences of

isolates representative of MLSA7 PEs A5 and A9 (Olsen et al., 2015; and see Supplemental Data Sheet Section X) allowed us to associate *rbsK* and *psaA* sequences corresponding to the same PEs. In these cases we could demonstrate correspondence between *rbsK* distributions and the *psaA* distributions observed in single-locus analysis of the same samples by Becraft et al. (2015) (see also **Figures 3B,C**, where MLSA7 PE 5 = *psaA* PE A6 and MLSA7 PE 9 = *psaA* PE A14). Both of these PEs have been shown to be most prevalent toward the bottom portion of the upper green layer of the mat at this temperature (Becraft et al., 2015), and representative isolates have been shown to possess genes that enable harvesting of lower amounts of light, characteristic of *in situ* light conditions (Becraft et al., 2015; Nowack et al., 2015; Olsen et al., 2015; also see Supplemental Data Sheet Section X).

eBURST Analyses

The eBURST algorithm assembles STs into clonal complexes (Maiden et al., 1998; Feil et al., 1999, 2000a, 2004) that are typically composed of a single predominant genotype (consensus group or here, dominant variant), plus variants that are identical to the predominant genotype at all but one locus (single-locus variants; Feil and Spratt, 2001; Feil et al., 2004). Single-locus variants can differ from the dominant variant by any number of SNPs as long as those SNPs occur in only one locus (Feil et al., 1999, 2000b, 2003). Only 3 clonal complexes were observed with eBURST analyses of the MLSA7 dataset (**Figure 4**, **Table 5**), and the correspondence between eBURST's clonal complexes and Ecotype Simulation's PEs was imprecise (see **Figure 4** and Supplemental Data Sheet Section VIII for details of these analyses and results). In the eBURST analyses of the MLSA5 datasets, Fisher's exact tests did not indicate differences among clonal complexes in their habitat associations (Supplemental Data Presentation Figure 17, *p*-value 0.943; Supplemental Data Presentation Figure 18, *p*-value 0.734).

FIGURE 3 | *rbsK* vertical distributions in the 63–65°C Mushroom Spring microbial mat of dominant *rbsK* and *psaA* variants associated with *Synechococcus* putative ecotypes (PE) demarcated from multi-locus sequence analyses (MLSA) by Ecotype Simulation, shown as colored solid lines. **(A)** *rbsK* distributions of two MLSA PEs that are demarcated as a single PE in *rbsK* analysis. **(B,C)** *rbsK* (solid line) and *psaA* (dotted line) distributions of two MLSA PEs connected through genomes of representative isolates. Bars represent standard error ($n = 3$). PE colors correspond across panels.

FIGURE 4 | eBURST population snapshot of A-like *Synechococcus* BACs for the 7-locus analysis shown in Figure 2A. Clonal complexes enclosed by solid black lines. PE demarcation from Ecotype Simulation analysis are overlaid, using different colors corresponding to **Figure 2A** to represent distinct PEs. STs are represented by numbers and those colored in gray belong to PEs demarcated from a single sequence with a unique ST. Reference genome indicated by "SynA" next to ST10.

TABLE 5 | eBURST analysis of clonal complexes for the 7-locus MLSA of A-like *Synechococcus* BACs.

							Clonal Complex A7-I										
Locus	Consensus DV-ST1 (14)	ST2	ST3	ST4	ST6	ST8	ST11	ST12	ST14	ST15	ST33	ST36	ST39	ST41	ST43	ST48	ST49
rbsK	1	1	1	1	3[1]	1	1	1	1	1	1	7[2]	8[2]	10[5]	12[1]	17[2]	1
PK	1	1	1	1	1	2[1]	1	1	8[1]	10[11]	1	1	1	1	1	1	1
hisF	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
lepB	1	1	2[1]	3[13]	1	1	1	1	1	1	1	1	1	1	1	1	1
CHP	1	1	1	1	1	1	2[20]	1	1	1	1	1	1	1	1	1	1
aroA	1	1	1	1	1	1	1	1	1	1	7[2]	1	1	1	1	1	1
dnaG	1	2[1]	1	1	1	1	1	5[19]	1	1	1	1	1	1	1	1	3[2]

				Clonal Complex A7-II				
Locus	Consensus DV-ST5 (2)	ST11	ST18	ST19	ST21	ST31	ST45	ST46
rbsK	2	1[20]	2	2	2	18[2]	14[8]	15[13]
PK	1	1	1	1	8[1]	1	1	1
hisF	1	1	1	1	1	1	1	1
lepB	1	1	2[1]	1	1	1	1	1
CHP	2	2	2	3[1]	2	2	2	2
aroA	1	1	1	1	1	1	1	1
dnaG	1	1	1	1	1	1	1	1

		Clonal Complex A7-III			
Locus	Consensus DV-ST6 (2)	ST1	ST7	ST22	ST23
rbsK	3	1[1]	3	3	3
PK	1	1	1	1	1
hisF	1	1	1	1	1
lepB	1	1	1	1	1
CHP	1	1	2[20]	1	1
aroA	1	1	1	1	5[2]
dnaG	1	1	1	4[1]	1

*The number of BACs within an ST is in parentheses when greater than 1. Superscripts next to the allele number denote number of nucleotide differences compared to the consensus sequence. Corresponding to **Figure 4**.*
DV, dominant variant.
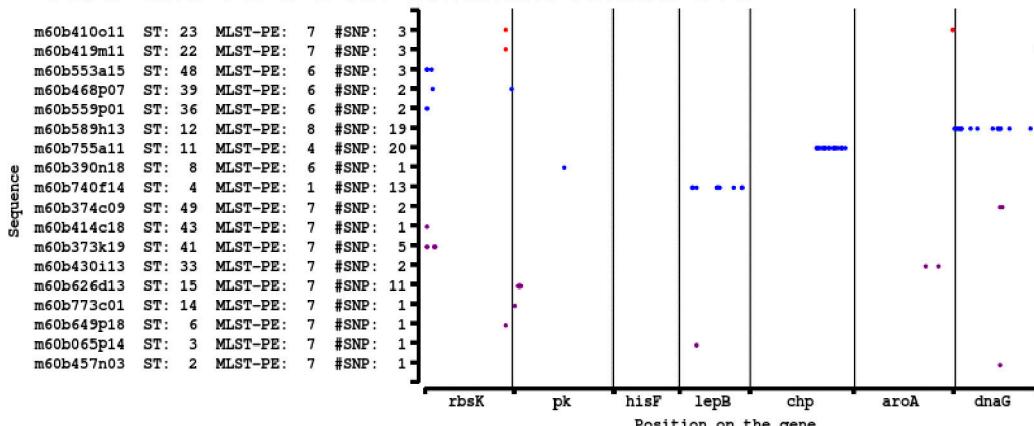
Evidence of Historic and Recent Recombination Events

The presence of historic recombination events was further investigated after it was initially found through SNP analysis that identical SNP differences were observed in two different MLSA7 PEs. **Figure 5** shows SNP differences between variants that were grouped into MLSA7 PEs and clonal complexes sharing the same dominant variant. Most differences resulted either from the exclusion of STs containing SNPs in multiple loci from clonal complexes by eBURST, or from the exclusion of STs containing a large number of SNPs from phylogenetic clusters demarcated as PEs by Ecotype Simulation. Of particular interest, however, was the observation of identical SNP differences in the *CHP* locus in two different MLSA7 PE A4 STs relative to two different dominant variants of MLSA7 PE A7 (ST7 vs. ST6 and ST11 vs. ST1). This prompted us to consider the possibility that these SNP patterns were clade-specific. We investigated recombination events occurring on internal nodes

(suggestive of historic recombination) using Clonal Frame. Clonal Frame predicted several internal nodes in both the *Synechococcus* A-like and B'-like lineages where there was a high probability of recombination having occurred in one or more loci (closed colored stars in **Figure 2A** and Supplemental Data Presentation Figure 10). This revealed a historical record of the impact recombination has had on the diversification of these organisms. Detailed manual inspection of SNP patterns in all clades allowed us to suggest additional putative recombination events in the phylogeny of these *Synechococcus* lineages (open colored stars in **Figure 2A** and Supplemental Data Presentation Figure 10; also see Supplemental Data Sheet Section XI and Supplemental Data Presentation Figure 21). For instance, all variants grouped into the clade containing MLSA7 PEs A2-A4 appear to have vertically inherited a recombinant *CHP* gene after an early recombination event (closed green star) and variants within MLSA7 PEs A2, A3, and a portion of PEA4, appear to have vertically inherited an *rbsK* sequence from a
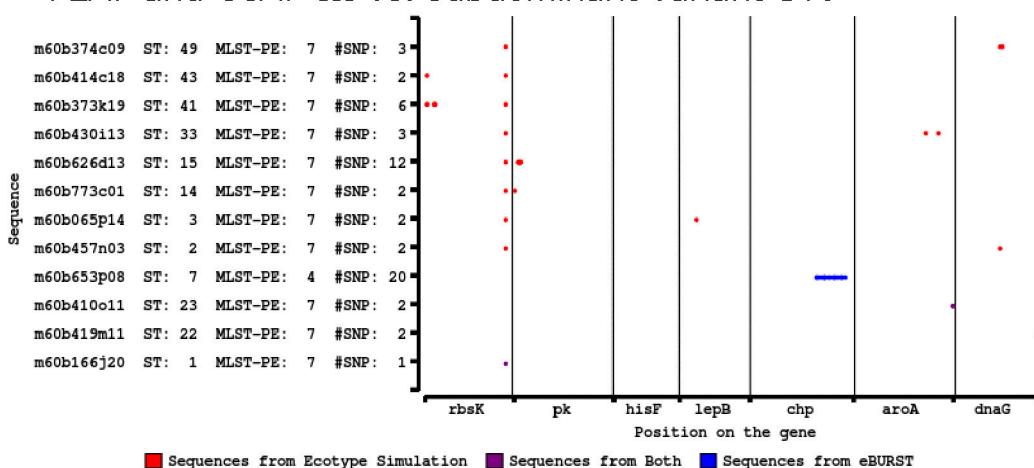
FIGURE 5 | Single nucleotide polymorphism patterning of A-like *Synechococcus* BACs grouped around the same dominant variant (also consensus sequence) by Ecotype Simulation and eBURST. Variants detected only by eBURST (blue), only by Ecotype Simulation (red) or by both (purple) are compared to the shared dominant variant (consensus sequence). **(A)** Dominant variant ST1 in PEA7 and clonal complex A7-I and **(B)** subdominant variant ST6 in PE7 and clonal complex A7-III. STs correspond with STs in **Figure 2A**.

subsequent recombination event (closed blue star). A third putative recombination event involving the *rbsK* locus (open blue star) may distinguish MLSA7 PEs A2 and A3 from MLSA7 PE A4.

These SNP analyses also revealed several examples of singleton MLSA7 PEs associated with recombination patterns involving a large number of SNPs [MLSA7 PEs A1 (ST4), A2 (ST27), A8 (ST12), and A11 (ST25); see **Figure 2A**]. These singleton-PE variants formed long-branches and were closely related to sister PEs comprised of multiple variants, but different in many SNPs from the dominant variant of that PE. For example, in **Figures 2A**, **5A**, singleton-PE A8 (ST12) was closely related to PE A7, but differed by 19 SNPs in the *dnaG* locus, suggesting that a recent recombination event might have caused enough sequence difference to prevent its accurate demarcation by Ecotype Simulation. There was only one case in which an identical SNP pattern shared by two STs (ST25 in singleton MLSA7 PE A11 and ST 34 in MLSA7 PE A9; *lepB*) was not

clade-specific (**Figure 2** and Supplemental Data Presentation Figure 21 panel I).

Similar analysis was performed on the *Synechococcus* B'-like BACs with the interesting result that 2–3 recombination events were associated with some divergences, even though only 4 loci were included in the B'-like MLSA4 phylogeny (Supplemental Data Sheet Section VIII and Supplemental Data Presentation Figures S10, S12, S13).

DISCUSSION

Recombination within and Between Lineages of *Synechococcus*

There is abundant evidence that genomic rearrangements and recombination have influenced the evolution of the *Synechococcus* populations inhabiting the Mushroom Spring microbial mat. As in previous investigations of *Sulfolobus*

islandicus (Cadillo-Quiroz et al., 2012), *Wolbachia* (Ellegaard et al., 2013), and *Vibrio cyclitrophicus* (Shapiro et al., 2012) mentioned above, recombination between the divergent A and B' lineages of *Synechococcus* appears to be less frequent than recombination within each lineage. Specifically, only approximately 2% of BACs exhibited evidence of recombination between A- and B'-like *Synechococcus* BACs (see Results, Supplemental Data Sheet Section VI and Supplemental Data Table 14). This corroborates our previous metagenomic analyses, in which we found a low percentage of metagenomic clones with end sequences stemming from both the A-like and B'-like lineages (Klatt et al., 2011) (Supplemental Data Sheet Section VI). In contrast, within-lineage recombination was greater, with 5.5% of A-like and 33.3% of B'-like *Synechococcus* BACs exhibiting evidence of recombination for approximately equal numbers of loci analyzed (MLSA5, 145 sequences and MLSA4, 72 sequences for *Synechococcus* A- and B'-like BACs respectively; **Table 3**).

Recombination events have occurred at various times in the history of the A-like and B'-like clades: some recombinant segments are fixed in large subclades (i.e., evidenced by clustering of SNPs in MLSA PEs 2, 3, and 4 in Supplemental Data Presentation Figures 21A,D,G or MLSA PEs 10, 11, 12, and 13 in Supplemental Data Presentation Figures 21C,F,I; for analogous B'-like fixed recombinant segments see Supplemental Data Presentation Figure 13), indicating a recombination event early in the clade's history followed by vertical inheritance of the recombined gene, while others are specific only to singleton (one doubleton) variants demarcated as PEs based on recent recombination in single loci (i.e., MLSA PEs 1, 2, 8, 10, 11, and 13 in **Figure 2A**). These results suggest that recent recombination events might cause overestimation of PEs by Ecotype Simulation. That is, a single recombination event, if involving a long enough fragment, can cause Ecotype Simulation to split a PE. In the case of eBURST, a single recombination event of any size cannot split a consensus group; however, two or more recombinants of any size will separate a recipient organism into a different consensus group.

The rates of recombination within the A and B' lineages were estimated to be less than or about the same as that of mutation (**Tables 1**, **2**). However, we determined that recombination had a somewhat greater impact than mutation on sequence change within these populations (**Tables 1**, **2**; Clonal Frame, r/m range 1.58–4.92). The impact of recombination (r/m) was estimated at 2.6 and 2.4, for the *Synechococcus* A-like and B'-like lineages, respectively. These r/m ratios are similar to values reported for other cyanobacteria (Vos and Didelot, 2009) and are in the same range as those reported for *S. islandicus*, *Wolbachia*, and *Halorubrum* (Papke et al., 2004; Whitaker et al., 2005; Ellegaard et al., 2013). The recent study on *Synechococcus* by Rosen et al. (2015), did not provide r/m, but their estimate of the ratio of recombination to mutation rates (ρ/θ) was close to 1, which fell within the R/θ range provided by our LDHat analysis (**Tables 1**, **2**). Why do such different interpretations arise from such similar results? Although, Rosen et al. interpreted from a very similar recombination/mutation ratio that recombination is "ubiquitous," we note that this ratio is not extraordinarily high when compared against the many groups of bacteria whose

recombination rates were quantified by Vos and Didelot (2009). Furthermore, Rosen et al. have not considered the theoretical work of Haldane (1932), which shows that such low rates of recombination cannot prevent ecological diversification.

Given the rates of mutation estimated for various bacteria (Drake, 2009; Vos and Didelot, 2009), we may conclude that a typical gene in the *Synechococcus* populations is experiencing recombination at an extremely low *absolute* rate, so that recombination between nascent species is unlikely to hinder their divergence (Haldane, 1932; Vos, 2011; Wiedenbeck and Cohan, 2011). Low-frequency recombination may allow shuffling of niche-neutral alleles, which do not determine ecological niche, but low-frequency recombination of *niche-determining* alleles from other populations can be countered by natural selection (Haldane, 1932). Maladaptive niche-determining alleles from other populations will be kept at negligible levels, maintaining the integrity of populations' adaptations (Wiedenbeck and Cohan, 2011). Rates of recombination observed in *Synechococcus* should not lead us to dismiss *a priori* the possibility of ecological diversification within either clade.

We therefore disagree with Rosen et al.'s conclusion (Rosen et al., 2015) that frequent recombination has caused each of the A and B' clades of *Synechococcus* to be a "quasisexual population," where close relatives are prevented from diversifying into long-standing, ecologically homogeneous populations, or ecotypes. While Rosen et al. (2015) have shown a shuffling of alleles (really gene segments) within the A and B' clades, they have not shown that the recombination has randomized associations between niche-determining alleles, which are responsible for ecotype divergence. Moreover, we do not see how they could rule out ecological diversification without studying ecology. As we have shown in many previous papers, as well as in the present study, there is ample evidence for the coexistence of long-standing, ecologically divergent populations within both the A and B' clades, as we discuss below. Rosen et al. mentioned some of these studies, but did not use an ecological model in their work, as we have done here.

As with most (if not all) bacteria, recombination has been sufficient in *Synechococcus* to create new ecological diversity. Within the A lineage, for example, low-light PEs differ from high-light PEs in having acquired a gene cluster likely coding for an additional photoreceptor (Olsen et al., 2015). It does not take a recombination rate much higher than that of mutation to transfer a gene that is adaptive in a new niche. Only one adaptive transfer of a gene (or one mutation event); see Bantinaki et al. (2007) is required to found a new ecotype and thereby bring about ecological diversification. On the other hand, a much higher rate of recombination is required to prevent adaptive divergence between nascent populations; this requires recurrent inter-population transfer of genes poorly adapted to their new niche (Wiedenbeck and Cohan, 2011).

Ecological Diversification within the A-like and B'-like *Synechococcus* Lineages

The Ecotype Simulation analysis of concatenated MLSA sequences hypothesized 13 and 29 PEs within the A-like and B'-like clades, respectively. This was greater than the

number of PEs predicted from single-locus analyses of the most divergent locus, *rbsK*. This is likely because of greater resolution provided by MLSA7 (as well as MLSA4; see Supplemental Data Presentation Figure 10), as evidenced by the fact that MLSA7 split numerous PEs that were lumped by *rbsK* analysis. We were able to demonstrate the ecological distinctness of some of the A-like putative ecotypes, first with respect to the two habitat types investigated in this study (60 and 65°C). That is, some hypothesized A-like ecotypes were sampled entirely from different habitats, while others were quantitatively different in their habitat associations. Also, pyrosequencing of PCR-amplified *rbsK* genes typical of different MLSA7 PEs demonstrated fine-scale distribution differences of MLSA7 PEs along vertical gradients. Additionally, the availability of genomes of isolates representative of MLSA7 PEs permitted us to demonstrate correspondence of *rbsK* distributions with distributions observed earlier in *psaA* analyses (Becraft et al., 2015). This led to demonstration of adaptation consistent with distribution patterns (Nowack et al., 2015) and genomic evidence of mechanisms likely to underlie these adaptations (Olsen et al., 2015). For instance, MLSA PEs 5 and 9 correspond to *psaA* PEs A6 and A14, whose distribution deep in the upper green layer of the mat (Becraft et al., 2015) is consistent with the presence of auxiliary photosystem genes that are likely to be associated with the ability to acclimate to low irradiance (Nowack et al., 2015; Olsen et al., 2015); and see Supplemental Data Sheet Section X). Becraft et al. (2011, 2015) also describe the existence and ecological distinction of B'-like *Synechococcus* ecotypes, as well as the ecological interchangeability of the members of each ecotype.

We conclude that both the A-like and B'-like *Synechococcus* lineages have clearly diversified into many younger species populations, whose niches correspond in part to differences along temperature and vertical gradients (Becraft et al., 2015). Our approach did not permit us to evaluate the role of positive selection within our dataset as our genes were under neutral/purifying selection. Evaluation of full genomes of strains of closely related species will allow us to further examine the roles that recombination, mutation, selection and neutral processes have played in the diversification of *Synechococcus*.

Whither the Biological Species Concept?

That recombination has occurred more frequently within closely related clades than between them has previously been taken as support for the Biological Species Concept in bacteria and archaea (Cadillo-Quiroz et al., 2012; Shapiro et al., 2012; Ellegaard et al., 2013). However, the quintessential idea of the Biological Species Concept is that sexual isolation is required for irreversible divergence and ecological distinctness in the origin of species (Cohan, 2013). Support for the Biological Species Concept requires more than a correlation between sexual isolation and divergence among clades; one must demonstrate that reduced recombination was necessary for their divergence.

We argue that such evidence is lacking in the above-cited studies on *Vibrio, Wolbachia,* and *Sulfolobus*. As seen in the present work, *Synechococcus* lineages have been noted to have

recombination levels similar to those reported for *Halorubrum* and *Sulfolobus*. Like these organisms, major *Synechococcus* clades that are highly divergent (3.6% diverged at the 16S rRNA locus and 18% diverged in average nucleotide identity (ANI), Bhaya et al., 2007) are ecologically distinct. However, our high-resolution analysis of closest relatives within each clade has shown ecological speciation among organisms that have identical or nearly identical 16S rRNA sequences and have diverged only on the order of 1% ANI (Olsen et al., 2015; also see Supplemental Data Table 25) and show minimal sexual isolation. Similarly, in work on another system we have found ecotype differentiation among *Bacillus* clades that show very little sexual isolation (Majewski and Cohan, 1999; Connor et al., 2010). We hypothesize that analysis of fine-scale ecological divergence within *Halorubrum* or *Sulfolobus* may yield similar results (Papke et al., 2007; Cadillo-Quiroz et al., 2012). Recombination does not appear to have impeded ecological diversification among close relatives that are not sexually isolated. Thus, sexual isolation, in this context, is unnecessary for the origin of species (Wiedenbeck and Cohan, 2011).

Instead, sexual isolation is more likely to be the result of divergence among closely related groups than the cause. Not only do ecologically distinct populations tend to diverge over time into separate sequence clusters (Cohan and Perry, 2007), but ecological divergence may also cause occupancy of different microhabitats, reducing opportunities for genetic exchange (Shapiro et al., 2012); in addition, functional divergence may result in reduced fitness of recombinants (Retchless and Lawrence, 2010). For other mechanisms of sexual isolation, also see Wiedenbeck and Cohan (2011). Regarding evidence for the Biological Species Concept provided by previous bacterial and archaeal studies, we note that two of these studies have provided no direct evidence that the groups of sequences studied have distinct ecological properties (Cadillo-Quiroz et al., 2012; Ellegaard et al., 2013), and rely primarily on the coexistence of sequence clusters as evidence that they utilize different resources. A third study by Shapiro et al looking at genetic exchange between two recently diverged populations of *Vibrio* (Shapiro et al., 2012) reported similar patterns of sub-clustering within the *Vibrio* populations (their Figure S2), and we predict that these sub-clusters would show ecological diversification if the authors were to test this. We predict each subgroup would represent an ecotype, subject to genomic sweeps. The fourth study (Rosen et al., 2015) claimed that there cannot be discrete ecotypes within the *Synechococcus* A and B' clades, owing to recombination, yet the existence of such discrete ecotypes has been amply demonstrated here and in many earlier papers (Becraft et al., 2015; Nowack et al., 2015; Olsen et al., 2015).

Most importantly, an argument for the Biological Species Concept should provide evidence that ecological diversification was prevented among groups hypothesized to recombine more frequently. This would require a search for ecological diversification among the close relatives recombining at the highest rates. While previous studies have not attempted to search for ecological diversity among closest relatives (within the major clades); this has been a principal aim of our study.

CONCLUSION

In conclusion, it appears that we have found some of the most newly divergent ecotypes of hot spring *Synechococcus*. They do recombine, and recombination has not prevented their divergence, as had been suggested by Doolittle and Zhaxybayeva (2009). Because a reduction in recombination does not appear necessary for ecological divergence, and because ecological patterning of bacterial and archaeal diversity is common, the Biological Species Concept does not appear appropriate for bacterial and archaeal speciation. While recombination has not hindered speciation, ongoing genomic and metagenomic analyses have begun to reveal evidence suggesting that (i) horizontal genetic transfer and recombination have likely led to ecological diversification by introducing niche-transcending adaptations to new lineages (Gogarten et al., 2002; Lawrence, 2002; Doolittle and Zhaxybayeva, 2009; Wiedenbeck and Cohan, 2011; Olsen et al., 2015), and (ii) mutation and selection have also played a role in the evolutionary ecology of *Synechococcus* ecological species (Olsen et al., 2015). Sub-clustering within major clades predicted to be ecologically distinct has been observed across a variety of taxa and habits (Smith et al., 2006; Zinser et al., 2006; Hunt et al., 2008; Connor et al., 2010; Denef et al., 2010; Mazard et al., 2012), suggesting that our observations may have general importance to the issue of species and speciation in bacteria and archaea. It is important to keep in mind that the molecular dimension of microbial species may be smaller than we have previously thought, and that it is important to understand theories underlying species and speciation.

MATERIALS AND METHODS

Samples were collected on 2 October 2003 from the effluent channel of Mushroom Spring (44.5386°N, 110.7979°W), an alkaline siliceous hot spring in the Lower Geyser Basin of Yellowstone National Park, WY, at two temperatures, 60 and 65°C. DNA extraction and construction of bacterial artificial chromosome (BAC) clones and oligonucleotide probe screening to identify BACs containing a *Synechococcus* A/B lineage-specific 16S rRNA gene are described in the Supplemental Data Sheet Section I. DNA from parallel samples was used for analyses of the 16S rRNA and ITS region on the BACs (Ward et al., 2006) and PCR-amplified single protein-encoding loci (*rbsK, aroA,* and *apcAB*) (Melendrez et al., 2011), as well as for construction of small-insert (2–12 kb) metagenomic libraries (Bhaya et al., 2007; Klatt et al., 2011).

Locus Selection

From one hundred genes that were upstream and downstream of the two unlinked 16S rRNA genes in the *Synechococcus* strain A and B' genomes, loci were selected based on the following characteristics: (i) presence in both genomes, (ii) range of distances from the 16S rRNA locus, (iii) high degree of nucleotide divergence between *Synechococcus* strain A and B' homologs, (iv) high average nucleotide divergence and variance of metagenomic homologs from *Synechococcus* strain A and B' homologs, (v) not under positive evolutionary selection (dN/dS values < 1) and (vi)

functionally useful for gene expression studies. In addition, loci adjacent to transposons or mobile elements were avoided due to the greater possibility of co-migration of adjacent loci. Loci used in this study are presented in Supplemental Data Tables 1, 2.

PCR Amplification and Sequencing of BAC MLSA Loci

Primers for the separate amplification of *Synechococcus* A-like and B'-like BAC target genes (Supplemental Data Tables 1, 2) were designed and tested as described for amplification of *rbsK*, *aroA,* and *apcAB* gene segments by Melendrez et al. (2011), which also provides details of PCR conditions and gel-based size verification and purification of amplicons. Cycling conditions for the *dnaG, pcrA, ispE, sufB,* and *argD* genes were the same used for *aroA* and *rbsK* genes, and cycling conditions for the protein kinase (*PK*), *lepB*, and *accC* genes were the same as for the *apcAB* gene, except for the use of 40 cycles instead of 30 cycles. Cycling conditions for *hisF* and the conserved hypothetical protein (*CHP* [locus tag CYA_2291]) gene were: an initial denaturing step at 94°C (2 min) followed by 20 cycles of 94°C (1 min), 60°C (1 min), and 72°C (1 min); then 20 cycles of 94°C (1 min), 55°C (1 min), and 72°C (1 min) with a final extension at 72°C for 10 min and storage at 4°C. BAC clones exhibiting no PCR product for any gene were amplified two more times (directly from the BAC clone sample) to increase confidence that the gene was absent.

Sequencing

Purified PCR products were sequenced using the forward and reverse primers for each gene described in Supplemental Data Tables 1, 2 and the BigDye v.3.1 cycle sequencing kit (Applied Biosystems) at the University of Nevada-Reno Sequence Center (Reno, NV). The sequences have been submitted to GenBank, via the BankIt submission tool; Accession numbers: *pcrA*; KT425377-KT425447, *rbsK;* HQ662694-HQ662843, HQ187926-187996, KT425519-KT425589, and KT426096-KT426240, 16S rRNA; KT425590-KT425660, *PK* (protein kinase); KT425661-KT425805, *CHP* (conserved hypothetical protein); KT425806-KT425950, *lepB*; KT425951-KT426095, *aroA*; HQ187854-HQ187925, KT425447-KT425518, and KT426241-426385, *dnaG*; KT426386-KT426455, *hisF*; KT426456-KT426525.

Sequence Alignment and Phylogenetic Analysis

Bidirectional sequence data for each locus were analyzed using Sequencher v.4.8. Sequence data were then analyzed with NCBI-BLAST nr/nt (Altschul et al., 1990) to determine whether the top match was with genomic homologs of *Synechococcus* strains JA-3-3Ab [accession: CP000239.1], JA-2-3B'a(2-13) [accession: CP000240.1], or neither. Sequences used in MLSA were concatenated using a custom perl script (available from J.M. Wood) and uploaded in MEGA4. Alignments were made using the ClustalW algorithm in the MEGA4 software (Tamura et al., 2007) or the MUSCLE (Edgar, 2004) algorithm implemented in Seaview (Gouy et al., 2010). The *Synechococcus* strain A and B' genomes were included as references (Bhaya et al.,

2007). MLSA including newly obtained isolates are described in Supplemental Data Sheet Section X. Alignments were analyzed using jModeltest2 (Darriba et al., 2012) to determine the best model fit for maximum likelihood tree construction. Maximum likelihood trees were constructed using PhyML (Guindon et al., 2010) as implemented in Seaview (Gouy et al., 2010) with aLRT support and edited using MEGA4 (Tamura et al., 2007) or Figtree (Rambaut, 2009). Estimates of average evolutionary divergence for single and multi-locus phylogenies were computed over all sequence pairs using the Maximum Composite Likelihood method in MEGA4 as previously described (Tamura et al., 2004, 2007; Melendrez et al., 2011).

Ecotype Simulation and Demarcation

Concatenated and single-locus sequence alignments were analyzed using Ecotype Simulation to predict the number of PEs (n), rates of periodic selection (sigma), ecotype formation (omega), and 95% confidence intervals (CI) for all parameters at the best precision match between observed and simulated data for that sequence dataset (between 1.25x and 2x). Ecotypes were manually demarcated conservatively as previously described (Cohan and Perry, 2007; Koeppel et al., 2008; Becraft et al., 2011; Melendrez et al., 2011) (http://fcohan.web.wesleyan.edu/ecosim/). Statistical tests were conducted using the R statistical package (http://cran.r-project.org/). Groups (rows) were defined as PEs that contained >5 sequences. The variables being tested were temperature (60 and 65°C) and vertical (80 μm intervals) distributions. Significance level was set at 0.05.

Multi-Locus and eBURST Analyses

Alignments for each gene used in MLSA were organized into groups that were 100% identical at the nucleotide level using Sequencher v 4.8. Allele types were assigned for each unique sequence and were used to generate allelic profiles; BACs with identical allelic profiles at all loci were assigned as unique sequence types (STs), as described in Supplemental Data Tables 8, 9, 19, 20. Allelic profiles and their unique ST designation were uploaded into eBURST (Feil et al., 2004; Spratt et al., 2004) and population snapshots were generated to view A-like and B'-like *Synechococcus* diversity. Clonal complexes (CC) were defined as a consensus group of BACs and at least 3 single-locus variants, as suggested (Feil et al., 2004). Population snapshots to visualize CCs with less stringent criteria were generated by defining CCs as a consensus group with at least 2 single-locus variants and/or allowing for double-locus variants to be included in the CC and are defined as sub-clonal complexes (see Supplemental Data Sheet Section XII).

Single Nucleotide Polymorphism Analysis

SNPs were analyzed by comparing sequences of PE clade variants and single-locus variants with that of the dominant variant of the same PE clade or consensus group of the same CC (often the same as the dominant variant) using the Perl program, Pigeon (https://github.com/sandain/pigeon). Pigeon reads in the FASTA file and compares each nucleotide in the dominant variant to the corresponding nucleotide in each PE variant (or single-locus variant), locating and reporting the position of SNPs.

Detection of Recombination Signals

Five methods were utilized to detect recombination in single-gene and concatenated datasets; (i) outlier detection on phylogeny followed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), (ii) RDP version 4 (RDP4) (Martin et al., 2015), (iii) Clonal Frame version 1.1 (Didelot and Falush, 2007), (iv) SNP analysis (PIGEON; https://github.com/sandain/pigeon), and (v) LDHat v2.2 (McVean et al., 2002).

(i) BLAST. Phylogenies were manually inspected for long branch lengths. Those STs/clones which fell on long branch lengths were analyzed using BLAST (bl2seq program) against isolate genomes (CP000239.1 and CP000240.1) to ensure that all genes within the concatenation had the highest match to the appropriate isolate genome as determined by 16S rRNA analysis (also see Supplemental Data Sheet Section VI).

(ii) RDP identifies recombinants in either multi-locus or single-gene sequence datasets. All loci for A-like and B'-like *Synechococcus* populations were tested for putative recombination events using Recombination Detection Program, version 4 (RDP4) (Martin et al., 2015). The loci were tested individually and as concatenated sequences. In the RDP suite of programs a number of different methods are implemented. The methods used for recombination detection in this study included the RDP method (Martin et al., 2005), GENECONV (Sawyer, 1989), Maximum Chi Square (Smith, 1992; Posada and Crandall, 2001), Chimera (Posada and Crandall, 2001), Sister Scanning (Siscan) (Gibbs et al., 2000), 3SEQ (Boni et al., 2007), and Likelihood Assisted Recombination Detection (LARD), which constitute some of the most powerful methods currently available (RDP4 manual, Martin et al., 2005). The general settings within RDP4 were as follows: the highest acceptable *p*-value was set to 0.05 with Bonferroni corrections. For the individual methods default parameters were used for all methods with the following exceptions:

In RDP, the window size was set to 30 as recommended (RDP4 Instruction Manual, available at http://web.cbio.uct.ac.za/~darren/RDP4Manual.pdf). In MaxChi and Chimera the 'variable window size' was used. In Siscan the window size was set to 200 bp with a step size of 20.

Recombination signals were considered present if they could be detected by at least 3 methods within the RDP4 package at significant *p*-values as suggested (RDP4 manual).

(iii) Clonal Frame identifies evidence of recombination in multi-locus sequence datasets (Didelot and Falush, 2007). Extended multi-FASTA formatted alignments (XMFA) were generated using the MAUVE program (Darling et al., 2004, 2007) and given file extensions of.dat. DAT files were loaded into Clonal Frame and analyzed using default settings with 1,000,000 iterations after burn in, 250,000 burnin,

sampling every 100 iterations and verbose mode (-v,-x,-y,-z options). OUT files with phylogenies and statistics (mutation events, recombination events, rho/theta and r/m) were visualized using the cfgui.bat program included in the Clonal Frame installation. Recombination events identified along branches are noted with closed stars for MLSA datasets in **Figure 2A** and Supplemental Data Presentation Figure 10.

(iv) SNP analysis. The patterning of PE variant and single-locus variant SNPs against the dominant variant sequence was also considered in interpreting possible evidence of recombination events (method described above). Recombination events inferred by SNP analysis are noted with open stars on branches for MLSA datasets in **Figure 2A** and Supplemental Data Presentation Figure 10.

(v) LDHat estimates a per-locus population recombination parameter (R), the average per-site population mutation rate (Watterson's θ), average pairwise distance (avePWD) and variance within the average pairwise difference (varPWD). These were determined using three methods within the LDHat v2.2a package (McVean et al., 2002); the composite-likelihood method, Rmin calculation and Wakeley's moment method (all implemented using convert, pairwise and associated lookup tables generated either from precompiled tables included with package distribution or generated directly from the sequence data). All analyses were run using default parameters. The recombination rate calculated by LDHat assumes a constant recombination rate over the region and a gene conversion model with either a finite sites model (composite likelihood) or infinite sites model (Rmin calculation). We note that the linkage of our loci caused by sampling a portion of the genome as well as sequencing only a portion of each gene may possibly cause underestimation or large range of recombination ratios if recombined segments tend to span several genes or if the recombined segment was larger than the amplified gene segment thereby capturing one 'breakpoint' but not the other (see Supplemental Data Sheet Section VI).

Pyrosequencing

PCR primers and protocols described by Melendrez et al. (2011) or in Supplemental Data Table 1 were used to amplify *rbsK* sequences from Mushroom Spring samples collected at ∼63–65°C and sectioned along vertical gradients (∼80 μm intervals using a cryotome). Samples were collected on 13 and 14 September 2008 and DNA was extracted as described in Becraft et al. (2011). Barcoding and Ti454-sequencing were completed at the J. Craig Venter Institute according to the GS FLX Titanium Series Rapid Library Preparation Method. DNA was

sheared using the Covaris S2 System, and qPCR was used to accurately estimate the number of molecules needed for emPCR. BAC clone MLSA sequences were trimmed at the 5′ and 3′ ends to match the average sequence length, which collapsed some SNP variation contained in the *rbsK* single-locus analysis and caused a decrease in molecular resolution. Homopolymer inserts were removed by aligning sequences to the *rbsK* reading frame of genomes from the A or B' *Synechococcus* isolates. High-frequency sequences (sequences totaling ≥50 identical copies in all combined samples) were then identified and PEs were demarcated from high-frequency sequences using Ecotype Simulation. Population percentages based on high-frequency sequences that matched STs of a single MLSA PE were calculated as described in Becraft et al. (2015). Sequence data for *rbsK* was deposited into the MG-RAST version 4 database (https://metagenomics.anl.gov/) under accession numbers 4642340.3-4642367.3.

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01540

REFERENCES

Allewalt, J. P., Bateson, M. M., Revsbech, N. P., Slack, K., and Ward, D. M. (2006). Effect of temperature and light on growth of and photosynthesis by *Synechococcus* isolates typical of those predominating in the octopus

spring microbial mat community of Yellowstone National Park. *Appl. Environ. Microb.* 72, 544–550. doi: 10.1128/AEM.72.1.544-550.2006

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Bantinaki, E., Kassen, R., Knight, C. G., Robinson, Z., Spiers, A. J., and Rainey, P. B. (2007). Adaptive divergence in experimental populations of Pseudomonas fluorescens. III. Mutational origins of wrinkly spreader diversity. *Genetics* 176, 441–453. doi: 10.1534/genetics.106.069906

Becraft, E. D., Cohan, F. M., Kühl, M., Jensen, S. I., and Ward, D. M. (2011). Fine-scale distribution patterns of *Synechococcus* ecological diversity in microbial mats of Mushroom Spring, Yellowstone National Park. *Appl. Environ. Microb.* 77, 7689–7697. doi: 10.1128/AEM.05927-11

Becraft, E. D., Wood, J. M., Rusch, D. B., Kühl, M., Jensen, S. I., Bryant, D. A., et al. (2015). The molecular dimension of microbial species: 1. Ecological distinctions among, and homogeneity within, putative ecotypes of *Synechococcus* inhabiting the cyanobacterial mat of Mushroom Spring, Yellowstone National Park. *Front. Microbiol.* 6:590. doi: 10.3389/fmicb.2015.00590

Bhaya, D., Grossman, A. R., Steunou, A. S., Khuri, N., Cohan, F. M., Hamamura, N., et al. (2007). Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. *ISME J.* 1, 703–713. doi: 10.1038/ismej.2007.46

Boni, M. F., Posada, D., and Feldman, M. W. (2007). An exact nonparametric method for inferring mosaic structure in sequence triplets. *Genetics* 176, 1035–1047. doi: 10.1534/genetics.106.068874

Cadillo-Quiroz, H., Didelot, X., Held, N. L., Herrera, A., Darling, A., Reno, M. L., et al. (2012). Patterns of gene flow define species of thermophilic Archaea. *PLoS Biol.* 10:e1001265. doi: 10.1371/journal.pbio.1001265

Cesarini, S., Bevivino, A., Tabacchioni, S., Chiarini, L., and Dalmastri, C. (2009). RecA gene sequence and Multilocus Sequence Typing for species-level resolution of *Burkholderia cepacia* complex isolates. *Lett. Appl. Microbiol.* 49, 580–588. doi: 10.1111/j.1472-765X.2009.02709.x

Cohan, F. M. (2013). "Species," in *Brenner's Encyclopedia of Genetics, 2nd Edn.*, eds S. Maloy and K. Hughes (Amsterdam: Elsevier), 506–511.

Cohan, F. M. (2016). "Prokaryotic species concepts," in *Encyclopedia of Evolutionary Biology*, ed R. M. Kliman (Oxford: Elsevier).

Cohan, F. M., and Perry, E. B. (2007). A systematics for discovering the fundamental units of bacterial diversity. *Curr. Biol.* 17, R373–R86. doi: 10.1016/j.cub.2007.03.032

Connor, N., Sikorski, J., Rooney, A. P., Kopac, S., Koeppel, A. F., Burger, A., et al. (2010). Ecology of speciation in the genus *Bacillus*. *Appl. Environ. Microb.* 76, 1349–1358. doi: 10.1128/AEM.01988-09

Coyne, J. A., and Orr, H. A. (2004). *Speciation*. Sunderland: Sinauer Associates.

Darling, A. C., Mau, B., Blattner, F. R., and Perna, N. T. (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 14, 1394–1403. doi: 10.1101/gr.2289704

Darling, A. E., Treangen, T. J., Messeguer, X., and Perna, N. T. (2007). Analyzing patterns of microbial evolution using the mauve genome alignment system. *Methods Mol. Biol.* 396, 135–152. doi: 10.1007/978-1-59745-515-2_10

Darriba, D., Taboada, G. L., Doallo, R., and Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods.* 9, 772. doi: 10.1038/nmeth.2109

Denef, V. J., Kalnejais, L. H., Mueller, R. S., Wilmes, P., Baker, B. J., Thomas, B. C., et al. (2010). Proteogenomic basis for ecological divergence of closely related bacteria in natural acidophilic microbial communities. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2383–2390. doi: 10.1073/pnas.0907041107

Didelot, X., and Falush, D. (2007). Inference of bacterial microevolution using multilocus sequence data. *Genetics* 175, 1251–1266. doi: 10.1534/genetics.106.063305

Didelot, X., and Maiden, M. C. (2010). Impact of recombination on bacterial evolution. *Trends Microbiol.* 18, 315–322. doi: 10.1016/j.tim.2010.04.002

Dobzhansky, T. (1951). *Genetics and the Origin of Species, 3rd Edn.* New York, NY: Columbia University Press.

Doolittle, W. F., and Zhaxybayeva, O. (2009). On the origin of prokaryotic species. *Genome Res.* 19, 744–756. doi: 10.1101/gr.086645.108

Drake, J. W. (2009). Avoiding dangerous missense: thermophiles display especially low mutation rates. *PLoS Genet.* 5:e1000520. doi: 10.1371/journal.pgen.1000520

Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5:113. doi: 10.1186/1471-2105-5-113

Ellegaard, K. M., Klasson, L., Naslund, K., Bourtzis, K., and Andersson, S. G. (2013). Comparative genomics of *Wolbachia* and the bacterial species concept. *PLoS Genet.* 9:e1003381. doi: 10.1371/journal.pgen.1003381

Eren, A. M., Maignien, L., Sul, W. J., Murphy, L. G., Grim, S. L., Morrison, H. G., et al. (2013). Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data. *Methods Ecol. Evol.* 4, 1111–1119. doi: 10.1111/2041-210X.12114

Feil, E. J., Cooper, J. E., Grundmann, H., Robinson, D. A., Enright, M. C., Berendt, T., et al. (2003). How clonal is *Staphylococcus aureus*? *J. Bacteriol.* 185, 3307–3316. doi: 10.1128/JB.185.11.3307-3316.2003

Feil, E. J., Enright, M. C., and Spratt, B. G. (2000a). Estimating the relative contributions of mutation and recombination to clonal diversification: a comparison between *Neisseria meningitidis* and *Streptococcus pneumoniae*. *Res. Microbiol.* 151, 465–469. doi: 10.1016/S0923-2508(00)00168-6

Feil, E. J., Holmes, E. C., Bessen, D. E., Chan, M. S., Day, N. P. J., Enright, M. C., et al. (2001). Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences. *Proc. Natl. Acad. Sci. U.S.A.* 98, 182–187. doi: 10.1073/pnas.98.1.182

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P., and Spratt, B. G. (2004). eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.* 186, 1518–1530. doi: 10.1128/JB.186.5.1518-1530.2004

Feil, E. J., Maiden, M. C. J., Achtman, M., and Spratt, B. G. (1999). The relative contributions of recombination and mutation to the divergence of clones of *Neisseria meningitidis*. *Mol. Biol. Evol.* 16, 1496–1502. doi: 10.1093/oxfordjournals.molbev.a026061

Feil, E. J., Smith, J. M., Enright, M. C., and Spratt, B. G. (2000b). Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. *Genetics* 154, 1439–1450. Available online at: http://www.genetics.org/content/154/4/1439.long

Feil, E. J., and Spratt, B. G. (2001). Recombination and the population structures of bacterial pathogens. *Annu. Rev. Microbiol.* 55, 561–590. doi: 10.1146/annurev.micro.55.1.561

Ferris, M. J., Kühl, M., Wieland, A., and Ward, D. M. (2003). Cyanobacterial ecotypes in different optical microenvironments of a 68 degrees C hot spring mat community revealed by 16S-23S rRNA internal transcribed spacer region variation. *Appl. Environ. Microb.* 69, 2893–2898. doi: 10.1128/AEM.69.5.2893-2898.2003

Ferris, M. J., and Ward, D. M. (1997). Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl. Environ. Microb.* 63, 1375–1381.

Gibbs, M. J., Armstrong, J. S., and Gibbs, A. J. (2000). Sister-Scanning: a monte carlo procedure for assessing signals in recombinant sequences. *Bioinformatics* 16, 573–582. doi: 10.1093/bioinformatics/16.7.573

Gogarten, J. P., Doolittle, W. F., and Lawrence, J. G. (2002). Prokaryotic evolution in light of gene transfer. *Mol. Biol. Evol.* 19, 2226–2238. doi: 10.1093/oxfordjournals.molbev.a004046

Gouy, M., Guindon, S., and Gascuel, O. (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* 27, 221–224. doi: 10.1093/molbev/msp259

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010

Haldane, J. B. S. (1932). *The Causes of Evolution*. London: Longmans, Green and Co.

Hanage, W. P., Fraser, C., and Spratt, B. G. (2005). Fuzzy species among recombinogenic bacteria. *BMC Biol.* 3:6. doi: 10.1186/1741-7007-3-6

Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J., and Polz, M. F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science* 320, 1081–1085. doi: 10.1126/science.1157890

Kashtan, N., Roggensack, S. E., Rodrigue, S., Thompson, J. W., Biller, S. J., Coe, A., et al. (2014). Single-cell genomics reveals hundreds of coexisting subpopulations in wild *Prochlorococcus*. *Science* 344, 416–420. doi: 10.1126/science.1248575

Klatt, C. G., Wood, J. M., Rusch, D. B., Bateson, M. M., Hamamura, N., Heidelberg, J. F., et al. (2011). Community ecology of hot spring cyanobacterial mats:

predominant populations and their functional potential. *ISME J.* 5, 1262–1278. doi: 10.1038/ismej.2011.73

Koeppel, A., Perry, E. B., Sikorski, J., Krizanc, D., Warner, A., Ward, D. M., et al. (2008). Identifying the fundamental units of bacterial diversity: a paradigm shift to incorporate ecology into bacterial systematics. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2504–2509. doi: 10.1073/pnas.0712205105

Kopac, S., and Cohan, F. M. (2011). "A theory-based pragmatism for discovering and classifying newly divergent bacterial species," in *Genetics and Evolution of Infectious Diseases,* ed M. Tibayrenc (London: Elsevier), 21–41.

Kopac, S., Wang, Z., Wiedenbeck, J., Sherry, J., Wu, M., and Cohan, F. M. (2014). Genomic heterogeneity and ecological speciation within one subspecies of *Bacillus subtilis. Appl. Environ. Microb.* 80, 4842–4853. doi: 10.1128/AEM.00576-14

Lawrence, J. G. (2002). Gene transfer in bacteria: speciation without species? *Theor. Popul. Biol.* 61, 449–460. doi: 10.1006/tpbi.2002.1587

Levin, B. R., and Bergstrom, C. T. (2000). Bacteria are different: observations, interpretations, speculations, and opinions about the mechanisms of adaptive evolution in prokaryotes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6981–6985. doi: 10.1073/pnas.97.13.6981

Liles, M. R., Williamson, L. L., Rodbumrer, J., Torsvik, V., Goodman, R. M., and Handelsman, J. (2008). Recovery, purification, and cloning of high-molecular-weight DNA from soil microorganisms. *Appl. Environ. Microb.* 74, 3302–3305. doi: 10.1128/AEM.02630-07

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., et al. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. U.S.A.* 95, 3140–3145. doi: 10.1073/pnas.95.6.3140

Majewski, J., and Cohan, F. M. (1999). DNA sequence similarity requirements for interspecific recombination in Bacillus. *Genetics* 153, 1525–1533.

Mallet, J. (2008). Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 363, 2971–2986. doi: 10.1098/rstb.2008.0081

Martin, D. P., Murrell, B., Golden, M., Khoosal, A., and Muhire, B. (2015). RDP4: detection and analysis o recombination patterns in virus genoems. *Virus Evolution.* 1:vev003. doi: 10.1093/ve/vev003

Martin, D. P., Williamson, C., and Posada, D. (2005). RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics* 21, 260–262. doi: 10.1093/bioinformatics/bth490

Mayr, E. (1942). *Systematics and the Origin of Species from the Viewpoint of a Zoologist.* New York, NY: Columbia University.

Mayr, E. (1963). *Animal Species and Evolution.* Cambrige, MA: Belknap Press of Harvard University Press.

Mazard, S., Ostrowski, M., Partensky, F., and Scanlan, D. J. (2012). Multi-locus sequence analysis, taxonomic resolution and biogeography of marine *Synechococcus. Environ. Microbiol.* 14, 372–386. doi: 10.1111/j.1462-2920.2011.02514.x

McVean, G. A. T., Myers, S. R., Hunt, S., Deloukas, P., Bentley, D. R., and Donnelly, P. (2004). The fine-scale structure of recombination rate variation in the human genome. *Science* 304, 581–584. doi: 10.1126/science.1092500

McVean, G., Awadalla, P., and Fearnhead, P. (2002). A coalescent-based method for detecting and estimating recombination from gene sequences. *Genetics* 160, 1231–1241. Available online at: http://www.genetics.org/content/160/3/1231.long

Melendrez, M. C., Lange, R. K., Cohan, F. M., and Ward, D. M. (2011). Influence of molecular resolution on sequence-based discovery of ecological diversity among *Synechococcus* populations in an alkaline siliceous hot spring microbial mat. *Appl. Environ. Microb.* 77, 1359–1367. doi: 10.1128/AEM.02032-10

Nowack, S., Olsen, M. T., Schaible, G., Becraft, E. D., Shen, G., Klapper, I., et al. (2015). The molecular dimension of microbial species: 2. *Synechococcus* isolates representative of putative ecotypes inhabiting different depths in the Mushroom Spring microbial mat exhibit different adaptive and acclimative responses to light. *Front. Microbiol.* 6:626. doi: 10.3389/fmicb.2015.00626

Olsen, M. T., Nowack, S., Wood, J. M., Becraft, E. D., LaButti, K., Lipzen, A., et al. (2015). The molecular dimension of microbial species: 3. Comparative genomics of *Synechoccocus* isolates with different light responses and *in situ* diel transcription patterns of associated putative ecotypes in the Mushroom Spring microbial mat. *Front. Microbiol.* 6:604. doi: 10.3389/fmicb.2015.00604

Papke, R. T., Koenig, J. E., Rodriguez-Valera, F., and Doolittle, W. F. (2004). Frequent recombination in a saltern population of *Halorubrum. Science* 306, 1928–1929. doi: 10.1126/science.1103289

Papke, R. T., Zhaxybayeva, O., Feil, E. J., Sommerfeld, K., Muise, D., and Doolittle, W. F. (2007). Searching for species in haloarchaea. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14092–14097. doi: 10.1073/pnas.0706358104

Polz, M. F., Alm, E. J., and Hanage, W. P. (2013). Horizontal gene transfer and the evolution of bacterial and archaeal population structure. *Trends Genet.* 29, 170–175. doi: 10.1016/j.tig.2012.12.006

Posada, D., and Crandall, K. A. (2001). Evaluation of methods for detecting recombination from DNA sequences: computer simulations. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13757–13762. doi: 10.1073/pnas.241370698

Rambaut, A. (2009). *FigTree v1. 3.1: Tree Figure Drawing Tool.* Available online at: http://treebioedacuk/software/figtree/

Ramsing, N. B., Ferris, M. J., and Ward, D. M. (2000). Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. *Appl. Environ. Microb.* 66, 1038–1049. doi: 10.1128/AEM.66.3.1038-1049.2000

Retchless, A. C., and Lawrence, J. G. (2010). Phylogenetic incongruence arising from fragmented speciation in enteric bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 107, 11453–11458. doi: 10.1073/pnas.1001291107

Rosen, M. J., Davison, M., Bhaya, D., and Fisher, D. S. (2015). Microbial diversity. Fine-scale diversity and extensive recombination in a quasisexual bacterial population occupying a broad niche. *Science* 348, 1019–1023. doi: 10.1126/science.aaa4456

Rusch, D. B., Halpern, A. L., Sutton, G., Heidelberg, K. B., Williamson, S., Yooseph, S., et al. (2007). The sorcerer II global ocean sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. *PLoS Biol.* 5, 398–431. doi: 10.1371/journal.pbio.0050077

Salerno, A., Delétolle, A., Lefevre, M., Ciznar, I., Krovacek, K., Grimont, P., et al. (2007). Recombining population structure of *Plesiomonas shigelloides* (Enterobacteriaceae) revealed by multilocus sequence typing. *J. Bacteriol.* 189, 7808–7818. doi: 10.1128/JB.00796-07

Sawyer, S. (1989). Statistical tests for detecting gene conversion. *Mol. Biol. Evol.* 6, 526–538.

Schluter, D. (2009). Evidence for ecological speciation and its alternative. *Science* 323, 737–741. doi: 10.1126/science.1160006

Shapiro, B. J., Friedman, J., Cordero, O. X., Preheim, S. P., Timberlake, S. C., Szabó, G., et al. (2012). Population genomics of early events in the ecological differentiation of bacteria. *Science* 336, 48–51. doi: 10.1126/science.1218198

Shizuya, H., Birren, B., Kim, U. J., Mancino, V., Slepak, T., Tachiiri, Y., et al. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8794–8797. doi: 10.1073/pnas.89.18.8794

Smith, J. M. (1992). Analyzing the mosaic structure of genes. *J. Mol. Evol.* 34, 126–129. doi: 10.1007/BF00182389

Smith, N. H., Gordon, S. V., de la Rua-Domenech, R., Clifton-Hadley, R. S., and Hewinson, R. G. (2006). Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis. Nat. Rev. Microbiol.* 4, 670–681. doi: 10.1038/nrmicro1472

Spratt, B. G., Hanage, W. P., Li, B., Aanensen, D. M., and Feil, E. J. (2004). Displaying the relatedness among isolates of bacterial species - the eBURST approach. *Fems Microbiol. Lett.* 241, 129–134. doi: 10.1016/j.femsle.2004.11.015

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599. doi: 10.1093/molbev/msm092

Tamura, K., Nei, M., and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11030–11035. doi: 10.1073/pnas.0404206101

Tao, Q., Wang, A., and Zhang, H. B. (2002). One large-insert plant-transformation-competent BIBAC library and three BAC libraries of Japonica rice for genome research in rice and other grasses. *Theor. Appl. Genet.* 105, 1058–1066. doi: 10.1007/s00122-002-1057-3

Van Valen, L. (1976). Ecological species, multispecies, and oaks. *Taxon* 25, 233–239. doi: 10.2307/1219444

Vitorino, L. R., Margos, G., Feil, E. J., Collares-Pereira, M., Zé-Zé, L., and Kurtenbach, K. (2008). Fine-scale phylogeographic structure of borrelia

lusitaniae revealed by multilocus sequence typing. *PLoS ONE* 3:e4002. doi: 10.1371/journal.pone.0004002

Vos, M. (2011). A species concept for bacteria based on adaptive divergence. *Trends Microbiol.* 19, 1–7. doi: 10.1016/j.tim.2010.10.003

Vos, M., and Didelot, X. (2009). A comparison of homologous recombination rates in bacteria and archaea. *Isme J.* 3, 199–208. doi: 10.1038/ismej.2008.93

Ward, D. M., Bateson, M. M., Ferris, M. J., Kühl, M., Wieland, A., Koeppel, A., et al. (2006). Cyanobacterial ecotypes in the microbial mat community of Mushroom Spring (Yellowstone National Park, Wyoming) as species-like units linking microbial community composition, structure and function. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 361, 1997–2008. doi: 10.1098/rstb.2006.1919

Ward, D. M., Ferris, M. J., Nold, S. C., and Bateson, M. M. (1998). A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol. Mol. Biol. Rev.* 62, 1353–1370.

Whitaker, R. J., Grogan, D. W., and Taylor, J. W. (2005). Recombination shapes the natural population structure of the hyperthermophilic archaeon *Sulfolobus islandicus. Mol. Biol. Evol.* 22, 2354–2361. doi: 10.1093/molbev/msi233

Wiedenbeck, J., and Cohan, F. M. (2011). Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiol. Rev.* 35, 957–976. doi: 10.1111/j.1574-6976.2011.00292.x

Zinser, E. R., Coe, A., Johnson, Z. I., Martiny, A. C., Fuller, N. J., Scanlan, D. J., et al. (2006). *Prochlorococcus* ecotype abundances in the North Atlantic Ocean as revealed by an improved quantitative PCR method. *Appl. Environ. Microb.* 72, 723–732. doi: 10.1128/AEM.72.1.723-732.2006

Characterization of a single mutation in TraQ in a strain of *Escherichia coli* partially resistant to Qβ infection

Akiko Kashiwagi*, Hikari Kitamura and Fumie Sano Tsushima

Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Japan

Bacteria and virulent bacteriophages are in a prey–predator relationship. Experimental models under simplified conditions with the presence of bacteria and bacteriophages have been used to elucidate the mechanisms that have enabled both prey and predator to coexist over long periods. In experimental coevolution conducted with *Escherichia coli* and the virulent RNA bacteriophage Qβ in serial transfer, both coexisted for at least for 54 days, during which time they continued to change genetically and phenotypically. By day 16, an *E. coli* strain partially resistant to Qβ appeared and caused an approximately 10^4-fold decrease in Qβ amplification. Whole-genome analysis of this strain suggested that a single mutation in TraQ was responsible for the partially resistant phenotype. TraQ interacts with propilin, encoded by the *traA* gene and a precursor of pilin, which is a component of the F pilus. The present study was performed to elucidate the mechanism underlying the coexistence of *E. coli* and Qβ by investigating how a mutation in TraQ altered the physiological state of *E. coli*, and thus the amplification of Qβ. Overexpression of wild-type TraQ in the partially resistant *E. coli* strain resulted in recovery of both TraA protein content, including propilin and pilin, and Qβ amplification to levels comparable to those observed in the susceptible strain. Intriguingly, overexpression of the mutant TraQ in the partially resistant strains also increased the levels of TraA protein and Qβ amplification, but these increases were smaller than those observed in the wild-type strain or the partially resistant strain expressing wild-type TraQ. The results of this study represent an example of how *E. coli* can become partially resistant to RNA bacteriophage infection via changes in a protein involved in maturation of a receptor rather than in the receptor itself and of how *E. coli* can stably coexist with virulent RNA bacteriophages.

Keywords: coevolution, prey–predator, experimental evolution, virulent phage, partial resistance

INTRODUCTION

There have long been ecological and theoretical investigations regarding why predators do not eradicate their prey (Murdoch and Oaten, 1975; Anderson and May, 1978; Alexander, 1981; Berryman, 1992; Abrams, 2000; Briggs and Hoopes, 2004; Pettorelli et al., 2011; Brockhurst and Koskella, 2013). Bacteria and bacteriophages have been used as model systems of prey–predator interactions to elucidate the fundamental mechanism underlying this issue. Due to their short generation time, large population size, and ease of analyzing phenotypic and genomic changes, theoretical and experimental evolutionary research have been conducted extensively using these systems (Campbell, 1961; Levin et al., 1977; Lenski and Levin, 1985; Lenski, 1988; Schrag and Mittler, 1995; Sasaki, 2000; Paterson et al., 2010; Dennehy, 2012; Brockhurst and Koskella, 2013; Bull et al., 2014). The stable coexistence of bacteria and virulent phages has been widely observed in experimental evolution (Horne, 1970; Chao et al., 1977; Levin et al., 1977; Buckling and Rainey, 2002; Lythgoe and Chao, 2003; Kerr et al., 2006; Kashiwagi and Yomo, 2011; Marston et al., 2012), even though virulent phages kill the host bacteria to release progeny phages.

The existence of refuges for sensitive bacteria or the occurrence of endless arms races were suggested to be necessary to explain the coexistence of bacteria and bacteriophages (Chao et al., 1977; Lenski, 1988; Schrag and Mittler, 1995; Brockhurst et al., 2006). The numerical refuge in which density-dependent protection of susceptible cells from over-predation (Chao et al., 1977), spatial refuges such as wall populations on flasks in continuous culture or solid media used in serial passage (Chao et al., 1977; Lenski, 1988; Schrag and Mittler, 1995), and physiological refuges in which cells become transiently resistant or susceptible have been discussed (Lenski, 1988). In most of these previous studies, DNA bacteriophages, such as T2, T5, T7, λ, and Φ2, were used (Bohannan and Lenski, 2000; Buckling and Rainey, 2002; Dennehy, 2012). Although a great deal of knowledge has been accumulated regarding DNA bacteriophages, little is known about RNA bacteriophages in terms of stable coexistence.

In our previous study, *Escherichia coli* and the lytic RNA bacteriophage Qβ (Qβ) were shown to coevolve for at least 54 days, equivalent to 165 generations, under conditions of serial passage with shaking (Kashiwagi and Yomo, 2011). Qβ is a bacteriophage with a single-stranded RNA genome that specifically infects and lyses *E. coli* cells to release progeny phages (Van Duin and Tsareva, 2006). Phenotypic and genomic analyses indicated the coevolution of both *E. coli* and Qβ. In the course of experimental coevolution,

partially resistant *E. coli* appeared in the 54th generation (16th day). Detailed analysis of the partially resistant *E. coli* is necessary to determine how *E. coli* and Qβ coexist in this experimental coevolution system. Genetic analysis revealed a single mutation in the *traQ* gene in the day-16 *E. coli* population (Kashiwagi and Yomo, 2011). It was reported that TraQ protein is a chaperone for insertion of propilin encoded by the *traA* gene (Moore et al., 1982; Kathir and Ippen-Ihler, 1991), and propilin was also reported to be unstable in *traQ*⁻ cells (Maneewannakul et al., 1993). The 13-kDa propilin is processed by peptidase to a 7-kDa pilin and pilin proteins are assembled into filaments (i.e., the F pilus). Qβ adsorbs the F pilus of *E. coli* at the first step of infection (Van Duin and Tsareva, 2006), and the adsorption rate of Qβ on partially resistant cells estimated by first-order kinetics decreased markedly (Kashiwagi and Yomo, 2011). Therefore, the partially resistant phenotype of *E. coli* to Qβ infection observed in coevolution may be correlated with F pilus biosynthesis, especially TraA, and we focused on the relationships among mutation in TraQ, TraA content, and Qβ amplification.

Here, we report that a single amino acid change in TraQ was linked to the reduction of TraA content in the *E. coli* population. In addition, this decrease was recovered by supplying ancestral (wild-type) or mutant-type TraQ from an expression vector, and the ability of Qβ to amplify in the cell also recovered. These results represent one example of how *E. coli* can become partially resistant to RNA bacteriophage infection, which involves changes in a protein related to the maturation of a receptor, in this case the F pilus, rather than changes to the receptor itself. These results suggest that the mutation in TraQ may cause heterogeneity within the *E. coli* population, with a small number of cells supporting the phage population and a large number of cells supporting the *E. coli* population without Qβ infection, even though the *E. coli* cells were genetically identical.

MATERIALS AND METHODS

STRAINS, CULTURE MEDIA, AND PLASMID DNA

Escherichia coli Anc(C), the partially Qβ infection-resistant mutant strain, M54(C) (Kashiwagi and Yomo, 2011), and DH1 $\Delta leuB$::($gfpuv5$-Km^r) (hereafter called LKG; Kishimoto et al., 2010) were used to characterize the effects of S21P mutation in TraQ protein and a control F⁻ strain. *E. coli* A/λ (Watanabe et al., 1979) was used as the host strain for titer assay. LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; Nakalai Tesque, Kyoto, Japan) was used.

To construct TraQ with a Strep-tag II sequence (Schmidt and Skerra, 2007) at the C-terminus of the ancestral-type TraQ (TraQ$_{Anctag}$) and mutant-type TraQ (TraQ$_{S21Ptag}$), the *traQ* gene was amplified by PCR with Anc(C) and M54(C) genome as the template, the primers traQ_XbaI and traQ_strep_HindIII, and Phusion® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). The oligonucleotide DNAs used in this study are listed in **Table 1**. The *traQ*$_{Anctag}$ and *traQ*$_{S21Ptag}$ genes with *Xba*I and *Hin*dIII sites at both ends were subcloned into the *Xba*I/*Hin*dIII sites of pASK-IBA3plus (IBA Biologics GmbH, Goettingen, Germany). The resulting plasmid DNAs were designated as pASK-traQ$_{Anctag}$ and pASK-traQ$_{S21Ptag}$, respectively.

Table 1 | Oligonucleotide DNA sequence.

Primer name	Sequence (5′→3′)
F_4f_2	ATCAGCGCAATAATTGCCGC
F_4r_2	CGATTATTCCCGTCACGATG
Linker_r	ATTGATGGTGCCTACAG
pACYC_rev2	CCACACATTATACGAGCCG
traA1	GACGAGTGAATTTGGAAAAA
	AACGACTTCTTTTTGACGGGC
	GCAGAAGCACCCTGAACAC
traA_f	ATGAATGCTGTTTTAAGTGT
traA_r	TCAGAGGCCAACGACGGCCA
traA_r2	GGCCATACCCACAGCAATAA
traQ_XbaI	TCTAGAAGGAGATATACAATG
	ATAAGTAAACGCAGATT
traQ_strep_HindIII	AAGCTTATTATTTTTCGAACTG
	CGGGTGGCTCCAGTGAGAGA
	CATGTCCGCCCT
5PpACYC_rev	pTCGGCTCGTATAATGTGTGG
16SrRNA_1	GCTGCCTCCCGTAGGAGT

DNA SEQUENCING OF THE *traQ* GENE OF ANCESTRAL AND PARTIALLY RESISTANT *E. coli*

To determine the *traQ* gene sequneces of Anc(C) and M54(C), the *traQ* region in 10 colonies each of Anc(C) and M54(C) was amplified by PCR with the primers F_4f_2 and F_4r_2 and Phusion® High-Fidelity DNA polymerase (New England BioLabs), and PCR products were directly sequenced by the dideoxynucleotide chain termination sequencing method (Sanger et al., 1977).

ESTIMATION OF Qβ AMPLIFICATION

Anc(C)/pASK-traQ$_{Anctag}$, Anc(C)/pASK-traQ$_{S21Ptag}$, Anc(C)/pASK-IBA3plus, M54(C)/pASK-traQ$_{Anctag}$, M54(C)/pASK-traQ$_{S21Ptag}$, and M54(C)/pASK-IBA3plus were cultured in 5 mL of LB with 100 μg/mL ampicillin overnight and 50 μL of the culture was inoculated into 5 mL of LB with 100 μg/mL ampicillin for approximately 2 h. Aliquots of 1 mL of the culture were transferred into 4 mL of LB medium with 100 μg/mL ampicillin and 100 nM doxycycline-HCl (Dox) and cultured for a further 2 h. Qβ was added to infect the bacterial cells and the free phage was separated immediately or 4 h after infection by centrifugation at 13400 × *g* for 1 min. The free phage in the supernatant was diluted, the number of plaque forming units per milliliter was determined (PFU/mL), and the amplification ratio was calculated as $x = N_4/N_0$, where N_4 and N_0 represent 4 h after and initial (0 h) free phage density, respectively. The titer assay was conducted according to the standard method described previously (Carlson, 2005).

WESTERN BLOTTING ANALYSIS

A polyclonal antibody to TraA protein raised against the keyhole limpet hemocyanin-conjugated peptide (CDLMASGNTTVKAT-FGKDSS) was obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Cell preculture was conducted as described in the Section "Estimation of Qβ Amplification." For induction with 100 nM Dox, Dox was added to the 2-h culture and cells were cultured for a further 5.75 h. Without Dox induction, the cells were cultured for 7.25 h in LB medium. Proteins from the cells obtained from 0.1 mL of culture with $OD_{600} = 2.0$ were subjected to SDS-PAGE using Any kD^{TM} Mini-PROTEAN® TGX^{TM} precast gels (Bio-Rad Laboratories, Hercules, CA, USA), and the TraA protein was determined by Western blotting analysis with anti-TraA antibody and HRP-conjugated goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as the primary and secondary antibodies, respectively, diluted with Can Get Signal® immunoreaction enhancer solution (Toyobo Co. Ltd., Osaka, Japan). TraQ with a Strep-tag II at the C-terminus was detected using Precision Protein^{TM} Strep-Tactin-HRP conjugate (Bio-Rad Laboratories). The signals were detected with Chemi-Lumi One L (Nakalai Tesque).

NORTHERN HYBRIDIZATION ANALYSIS

To compare the *traA* mRNA contents by Northern hybridization, Anc(C), M54(C), and LKG with pASK-IBA3plus were cultured in LB medium and total RNA was extracted from the cells in logarithmic phase using the SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Total RNA from cells obtained from 0.16 mL of culture with $OD_{600} = 0.27$ was subjected to Northern hybridization using a digoxigenin-labeled single-stranded DNA probe, i.e., traA1 for *traA* and 16SrRNA_1 for 16SrRNA, as an indicator of the amount of total RNA used (Moran et al., 1995). The signals were detected with CDP-Star (GE Healthcare UK Ltd., Little Chalfont, UK). DynaMarker® Prestain Marker for RNA High (BioDynamics Laboratory Inc., Tokyo, Japan) was used to obtain the standard curve for RNA length.

DETERMINATION OF 5′- AND 3′-TERMINAL SEQUENCES OF *traA* mRNA

To determine the 5′-terminal sequence of *traA* mRNA, cDNA was synthesized using total RNA of Anc(C) as the template, SuperScript® III reverse transcriptase (Life Technologies, Carlsbad, CA, USA), and traA_r primer. The 5′-phosphorylated DNA linker 5PpACYC_rev was ligated at the 3′-terminus of the first strand cDNA with T4 RNA ligase 1 (New England Biolabs). PCR was performed using the resultant cDNA as the template, PrimeSTAR® HS DNA polymerase (Takara Bio Inc., Shiga, Japan), and the primers pACYC_rev2 and traA_r2. To determine the 3′-terminal sequence of *traA* mRNA, universal miRNA cloning linker (5′-rAppCTGTAGGCACCATCAAT-NH2-3′; New England Biolabs) was ligated with the 3′-terminus of total RNA of Anc(C) using T4 RNA ligase 1 (New England Biolabs). The first strand cDNA was synthesized using the primer Linker_r and SuperScript® III reverse transcriptase (Life Technologies), and then purified cDNA was subjected to PCR using the primers Linker_r and traA_f. Three and two bands of PCR products for 5′- and 3′-terminal sequence determination, respectively, were sliced from the gel and subcloned using a Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (Life Technologies). Three to six clones were randomly picked and sequenced by the dideoxynucleotide chain termination sequencing method.

STATISTICAL ANALYSIS

Amplification ratios were compared by one-way ANOVA with the *post hoc* Tukey's test (Zar, 2010). In all analyses, values of $\log_{10}(N_4/N_0)$ of each strain were used for statistical analysis. The Studentized range, *q*, is shown in the text. In all analyses, $P < 0.01$ was taken to indicate statistical significance.
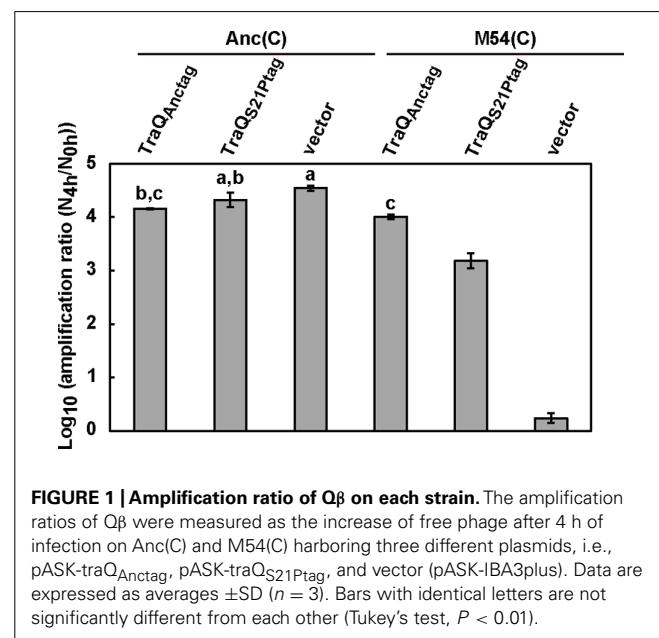
RESULTS

RECOVERY OF Qβ AMPLIFICATION IN RESISTANT *E. coli* BY SUPPLYING TraQ

We first analyzed the *traQ* gene sequences from 10 single colonies derived from the coevolved *E. coli* population to confirm that the majority harbored the T61C mutation. We picked 10 single colonies from each of Anc(C) and M54(C) populations, which were the initial and day-16 *E. coli* populations in the coevolution experiment (Kashiwagi and Yomo, 2011). All 10 colonies of Anc(C) had T and all 10 colonies of M54(C) had C at position 61, and this T61C mutation resulted in S21P in the TraQ protein.

We analyzed the amplification ratio of Qβ on Anc(C) and M54(C) that harbored only the vector (pASK-IBA3plus) to determine the extent of reduction in the amplification ratio of Qβ on M54(C). Anc(C) and M54(C) expressed inherent ancestral-type TraQ protein and mutant-type TraQ protein from the F plasmid, respectively. The amplification ratio of Qβ was calculated as described in the Section "Materials and Methods." The amplification ratios of Qβ on Anc(C)/pASK-IBA3plus and M54(C)/pASK-IBA3plus were 3.5×10^4 and 1.8, respectively (**Figure 1**). Although the amplification ratio on M54(C) was much lower than that on Anc(C), Qβ could undergo amplification on M54(C), indicating that M54(C) had a partially rather than fully resistant phenotype.

To analyze whether ancestral-type TraQ expression in M54(C) could compensate for Qβ amplification in this strain, ancestral-type TraQ with the Strep-tag II sequence at the C-terminus

FIGURE 1 | Amplification ratio of Qβ on each strain. The amplification ratios of Qβ were measured as the increase of free phage after 4 h of infection on Anc(C) and M54(C) harboring three different plasmids, i.e., pASK-traQ$_{Anctag}$, pASK-traQ$_{S21Ptag}$, and vector (pASK-IBA3plus). Data are expressed as averages ±SD (*n* = 3). Bars with identical letters are not significantly different from each other (Tukey's test, $P < 0.01$).

was expressed from the P_{tetA} promoter by doxycycline (Dox) induction. We designated ancestral and mutant-type TraQ with Strep-tag II sequence at the C-terminus expressed from the expression vector as $TraQ_{Anctag}$ and $TraQ_{S21Ptag}$ to allow them to be distinguished from the inherent $TraQ_{Anc}$ and $TraQ_{S21P}$ derived from the F plasmid, respectively. First, to examine whether $TraQ_{Anctag}$ overexpression altered the amplification ratio of Qβ on Anc(C), we compared the amplification ratios of Qβ on Anc(C)/pASK-traQ$_{Anctag}$ and Anc(C)/pASK-IBA3plus under conditions of Dox induction. The amplification ratio of Qβ on Anc(C) overexpressing $TraQ_{Anctag}$ was 1.4×10^4, which was lower than that on Anc(C) harboring only the vector, 3.5×10^4, suggesting that overexpression of TraQ may be slightly deleterious for Qβ amplification (one-way ANOVA $F_{5,12} = 920$, $P < 0.01$; *post hoc* Tukey's test $q = 7.3$, $P < 0.01$; **Figure 1**). Second, the amplification ratio of Qβ on M54(C) overexpressing $TraQ_{Anctag}$ was 1.0×10^4, which was greater than the value of 1.8 on M54(C) carrying only the vector (one-way ANOVA $F_{5,12} = 920$, $P < 0.01$; *post hoc* Tukey's test $q = 70.5$, $P < 0.01$), and the amplification ratio of M54(C) overexpressing $TraQ_{Anctag}$ was comparable to that of Anc(C)/pASK-TraQ$_{Anctag}$ (one-way ANOVA $F_{5,12} = 920$, $P < 0.01$; *post hoc* Tukey's test $q = 2.76$, $P = 0.42$; **Figure 1**). These results showed that the amplification ratio of Qβ on M54(C) was recovered by supplying $TraQ_{Anctag}$. Intriguingly, supplying mutant-type TraQ, $TraQ_{S21Ptag}$, to M54(C) also rescued the amplification of Qβ on this strain. When $TraQ_{S21Ptag}$ was overexpressed by Dox induction in M54(C), the amplification ratio of Qβ on the strain was 1.6×10^3, which was greater than that of M54(C) with the vector alone (one-way ANOVA $F_{5,12} = 920$, $P < 0.01$; *post hoc* Tukey's test $q = 55.1$, $P < 0.01$), but was lower than that of M54(C) overexpressing $TraQ_{Anctag}$ (one-way ANOVA $F_{5,12} = 920$, $P < 0.01$; *post hoc* Tukey's test $q = 15.4$, $P < 0.01$; **Figure 1**). These results indicated that overexpression of mutant-type TraQ in M54(C) could partially, but not completely, compensate for the decrease in amplification of Qβ on the partially resistant cells.

RECOVERY OF Qβ AMPLIFICATION LINKED TO AN INCREASE IN TraA
We analyzed the TraA content by Western blotting to investigate the links between the mutation in TraQ and TraA content.

Proteins derived from the same cell numbers calculated by the values of OD_{600} were subjected to SDS-PAGE. When we compared the levels of TraA produced by Anc(C) and M54(C) with vector (pASK-IBA3plus) and Anc(C) without the vector, the signal strength of TraA of Anc(C) with vector was almost the same as that of Anc(C) without the vector independent of Dox induction (**Figure 2**), but the TraA content of M54(C) with vector was extremely low independent of Dox induction (**Figure 2**). As no signal was detected in the lane for the F$^-$ control strain, LKG, M54(C) had not entirely lost TraA. These results showed that M54(C) had markedly decreased propilin and/or pilin content.

The expression levels of ancestral and mutant-type TraQ with the Strep-tag II sequence at its C-terminus were determined with Strep-Tactin. When $TraQ_{Anctag}$ was expressed in M54(C), the signal level for TraA was almost the same as that of Anc(C; **Figure 2**). In this case, the TraA contents were independent of Dox induction. Next, we supplied $TraQ_{S21Ptag}$ to M54(C) with and without Dox induction. The TraA content in M54(C) increased with Dox induction, but the level was lower than that in M54(C) supplied with $TraQ_{Anctag}$ (**Figure 2**). The TraA content of M54(C) also increased without Dox induction, but the strength of the signal was smaller than that under conditions of Dox induction. With supply of $TraQ_{S21Ptag}$, the TraA content was dependent on the expression level of $TraQ_{S21Ptag}$. These observations indicated that a small amount of ancestral-type TraQ in the partially resistant cells was sufficient to recover the TraA content, and a large amount of mutant-type TraQ protein increased the TraA content but the level was lower than that of the ancestral cells. Therefore, these results showed that a single mutation in TraQ resulted in a decrease of TraA content in the partially resistant cells.

traA mRNA EXPRESSION LEVEL IN M54(C)
To determine whether the decrease of TraA protein content in the partially resistant cells was due to a decrease in *traA* mRNA content, we analyzed the *traA* mRNA level by Northern hybridization. No signal was detected in the lane for the F$^-$ control strain LKG (**Figure 3A**), while signals were observed in the lanes for the Anc(C) and M54(C) strains (**Figure 3A**). The difference in
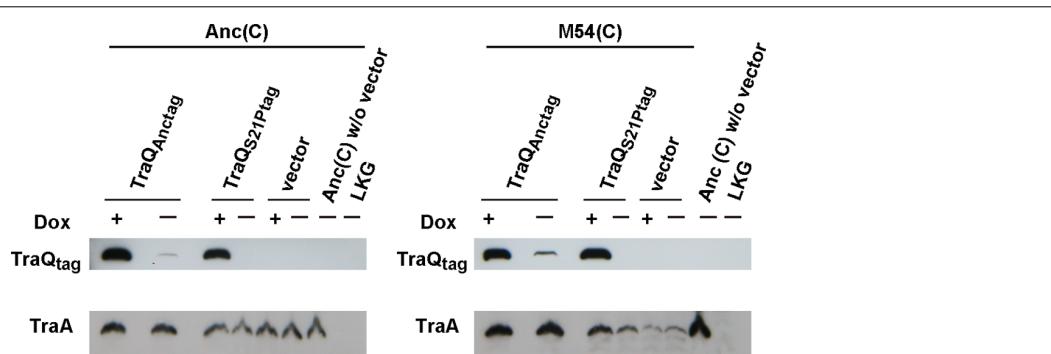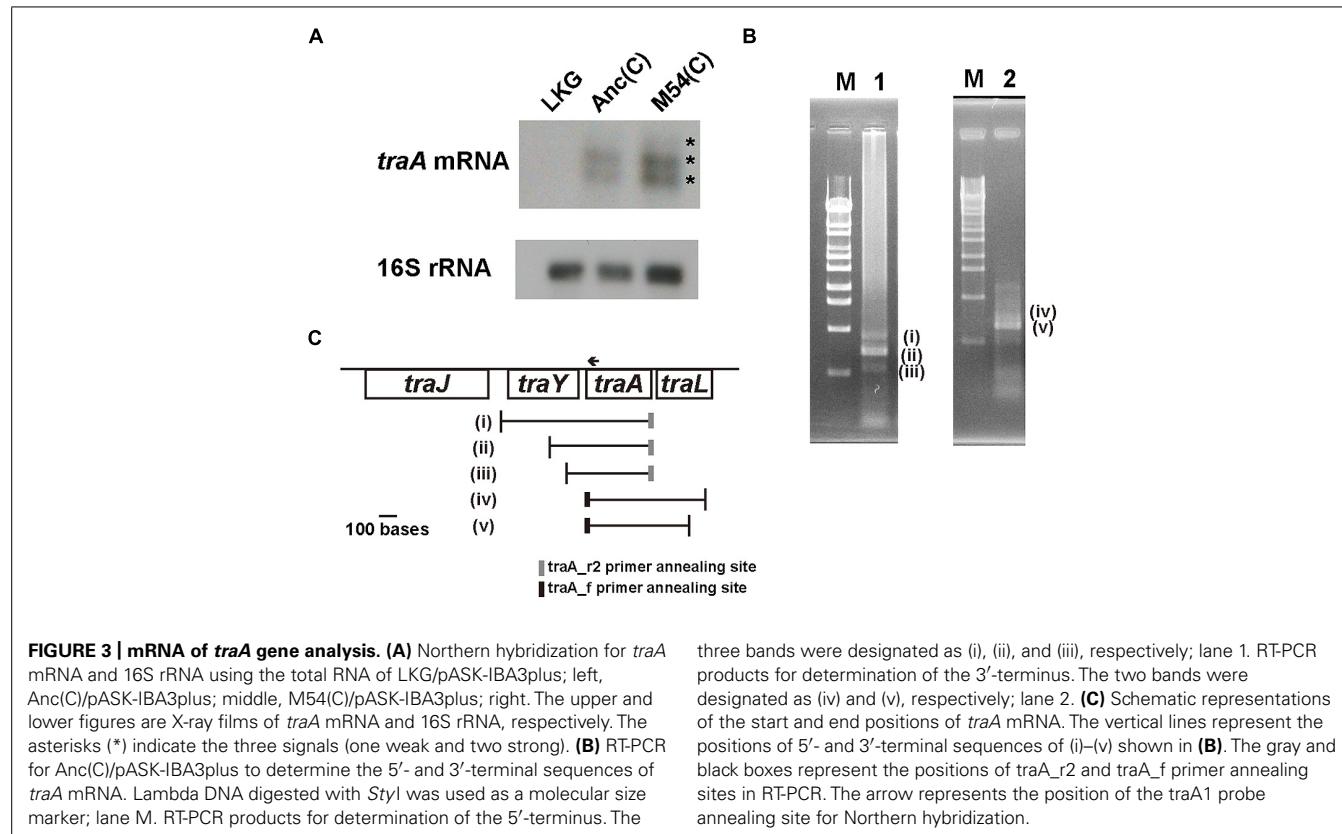
FIGURE 2 | TraA expression analysis. The TraA and TraQ with Strep-tag II sequence contents of Anc(C) and M54(C) harboring three different plasmids, i.e., pASK-traQ$_{Anctag}$, pASK-traQ$_{S21Ptag}$, and vector (pASK-IBA3plus), were analyzed. Anc(C) without vector and LKG were used as control strains that expressed and did not express TraA, respectively. (+) and (−) represent with and without 100 nM Dox, respectively. Top and bottom represent Strep-Tactin detection for TraQ with Strep-tag II sequence at the C-terminus and anti-TraA detection for TraA.

FIGURE 3 | mRNA of *traA* gene analysis. (A) Northern hybridization for *traA* mRNA and 16S rRNA using the total RNA of LKG/pASK-IBA3plus; left, Anc(C)/pASK-IBA3plus; middle, M54(C)/pASK-IBA3plus; right. The upper and lower figures are X-ray films of *traA* mRNA and 16S rRNA, respectively. The asterisks (*) indicate the three signals (one weak and two strong). **(B)** RT-PCR for Anc(C)/pASK-IBA3plus to determine the 5′- and 3′-terminal sequences of *traA* mRNA. Lambda DNA digested with *Sty*I was used as a molecular size marker; lane M. RT-PCR products for determination of the 5′-terminus. The three bands were designated as (i), (ii), and (iii), respectively; lane 1. RT-PCR products for determination of the 3′-terminus. The two bands were designated as (iv) and (v), respectively; lane 2. **(C)** Schematic representations of the start and end positions of *traA* mRNA. The vertical lines represent the positions of 5′- and 3′-terminal sequences of (i)–(v) shown in **(B)**. The gray and black boxes represent the positions of traA_r2 and traA_f primer annealing sites in RT-PCR. The arrow represents the position of the traA1 probe annealing site for Northern hybridization.

signal strength of *traA* mRNA between Anc(C) and M54(C) may have been due to differences in the amount of total RNA loaded per lane, which was determined based on the 16SrRNA signal strength, although equal amounts of total RNA were loaded in each lane as calculated from the OD_{600}. Therefore, we assumed that the mRNA levels were the almost same for Anc(C) and M54(C).

As shown in **Figure 3A**, three bands were observed on Northern hybridization; one was weak and two were strong. The three bands corresponded to approximately 800, 610, and 460 bases, respectively, calculated using the standard curve obtained with the molecular weight markers. As the *traA* gene is 366 bases in length, the mRNA may contain upstream and/or downstream sequences. To determine the 5′- and 3′-terminal sequences of *traA* mRNA, RT-PCR was performed as described in the Section "Materials and Methods." For 5′-terminal analysis of mRNA, we added a linker to the 3′-terminus of the first strand cDNA and conducted PCR. At least three PCR products, two of which were clear and the remaining one was weak, were obtained (**Figure 3B**, left). For 3′-terminal analysis of mRNA, we added a linker at the 3′-terminus of the mRNA and conducted RT-PCR. At least two PCR products were obtained, one of which was clearly observed and the other was weak (**Figure 3B**, right). The sequences of these PCR products, designated as (i)–(v), were analyzed and the starting and termination positions of the mRNA including *traA* were determined (**Figure 3C**). The starting and terminating positions described below are numbered according to GenBank accession number AP001918.1. Analysis of six clones of (i) showed that mRNAs

started from a position upstream of the *traY* gene; five started at position 67808 and one started at position 67754. Analysis of six clones of (ii) showed that it started from within the *traY* gene; four started at position 68000 and two started at position 67999. Analysis of five clones of (iii) showed that mRNAs started from within the *traY* gene; one started at position 68165, two at 68166, one at 68167, and one at 68189 (**Figure 3C**). Analysis of five clones of (v) showed that mRNAs terminated within the *traL* gene; four terminated at 68818 and one at 68819. Analysis of three clones of (iv) also showed that the mRNAs terminated within the *traL* gene; all three terminated at position 68902. These results indicated that the total RNA included mRNAs encoding *traA* of various lengths with different upstream and downstream sequences. Therefore, all three bands observed on Northern hybridization should contain the *traA* gene sequence.

DISCUSSION

We reported previously that *E. coli* and Qβ coexisted in serial passage and both continued to change genetically and phenotypically (Kashiwagi and Yomo, 2011). Here, we characterized partially resistant *E. coli* obtained in the previous study and demonstrated links among TraQ content, TraA content, and amplification of Qβ.

In this study, overexpression of mutant-type $TraQ_{S21Ptag}$ was shown to result in an increase in TraA content and recovery of Qβ amplification. These observations indicated that $TraQ_{S21P}$ had not entirely lost its function. The S21P mutation may alter the activity of TraQ, such as changing the binding affinity between TraQ and TraA, and may result in a decrease of TraA content in the

M54(C) population and reduce the possibility of Qβ infection by decreasing either the number of cells with F pili or the amount of F pili in each cell in the population, as TraQ protein binds propilin that is a precursor of mature TraA and the first 21 amino acids are important for this binding (Harris et al., 1999). In this study, not only TraQ$_{Anctag}$ but also the TraQ$_{S21Ptag}$ increased the TraA contents of M54(C) without Dox induction. The copy numbers of pASK-TraQ$_{S21Ptag}$, which has the ColE1 replication origin, and F plasmid in the cell are 15–20 and 1, respectively (Snyder and Champness, 2007). Therefore, introduction of pASK-TraQ$_{S21Ptag}$ into the cell increased the copy number of the *traQ* (T61C) gene and therefore may have increased the concentration of mutant-type TraQ in the cell.

It has been reported that F$^+$ cells in *E. coli* populations are heterogeneous in the number of F pili per cell and in the length of F pili through the cycles of extension and retraction (Clarke et al., 2008; Silverman and Clarke, 2010). In addition, it is widely accepted that even *E. coli* with the same genotype show phenotypic diversity due to the stochasticity in living organisms (Elowitz et al., 2002; Kashiwagi et al., 2006; Bressloff, 2014). Therefore, even in Anc(C), the population would be heterogeneous in both number and length of F pili per cell. The single mutation of TraQ would decrease the percentage of cells that could be infected by Qβ in the population, even though the M54(C) population was genetically identical throughout the community. The mutation was introduced into the region involved in binding with propilin (Harris et al., 1999) and protein–protein binding is one of the stochastic processes in a cell because it is a collision reaction and reducing numbers of interacting molecules in a cell would increase the fluctuation in number of bound proteins (Bressloff, 2014). Therefore, there would be at least three types of players in the community: a small proportion of cells supporting the phage population, a large proportion of cells supporting the *E. coli* population due to escape from Qβ infection, and Qβ itself. This heterogeneity would result in the partially resistant phenotype of the M54(C) population, as we assessed the phenotype based on the amplification ratio of Qβ in the population and not in single infected cells. As we evaluated the TraA and TraQ contents and Qβ amplification of the population and not of single cells, there are at least two plausible explanations for the partial resistance. The first is that every cell had low levels of TraA or F pili, and the second is that only a small portion of cells in the population had sufficient F pili for Qβ adsorption. In both cases, at least two types of cell—a minor population infected by Qβ and a major population that was not infected by Qβ—may emerge from *E coli* with the identical genotype.

The physiological refuge hypothesis has been reported as one of the mechanisms allowing the coexistence of bacteria and bacteriophages by providing phenotypic heterogeneity in resistance within the bacterial population (Lenski, 1988; Schrag and Mittler, 1995). The results of the present study suggested that the coevolved *E. coli* in experimental evolution would generate phenotypic heterogeneity with both resistant and susceptible cells, as suggested by the physiological refuge hypothesis.

Many resistance mechanisms of bacteria for DNA bacteriophages have been reported, such as preventing phage adsorption, preventing phage DNA entry, cutting phage nucleic acids, abortive infection, and phase variation (Hancock and Reeves, 1975; Labrie et al., 2010; Bikard and Marraffini, 2012). In preventing phage adsorption, surface receptors of bacteria for phage infection were modified, masked by proteins, or blocked by exopolysaccharide (Labrie et al., 2010). However, there have been few discussions regarding the mechanisms of resistance to RNA bacteriophages. Here, we first reported one of the mechanisms underlying partial resistance of *E. coli* to the RNA bacteriophage Qβ that would be included in preventing phage adsorption: a decrease in chance of phage adsorption by reducing the receptor contents in the host population by changing a single amino acid on the protein related to production of the mature receptor (F pilus), not the receptor itself. In addition, the results of this study also suggested that the phenotypic fluctuation caused by changing a single amino acid on the protein would facilitate long-term coexistence of both predator (Qβ phage) and prey (*E. coli*).

AUTHOR CONTRIBUTIONS

AK designed the research. AK, HK, and FST carried out the experiments and analyzed the data. AK wrote the manuscript.

ACKNOWLEDGMENTS

REFERENCES

Abrams, P. A. (2000). The evolution of predator-prey interactions: theory and evidence. *Annu. Rev. Ecol. Syst.* 31, 79–105. doi: 10.1146/annurev.ecolsys.31.1.79

Alexander, M. (1981). Why microbial predators and parasites do not eliminate their prey and hosts. *Annu. Rev. Microbiol.* 35, 113–133. doi: 10.1146/annurev.mi.35.100181.000553

Anderson, R. M., and May, R. M. (1978). Regulation and stability of host-parasite population interactions I. Regulatory processes. *J. Anim. Ecol.* 47, 219–247. doi: 10.2307/3933

Berryman, A. A. (1992). The origins and evolution of predator-prey theory. *Ecology* 73, 1530–1535. doi: 10.2307/1940005

Bikard, D., and Marraffini, L. A. (2012). Innate and adaptive immunity in bacteria: mechanisms of programmed genetic variation to fight bacteriophages. *Curr. Opin. Immunol.* 24, 15–20. doi: 10.1016/j.coi.2011.10.005

Bohannan, B. J., and Lenski, R. E. (2000). Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. *Ecol. Lett.* 3, 362–377. doi: 10.1046/j.1461-0248.2000.00161.x

Bressloff, P. C. (2014). *Stochastic Processes in Cell Biology.* Heidelberg: Springer. doi: 10.1007/978-3-319-08488-6

Briggs, C. J., and Hoopes, M. F. (2004). Stabilizing effects in spatial parasitoid-host and predator-prey models: a review. *Theor. Popul. Biol.* 65, 299–315. doi: 10.1016/j.tpb.2003.11.001

Brockhurst, M. A., Buckling, A., and Rainey, P. B. (2006). Spatial heterogeneity and the stability of host-parasite coexistence. *J. Evol. Biol.* 19, 374–379. doi: 10.1111/j.1420-9101.2005.01026.x

Brockhurst, M. A., and Koskella, B. (2013). Experimental coevolution of species interactions. *Trends Ecol. Evol.* 28, 367–375. doi: 10.1016/j.tree.2013.02.009

Buckling, A., and Rainey, P. B. (2002). Antagonistic coevolution between a bacterium and a bacteriophage. *Proc. Biol. Sci.* 269, 931–936. doi: 10.1098/rspb.2001.1945

Bull, J. J., Vegge, C. S., Schmerer, M., Chaudhry, W. N., and Levin, B. R. (2014). Phenotypic resistance and the dynamics of bacterial escape from phage control. *PLoS ONE* 9:e94690. doi: 10.1371/journal.pone.0094690

Campbell, A. (1961). Conditions for the existence of bacteriophage. *Evolution* 15, 153–165. doi: 10.2307/2406076

Carlson, K. (2005). "Working with bacteriophages: common techniques and methodological approaches," in *Bacteriophages, Biology and Applications*, eds E. Kutter and A. Sulakvelidze (Boca Raton, FL: CRC Press), 437–494.

Chao, L., Levin, B. R., and Stewart, F. M. (1977). A complex community in a simple habitat: an experimental study with bacteria and phage. *Ecology* 58, 369–378. doi: 10.2307/1935611

Clarke, M., Maddera, L., Harris, R. L., and Silverman, P. M. (2008). F-pili dynamics by live-cell imaging. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17978–17981. doi: 10.1073/pnas.0806786105

Dennehy, J. J. (2012). What can phages tell us about host-pathogen coevolution? *Int. J. Evol. Biol.* 2012, 12. doi: 10.1155/2012/396165

Elowitz, M. B., Levine, A. J., Siggia, E. D., and Swain, P. S. (2002). Stochastic gene expression in a single cell. *Science* 297, 1183–1186. doi: 10.1126/science.1070919

Hancock, R. E., and Reeves, P. (1975). Bacteriophage resistance in *Escherichia coli* K-12: general pattern of resistance. *J. Bacteriol.* 121, 983–993.

Harris, R. L., Sholl, K. A., Conrad, M. N., Dresser, M. E., and Silverman, P. M. (1999). Interaction between the F plasmid TraA (F-pilin) and TraQ proteins. *Mol. Microbiol.* 34, 780–791. doi: 10.1046/j.1365-2958.1999.01640.x

Horne, M. T. (1970). Coevolution of *Escherichia coli* and bacteriophages in chemostat culture. *Science* 168, 992–993. doi: 10.1126/science.168.3934.992-a

Kashiwagi, A., Urabe, I., Kaneko, K., and Yomo, T. (2006). Adaptive response of a gene network to environmental changes by fitness-induced attractor selection. *PLoS ONE* 1:e49. doi: 10.1371/journal.pone.0000049

Kashiwagi, A., and Yomo, T. (2011). Ongoing phenotypic and genomic changes in experimental coevolution of RNA bacteriophage Qβ and *Escherichia coli*. *PLoS Genet.* 7:e1002188. doi: 10.1371/journal.pgen.1002188

Kathir, P., and Ippen-Ihler, K. (1991). Construction and characterization of derivatives carrying insertion mutations in F plasmid transfer region genes, trbA, artA, traQ, and trbB. *Plasmid* 26, 40–54. doi: 10.1016/0147-619X(91)90035-U

Kerr, B., Neuhauser, C., Bohannan, B. J., and Dean, A. M. (2006). Local migration promotes competitive restraint in a host-pathogen 'tragedy of the commons.' *Nature* 442, 75–78. doi: 10.1038/nature04864

Kishimoto, T., Iijima, L., Tatsumi, M., Ono, N., Oyake, A., Hashimoto, T., et al. (2010). Transition from positive to neutral in mutation fixation along with continuing rising fitness in thermal adaptive evolution. *PLoS Genet.* 6:e1001164. doi: 10.1371/journal.pgen.1001164

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327. doi: 10.1038/nrmicro2315

Lenski, R. E. (1988). "Dynamics of interactions between bacteria and virulent bacteriophage," in *Advances in Microbial Ecology*, ed. K. C. Marshall (New York: Plenum Publishing Corporation), 1–44.

Lenski, R. E., and Levin, B. R. (1985). Constraints on the coevolution of bacteria and virulent phage: a model, some experiments, and predictions for natural communities. *Am. Nat.* 125, 585–602. doi: 10.1086/284364

Levin, B. R., Stewart, F. M., and Chao, L. (1977). Resource-limited growth, competition, and predation: a model and experimental studies with bacteria and bacteriophage. *Am. Nat.* 111, 3–24. doi: 10.1086/283134

Lythgoe, K. A., and Chao, L. (2003). Mechanisms of coexistence of a bacteria and a bacteriophage in a spatially homogeneous environment. *Ecol. Lett.* 6, 326–334. doi: 10.1046/j.1461-0248.2003.00433.x

Maneewannakul, K., Maneewannakul, S., and Ippen-Ihler, K. (1993). Synthesis of F pilin. *J. Bacteriol.* 175, 1384–1391.

Marston, M. F., Pierciey, F. J. Jr., Shepard, A., Gearin, G., Qi, J., Yandava, C., et al. (2012). Rapid diversification of coevolving marine *Synechococcus* and a virus. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4544–4549. doi: 10.1073/pnas.1120310109

Moore, D., Sowa, B. A., and Ippen-Ihler, K. (1982). A new activity in the Ftra operon which is required for F-pilin synthesis. *Mol. Gen. Genet.* 188, 459–464. doi: 10.1007/BF00330049

Moran, M. A., Rutherford, L. T., and Hodson, R. E. (1995). Evidence for indigenous *Streptomyces* populations in a marine environment determined with a 16S rRNA probe. *Appl. Environ. Microbiol.* 61, 3695–3700.

Murdoch, W. W., and Oaten, A. (1975). "Predation and population stability," in *Advances in Ecological Research*, ed. A. Macfadyen (London: Academic press Inc.), 1–131.

Paterson, S., Vogwill, T., Buckling, A., Benmayor, R., Spiers, A. J., Thomson, N. R., et al. (2010). Antagonistic coevolution accelerates molecular evolution. *Nature* 464, 275–278. doi: 10.1038/nature08798

Pettorelli, N., Coulson, T., Durant, S. M., and Gaillard, J. M. (2011). Predation, individual variability and vertebrate population dynamics. *Oecologia* 167, 305–314. doi: 10.1007/s00442-011-2069-y

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467. doi: 10.1073/pnas.74.12.5463

Sasaki, A. (2000). Host-parasite coevolution in a multilocus gene-for-gene system. *Proc. Biol. Sci.* 267, 2183–2188. doi: 10.1098/rspb.2000.1267

Schmidt, T. G., and Skerra, A. (2007). The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nat. Protoc.* 2, 1528–1535. doi: 10.1038/nprot.2007.209

Schrag, S. J., and Mittler, J. E. (1995). Host-parasite coexistence: the role of spatial refuges in stabilizing bacteria-phage interactions. *Am. Nat.* 148, 348–377. doi: 10.1086/285929

Silverman, P. M., and Clarke, M. B. (2010). New insights into F-pilus structure, dynamics, and function. *Integr. Biol.* 2, 25–31. doi: 10.1039/B917761B

Snyder, L., and Champness, W. (2007). "Plasmids," in *Molecular Genetics of Bacteria* (Washington, DC: ASM Press), 197–242.

Van Duin, J., and Tsareva, N. (2006). "Single-stranded RNA phages," in *The Bacteriophages*, ed. R. Calendar (New York: Oxford University Press), 175–196.

Watanabe, I., Sakurai, T., Furuse, K., and Ando, A. (1979). "Pseudolysogenization" by RNA phage Qβ. *Microbiol. Immunol.* 23, 1077–1083. doi: 10.1111/j.1348-0421.1979.tb00539.x

Zar, J. H. (2010). *Biostatistical Analysis*, 5th Edn, Upper Saddle River, NJ: Pearson education Inc.

Complexities of cell-to-cell communication through membrane vesicles: implications for selective interaction of membrane vesicles with microbial cells

Yusuke Hasegawa, Hiroyuki Futamata and Yosuke Tashiro *

Department of Applied Chemistry and Biochemical Engineering, Graduate School of Engineering, Shizuoka University, Hamamatsu, Japan

Introduction

Interaction between microbes in multicellular communities contributes to the development of complex microbial ecosystems. The secretion of various substances such as extracellular toxic compounds for combatting predation, extracellular DNA for horizontal gene transfer (HGT) and quorum-sensing (QS) signals for cell density-dependent cooperation, greatly influences microbial interactions (Hibbing et al., 2010; Tashiro et al., 2013). Study of the diffusion of substances secreted from donor cells to the extracellular environment and their uptake by recipient cells offers valuable insights for understanding microbial interactions.

The existence of membrane vesicles (MVs) increases the complexity involved in the diffusion of secreted substances during microbial interactions. MVs are extracellular particle-like liposome structures ranging from 20 to 200 nm in diameter (**Figure 1A**) and are pinched off from the external membrane of the microbe. The phenomenon of MV secretion has been observed in Gram-negative bacteria; however, recent studies have indicated that MVs are also produced by other prokaryotes including Gram-positive bacteria and archaea (Beveridge, 1999; Tashiro et al., 2010a, 2012; Haurat et al., 2015). MVs encapsulate membranal, periplasmic, and cytoplasmic components and play a role in the transfer of several compounds to organisms including both prokaryotic and eukaryotic cells. MVs contain proteins, DNA, RNA and in some cases, quorum sensing signals, and these substances are transferred to cells. Compared with freely-diffused chemical compounds secreted from bacteria, MVs have the following unique characteristics: (1) several chemical substances are highly concentrated in MVs, (2) interior substances in MVs are protected against environmental stresses, and (3) MVs play a role in effectively delivering these substances to cells. In this opinion article, we highlight the characteristics of MVs stated above and discuss the possibility of MV-mediated selective delivery to target cells (**Figure 1B**).

Highly Concentrated Substances in MVs

The significant characteristic of MVs is their ability to encapsulate specific substances. Interior substances are maintained at high concentration and are protected from degradation by exterior stresses and enzymes. In the case of *Pseudomonas aeruginosa*, which is not only known as an
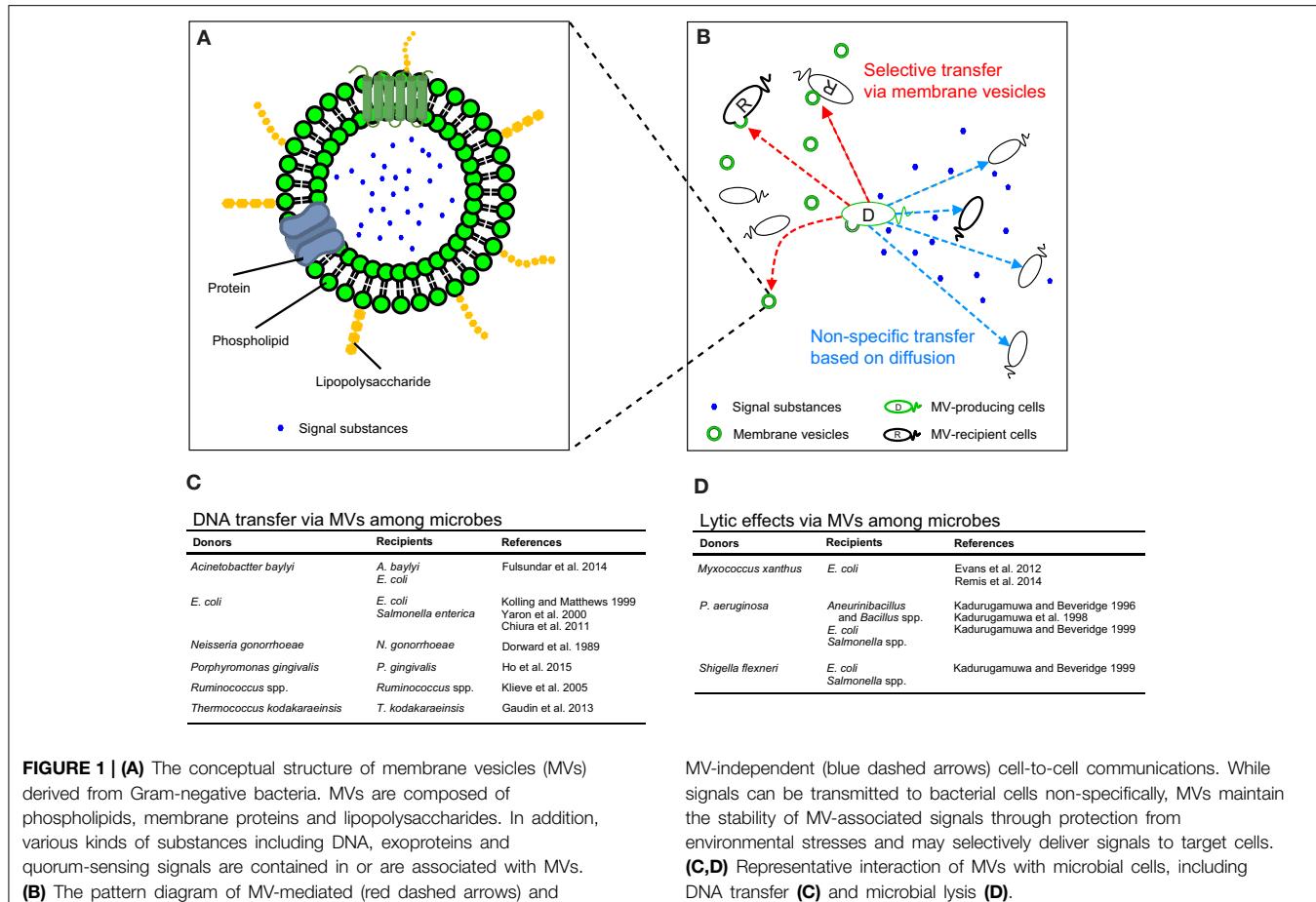
FIGURE 1 | (A) The conceptual structure of membrane vesicles (MVs) derived from Gram-negative bacteria. MVs are composed of phospholipids, membrane proteins and lipopolysaccharides. In addition, various kinds of substances including DNA, exoproteins and quorum-sensing signals are contained in or are associated with MVs. **(B)** The pattern diagram of MV-mediated (red dashed arrows) and

MV-independent (blue dashed arrows) cell-to-cell communications. While signals can be transmitted to bacterial cells non-specifically, MVs maintain the stability of MV-associated signals through protection from environmental stresses and may selectively deliver signals to target cells. **(C,D)** Representative interaction of MVs with microbial cells, including DNA transfer **(C)** and microbial lysis **(D)**.

opportunistic pathogen but also known to inhabit a variety of environments, a total of 68% of phospholipase C and 50% of alkaline phosphatase in the supernatant are localized in MVs along with the highly concentrated murein hydrolase (Kadurugamuwa and Beveridge, 1995, 1996). The encapsulation of toxic proteins provides an effective means for toxic transfer to not only eukaryotic cells but also other bacteria to counteract predation in the environment (Li et al., 1998; Evans et al., 2012). Furthermore, 86% of the *Pseudomonas* quinolone signal, which is one of the QS signals of *P. aeruginosa*, is localized in MVs, although smaller percentages (<1%) of other QS signals, including acyl-homoserine lactones, are localized in MVs (Mashburn and Whiteley, 2005). Such a highly concentrated QS signal is likely to effectively facilitate rapid alteration of gene expression in recipient cells. DNA is also highly concentrated in MVs, and MV-associated DNA is protected against extracellular DNase (Renelli et al., 2004). Several studies indicated that encapsulation of DNA contributes to HGT (Kolling and Matthews, 1999; Yaron et al., 2000; Fulsundar et al., 2014). Thus, the encapsulation of various signals in MVs plays an important role in microbial communications including QS and HGT, and increases effectiveness compared with diffusion-based interactions.

Association of Vesicles in Eukaryotic Cells

Whether MVs associate with a specific cellular surface remains unknown. In studies of eukaryotic intracellular vesicles, it has been shown that membrane-enveloped vesicles travel in between organelles in the cytoplasm, playing a role in transporting specific cargos to programmed locations via membrane fusion (Balch et al., 1984; Wilson et al., 1989; Sollner et al., 1993; Mcnew et al., 2000). In particular, a specific pairing between ligand and receptor allows vesicles to recognize the target compartment. In addition, the association between MVs and eukaryotic cells has been comprehensively studied in pathogenic bacteria, and MVs secreted from pathogens transfer virulent factors to cells (Parker et al., 2010; Chatterjee and Chaudhuri, 2011; Elmi et al., 2012; Rompikuntal et al., 2012; Bielaszewska et al., 2013). In particular, specific proteins localized on the surface of MVs increase the association with epithelial cells. For example, a heat-labile enterotoxin associated with MVs derived from enterotoxigenic *Escherichia coli* increases the association of MVs with cells (Kesty et al., 2004). The aminopeptidase of *P. aeruginosa* PaAP similarly increases the association of *P. aeruginosa* MVs with lung epithelial cells (Bauman and Kuehn, 2009). In addition, bacterial cytotoxin VacA increases

the adhesion of MVs with cells, most likely by increasing the association of MV lipopolysaccharide with cells (Parker et al., 2010). Thus, MVs can contribute to the virulence of eukaryotic cells, but little is understood regarding whether such associations are promoted by specific interactions between a ligand and a receptor. Understanding the specific association of MVs with cells would enable the development of applications involving MVs as vehicles for cell-specific drug delivery (Gujrati et al., 2014).

Selective Association between MVs and Microbial Cells

The association of MVs and microbial cells has been a subject of several studies on electric charge and hydrophobicity of cellular surfaces. The surfaces of bacterial cells are usually negatively charged and rich in divalent cations that stabilize surface charges such as Mg^{2+} or Ca^{2+}. When freely floating MVs encounter bacterial cells, these divalent cations act as a bridge between the negative charged surfaces, and the adhesion of negatively charged MVs on a bacterial cellular surface is stabilized (Kadurugamuwa and Beveridge, 1996; Tashiro et al., 2010b).

Hydrophobicity of a cellular surface is also an important factor for adhesion of MVs on bacterial cells. It has been shown that *P. aeruginosa* MVs can more easily attach to the hydrophilic surface of *Bacillus subtilis* than to other Gram-positive bacteria (Macdonald and Beveridge, 2002), indicating that liberated MVs from bacterial cells can selectively interact with bacterial cells. Nakao et al. developed a novel method to purify MVs derived from *Porphyromonas gingivalis* using epoxy-coated magnetic beads (Nakao et al., 2014) and the authors suggested that binding between MVs and epoxy beads is facilitated via a hydrophobic interaction.

In another report, MVs derived from *Myxococcus xanthus* adhered not only to the cells but also to MVs to form vesicle chains between cells (Remis et al., 2014). The detailed mechanism of the connection has not yet been elucidated, but it has been suggested that lipopolysaccharides (LPS) plays a role in MV-cell recognition because the distance between MVs and cellular surface was 5–10 nm, corresponding to the LPS.

Thus, MVs secreted from bacteria have characteristic surfaces and possess variable potential to attach to certain surfaces. Because specific proteins are selectively assimilated into MVs from bacterial cells, it is possible that the association of MVs with bacterial cells is a highly specific process based on a specific ligand-receptor interaction.

Transfer of MV Interior Substances to Microbial Cells

The importance of the contents of MVs has been exemplified by the critical role the transported material plays in the transfer of DNA (**Figure 1C**) (Dorward et al., 1989; Kolling and Matthews,

1999; Yaron et al., 2000; Klieve et al., 2005; Chiura et al., 2011; Gaudin et al., 2013; Fulsundar et al., 2014; Ho et al., 2015), bacterial lysis (**Figure 1D**) (Kadurugamuwa and Beveridge, 1996, 1999; Kadurugamuwa et al., 1998; Li et al., 1998; Evans et al., 2012; Remis et al., 2014) and QS-regulated gene expression (Tashiro et al., 2010b). It should be noted that membrane fusion does not always occur when interior substances in MVs are transferred to bacterial cells.

With regard to DNA transfer via MVs, the adhesion of MVs with bacterial cellular surface but not membrane fusion has been confirmed by transmission electron microscope observation in *E. coli* O157:H7 (Kolling and Matthews, 1999). Recently, however, it has been shown that MVs are integrated to recipient cells and DNA is transferred from *Acinetobacter baylyi* to *E. coli* and *A. baylyi* (Fulsundar et al., 2014).

Microbial predation using MVs occurs when virulent factors or peptidoglycan hydrolytic enzymes contained in MVs are transferred to other bacterial cells. It has been suggested that the mechanism of bacterial lysis via MVs secreted from Gram-negative bacteria differs in whether recipient cells are Gram-negative or positive. MVs derived from *P. aeruginosa* can attach to the surface of *E. coli* and *Staphylococcus aureus*, while they are able to fuse with *E. coli* but not *S. aureus* (Kadurugamuwa and Beveridge, 1996). The authors have suggested a possible mechanism described below. Because MVs have high curvature, negatively charged O-side chains of LPS are loosely packaged and they could form salt-bridging by cations such as Ca^{2+} and Mg^{2+}, with bacterial surfaces on which such cations are rich. For the association of MVs with a Gram-positive bacterial surface, this event would break apart the high curvature of MVs, and thereby open MVs, resulting in the liberation of interior lytic enzymes and the digestion of the cell wall. This event would enable the transition of the content from MVs to cells, possibly through permeation of the cellular membrane without fusion. On the other hand, MVs fuse into outer membrane of Gram-negative bacteria because they possess a compatible bilayer surface. Thus, it is considered that selective MV integration with recipient cells occurs, but interior substances in MVs can be transferred to microbial cells even when MVs just adhere on the cellular surfaces.

Concluding Remarks and Perspectives

Therefore, MVs encapsulate signaling substances secreted from cells, protect these substances from environmental stresses and maintain these substances at high concentrations. In addition, signals associated with MVs are likely transferred to specific bacterial cells. Such signal transfer via MVs is considerably different from diffusion-based cell-to-cell communication (**Figure 1B**). The transfer of substances via MVs increases accuracy, swiftness and effectiveness of responses in combating predation, HGT and QS. Thus, cell-to-cell communications are comprised of not only a simple method based on diffusive substances but also very complicated

aspects. Microbes intricately communicate through as-yet-unknown methods using MVs, thereby influencing interspecies networks, microbial community organization and ecosystem dynamics.

References

Balch, W. E., Glick, B. S., and Rothman, J. E. (1984). Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell* 39, 525–536. doi: 10.1016/0092-8674(84)90459-8

Bauman, S. J., and Kuehn, M. J. (2009). *Pseudomonas aeruginosa* vesicles associate with and are internalized by human lung epithelial cells. *BMC Microbiol* 9:26. doi: 10.1186/1471-2180-9-26

Beveridge, T. (1999). Structures of gram-negative cell walls and their derived membrane vesicles. *J. Bacteriol.* 181, 4725–4733.

Bielaszewska, M., Rüter, C., Kunsmann, L., Greune, L., Bauwens, A., Zhang, W., et al. (2013). Enterohemorrhagic *Escherichia coli* hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis. *PLoS Pathog.* 9:e1003797. doi: 10.1371/journal.ppat.1003797

Chatterjee, D., and Chaudhuri, K. (2011). Association of cholera toxin with *Vibrio cholerae* outer membrane vesicles which are internalized by human intestinal epithelial cells. *FEBS Lett.* 585, 1357–1362. doi: 10.1016/j.febslet.2011.04.017

Chiura, H. X., Kogure, K., Hagemann, S., Ellinger, A., and Velimirov, B. (2011). Evidence for particle-induced horizontal gene transfer and serial transduction between bacteria. *FEMS Microbial. Ecol.* 76, 576–591. doi: 10.1111/j.1574-6941.2011.01077.x

Dorward, D. W., Garon, C. F., and Judd, R. C. (1989). Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. *J. Bacteriol.* 171, 2499–2505.

Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D. C., Inglis, N. F., et al. (2012). Campylobacter jejuni outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect. Immun.* 80, 4089–4098. doi: 10.1128/IAI.00161-12

Evans, A. G. L., Davey, H. M., Cookson, A., Currinn, H., Cooke-Fox, G., Stanczyk, P. J., et al. (2012). Predatory activity of *Myxococcus xanthus* outer-membrane vesicles and properties of their hydrolase cargo. *Microbiology* 158, 2742–2752. doi: 10.1099/mic.0.060343-0

Fulsundar, S., Harms, K., Flaten, G. E., Johnsen, P. J., Chopade, B. A., and Nielsen, K. M. (2014). Gene transfer potential of outer membrane vesicles of *Acinetobacter baylyi* and effects of stress on vesiculation. *Appl. Environ. Microbiol.* 80, 3469–3483. doi: 10.1128/AEM.04248-13

Gaudin, M., Gauliard, E., Schouten, S., Houel-Renault, L., Lenormand, P., Marguet, E., et al. (2013). Hyperthermophilic archaea produce membrane vesicles that can transfer DNA. *Environ. Microbiol. Rep.* 5, 109–116. doi: 10.1111/j.1758-2229.2012.00348.x

Gujrati, V., Kim, S., Kim, S.-H., Min, J. J., Choy, H. E., Kim, S. C., et al. (2014). Bioengineered bacterial outer membrane vesicles as cell-specific drug-delivery vehicles for cancer therapy. *ACS Nano* 8, 1525–1537. doi: 10.1021/nn405724x

Haurat, M. F., Elhenawy, W., and Feldman Mario, F. (2015). Prokaryotic membrane vesicles: new insights on biogenesis and biological roles. *Biol. Chem.* 396, 95. doi: 10.1515/hsz-2014-0183

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* 8, 15–25. doi: 10.1038/nrmicro2259

Ho, M.-H., Chen, C.-H., Goodwin, J. S., Wang, B.-Y., and Xie, H. (2015). Functional advantages of *Porphyromonas gingivalis* vesicles. *PLoS ONE* 10:e0123448. doi: 10.1371/journal.pone.0123448

Kadurugamuwa, J., Mayer, A., Messner, P., Sára, M., Sleytr, U., and Beveridge, T. (1998). S-layered Aneurinibacillus and Bacillus spp. are susceptible to the lytic action of *Pseudomonas aeruginosa* membrane vesicles. *J. Bacteriol.* 180, 2306–2311.

Kadurugamuwa, J. L., and Beveridge, T. J. (1995). Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during

normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J. Bacteriol.* 177, 3998–4008.

Kadurugamuwa, J. L., and Beveridge, T. J. (1996). Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. *J. Bacteriol.* 178, 2767–2774.

Kadurugamuwa, J. L., and Beveridge, T. J. (1999). Membrane vesicles derived from *Pseudomonas aeruginosa* and *Shigella flexneri* can be integrated into the surfaces of other gram-negative bacteria. *Microbiology* 145, 2051–2060. doi: 10.1099/13500872-145-8-2051

Kesty, N., Mason, K., Reedy, M., Miller, S., and Kuehn, M. (2004). Enterotoxigenic *Escherichia coli* vesicles target toxin delivery into mammalian cells. *EMBO J.* 23, 4538–4549. doi: 10.1038/sj.emboj.7600471

Klieve, A. V., Yokoyama, M. T., Forster, R. J., Ouwerkerk, D., Bain, P. A., and Mawhinney, E. L. (2005). Naturally occurring DNA transfer system associated with membrane vesicles in cellulolytic *Ruminococcus* spp. of ruminal origin. *Appl. Environ. Microbiol.* 71, 4248–4253. doi: 10.1128/AEM.71.8.4248-4253.2005

Kolling, G. L., and Matthews, K. R. (1999). Export of virulence genes and Shiga toxin by membrane vesicles of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 65, 1843–1848.

Li, Z., Clarke, A. J., and Beveridge, T. J. (1998). Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. *J. Bacteriol.* 180, 5478–5483.

Macdonald, K. L., and Beveridge, T. J. (2002). Bactericidal effect of gentamicin-induced membrane vesicles derived from *Pseudomonas aeruginosa* PAO1 on gram-positive bacteria. *Can. J. Microbiol.* 48, 810–820. doi: 10.1139/w02-077

Mashburn, L. M., and Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437, 422–425. doi: 10.1038/nature03925

Mcnew, J. A., Parlati, F., Fukuda, R., Johnston, R. J., Paz, K., Paumet, F., et al. (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* 407, 153–159. doi: 10.1038/35025000

Nakao, R., Kikushima, K., Higuchi, H., Obana, N., Nomura, N., Bai, D., et al. (2014). A novel approach for purification and selective capture of membrane vesicles of the periodontopathic bacterium, *Porphyromonas gingivalis*: membrane vesicles bind to magnetic beads coated with epoxy groups in a noncovalent, species-specific manner. *PLoS ONE* 9:e95137. doi: 10.1371/journal.pone.0095137

Parker, H., Chitcholtan, K., Hampton, M. B., and Keenan, J. I. (2010). Uptake of *Helicobacter pylori* outer membrane vesicles by gastric epithelial cells. *Infect. Immun.* 78, 5054–5061. doi: 10.1128/IAI.00299-10

Remis, J. P., Wei, D., Gorur, A., Zemla, M., Haraga, J., Allen, S., et al. (2014). Bacterial social networks: structure and composition of *Myxococcus xanthus* outer membrane vesicle chains. *Environ. Microbiol.* 16, 598–610. doi: 10.1111/1462-2920.12187

Renelli, M., Matias, V., Lo, R. Y., and Beveridge, T. J. (2004). DNA-containing membrane vesicles of *Pseudomonas aeruginosa* PAO1 and their genetic transformation potential. *Microbiology* 150, 2161–2169. doi: 10.1099/mic.0.26841-0

Rompikuntal, P. K., Thay, B., Khan, M. K., Alanko, J., Penttinen, A.-M., Asikainen, S., et al. (2012). Perinuclear localization of internalized outer membrane vesicles carrying active cytolethal distending toxin from *Aggregatibacter actinomycetemcomitans*. *Infect. Immun.* 80, 31–42. doi: 10.1128/IAI.06069-11

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., et al. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318–324. doi: 10.1038/362318a0

Tashiro, Y., Ichikawa, S., Nakajima-Kambe, T., Uchiyama, H., and Nomura, N. (2010a). *Pseudomonas* quinolone signal affects membrane vesicle production

in not only Gram-negative but also Gram-positive bacteria. *Microb. Environ.* 25, 120–125. doi: 10.1264/jsme2.ME09182

Tashiro, Y., Ichikawa, S., Shimizu, M., Toyofuku, M., Takaya, N., Nakajima-Kambe, T., et al. (2010b). Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 76, 3732–3739. doi: 10.1128/AEM.02794-09

Tashiro, Y., Uchiyama, H., and Nomura, N. (2012). Multifunctional membrane vesicles in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 14, 1349–1362. doi: 10.1111/j.1462-2920.2011.02632.x

Tashiro, Y., Yawata, Y., Toyofuku, M., Uchiyama, H., and Nomura, N. (2013). Interspecies interaction between *Pseudomonas aeruginosa* and other microorganisms. *Microb. Environ.* 28, 13–24. doi: 10.1264/jsme2.ME12167

Wilson, D. W., Wilcox, C. A., Flynn, G. C., Chen, E., Kuang, W.-J., Henzel, W. J., et al. (1989). A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* 339, 355–359. doi: 10.1038/339355a0

Yaron, S., Kolling, G., Simon, L., and Matthews, K. (2000). Vesicle-mediated transfer of virulence genes from *Escherichia coli* O157:H7 to other enteric bacteria. *Appl. Environ. Microbiol.* 66, 4414–4420. doi: 10.1128/AEM.66.10.4414-4420.2000

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Mathematical modeling of dormant cell formation in growing biofilm

Kotaro Chihara[1][†], Shinya Matsumoto[2][†], Yuki Kagawa[3][†] and Satoshi Tsuneda[1,3]*

[1] Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan, [2] Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA, [3] Institute for Nanoscience and Nanotechnology, Waseda University, Tokyo, Japan

Understanding the dynamics of dormant cells in microbial biofilms, in which the bacteria are embedded in extracellular matrix, is important for developing successful antibiotic therapies against pathogenic bacteria. Although some of the molecular mechanisms leading to bacterial persistence have been speculated in planktonic bacterial cell, how dormant cells emerge in the biofilms of pathogenic bacteria such as *Pseudomonas aeruginosa* remains unclear. The present study proposes four hypotheses of dormant cell formation; stochastic process, nutrient-dependent, oxygen-dependent, and time-dependent processes. These hypotheses were implemented into a three-dimensional individual-based model of biofilm formation. Numerical simulations of the different mechanisms yielded qualitatively different spatiotemporal distributions of dormant cells in the growing biofilm. Based on these simulation results, we discuss what kinds of experimental studies are effective for discriminating dormant cell formation mechanisms in biofilms.

Keywords: biofilm, dormancy, persistence, individual-based modeling, mathematical model

Introduction

In natural environments or within the tissues of living organisms, some bacterial species form biofilms to ensure their long-term survival (Costerton et al., 1999; Hall-Stoodley et al., 2004). Bacteria attached to solid surfaces form mature biofilms by proliferating and producing extracellular polymeric substance (EPS), which captures planktonic bacteria (Stewart and Franklin, 2008). The EPS is composed of DNA molecules, proteins and polysaccharide. The thick biofilm structure impedes antibiotic diffusion and reduces the mobility of immune cells. Therefore, biofilms are responsible for the chronic and intractable characteristics of bacterial infectious disease (Stewart and Costerton, 2001; Stewart, 2002), which increase the morbidity and mortality of infections in immunocompromised patients. For example, intractable *Pseudomonas aeruginosa* infections have been reported in cystic fibrosis patients (Harrison, 2007).

One aspect of chronic characteristics of bacterial infectious disease is persister cells, which can tolerate sublethal concentrations of antibiotics. Unlike antibiotic resistant cells that carry genetic mutations, persister cells are nonheritable phenotypic variants. Some persister cells suppress their metabolism, including cell membrane formation, protein synthesis, and DNA replications (Lewis, 2007; Lewis et al., 2010). Such dormant bacteria can survive antibiotic exposure because their antibiotic target sites are deactivated. Actually, Balaban et al. (2004) investigated the single cell dynamics of the high persistence (*hip*) mutants (Moyed and Bertrand, 1983) of *Escherichia coli* by using microfluidic devices and found that preexisting subpopulations having reduced growth rates showed persistence under ampicillin exposure.

Several mechanisms of dormant cell formation have been proposed (Maisonneuve and Gerdes, 2014). Elowitz et al. (2002) posited that bacterial gene expressions related to physiological states are stochastically activated or inactivated. Expansion of this stochastic gene noise induces the formation of the stable subpopulation of some different bacterial phenotypes, such as dormant cell and active cell (Balaban et al., 2004). However, whether dormant cell is formed stochastically in a biofilm is unclear because previous studies were conducted by use of planktonic bacterial cell. Other researchers indicated that low nutrient concentration and diauxic shift of carbon initiate the ppGpp-controlled stress response, which activates the signal pathways for cell dormancy in planktonic bacterial cell (Nguyen et al., 2011; Amato et al., 2013). In a more recent study, Wakamoto et al. (2013) investigated persister cell dynamics of *Mycobacterium smegmatis* at a single cell level by microfluidic device in the presence of the drug isoniazid (INH) and time-lapse microscopy measurement. They showed that all persister cells did not necessarily repress their division, and persister cell did not always include dormant cell in the INH disposal of *M. smegmatis*, whereas dormant cell always included persister cell. Moreover, they showed that persister cells appeared due to the genetic stochastic expression encoded catalase-peroxidase (KatG), which activates INH.

During the biofilm formation, bacteria consume nutrients and oxygen, creating oxygen and nutrient concentration gradients within the developing biofilm (Stewart and Franklin, 2008). Therefore, dormant cells will emerge from the bottom of the biofilms, where the nutrient and oxygen are depleted to induce the dormant state due to the direct effect of nutrient or oxygen limitation, or the indirect effect of time-dependent growth arrest. To prevent the emergence of dormant cells, we need to elucidate the dynamics of dormant cell formation in growing biofilms. However, the spatiotemporal dynamics by which dormant cells emerge in growing biofilms are difficult to investigate, because experimental methods for specifically detecting dormant cells are not sufficiently developed. Consequently, which mechanisms of dormancy; stochastic, nutrient limitation, oxygen limitation, and time-dependent growth arrest, has a major impact against the bacterial dormancy within growing biofilm is unknown.

Experiments can be complemented by mathematical modeling, which has become one of the most promising tools for studying the emergence of dormant cells in growing biofilms. Many mathematical models of biofilm formation in specific environments have been developed. Multi-species biofilm formation has been modeled by one-dimensional partial differential equations describing a reaction–diffusion system (Rittmann et al., 2002). Other researchers have adopted two- and three-dimensional cellular automaton algorithms that replicate the complex structures of porous biofilms, such as mushroom-like structures with many voids and channels (Picioreanu et al., 1998a,b). An individual-based modeling (IbM) approach originally proposed for bacterial colony growth (Kreft et al., 1998), in which bacterial cells were represented as hard spheres, has been adapted to biofilms and microbial granule modeling by allowing continuous displacements and directions of the biomass

particles (Picioreanu et al., 2004; Matsumoto et al., 2010; Kagawa et al., 2015). The IbM algorithm is considered as a suitable basis for describing the dynamics of rare species such as dormant cells in growing biofilms.

Therefore, in the present study, we develop three-dimensional biofilm models based on the IbM algorithm to understand and predict the formation of dormant cells within growing biofilms. We propose four hypothetical mechanisms of dormant cell formation. Using the proposed model, we then simulate the spatiotemporal dynamics of dormant cell emergence under each hypothesis. Finally, we discuss what kinds of potential experimental design are effective for verifying the given hypotheses.

Methods

We first constructed a three-dimensional biofilm model based on the IbM algorithm. Next, we implemented four hypothetical mechanisms of dormant cell formation in the model. Here we proposed that dormant cells were formed by stochastic, nutrient-dependent, oxygen-dependent, or time-dependent processes. Using the developed model, we numerically simulated the spatiotemporal formation of dormant cells in a growing biofilm. Particularly, we perturbed the bulk nutrient or oxygen concentration, and observed the changes in the dormant cell distribution throughout the biofilm. Finally, based on these simulation results, we discussed what kinds of experimental approaches are effective for discriminating the above-mentioned hypotheses. The discussed experiments should provide new insights into the mechanisms of dormant cell formation.

Construction of a Three-dimensional Biofilm Model

Our three-dimensional (3D) mathematical model of biofilm formation is based on the IbM algorithm (Picioreanu et al., 2007). Parameter values used in this model are summarized in **Table 1**. Each bacterial cell is represented as a sphere of radius R positioned at (x, y, z) in 3D space. The radius is given by:

$$R = (3m/4\pi c)^{1/3}, \tag{1}$$

where m and c are the mass and density of the cells, respectively. Each particle undergoes the following three behaviors of real bacteria (Supplementary Figure 1A):

(i) *Growth*: Each bacterial cell consumes nutrient at a rate given by:

$$q = q^{\max} \cdot [S_S/(S_S + K_S)] \cdot [S_O/(S_O + K_O)], \tag{2}$$

where $q^{\max}$ is the maximum consumption rate, S_S and S_O are the local concentrations of the nutrient and oxygen, respectively, and K_S and K_O are the half-saturation constants of the nutrient and oxygen, respectively. Cells consume oxygen along with nutrient, and increase their masses according to the stoichiometric ratios defined in **Tables 2, 3**.

(ii) *Division*: When a cell reaches its maximum mass, it divides into two uniform daughter cells. The daughter cells do not

TABLE 1 | Parameter values used in 3D biofilm model.

Parameter	Symbol	Value	Unit	References
Time step	Δt	0.2	h	
System size	L_x, L_y, L_z	2.0×10^{-4}	m	Picioreanu et al., 2007
Grid number	N, M, L	33		
Thickness of boundary layer	N_{bl}	3	$\Delta x (= L_x/N)$	This study
Maximum consumption rate of active cell	q_A^{max}	75	(gCOD/gCOD)/day	Picioreanu et al., 2007
Maximum consumption rate of dormant cell	q_D^{max}	15	(gCOD/gCOD)/day	This study
Maximum cell density	C	1.5×10^5	gCOD/m^3	Picioreanu et al., 2007
Maximum cell mass	m_{max}	9.60×10^{-12}	gCOD	This study
Initial cell mass	m_{min}	4.80×10^{-12}	gCOD	This study
Bulk concentration of oxygen	C_O^{bulk}	4.0	g/m^3	Xavier et al., 2005
Bulk concentration of nutrient	C_S^{bulk}	100	gCOD/m^3	Xavier et al., 2005
Diffusion coefficient of oxygen	D_O	2.0×10^{-4}	m^2/day	Xavier et al., 2005
Diffusion coefficient of nutrient	D_S	4.5×10^{-6}	m^2/day	Picioreanu et al., 2007
Half saturation constant of oxygen	K_O	0.35	g/m^3	Xavier et al., 2005
Half saturation constant of nutrient	K_S	20	gCOD/m^3	Picioreanu et al., 2007

TABLE 2 | Stoichiometric matrix and kinetic rate expressions.

Process	Solute species		Particle species		Rate
	S_S	S_O	X_A	X_D	
Growth of active cell	−1	−(1-Y_A)	Y_A		$q_A^{max} \cdot [S_S/(S_S+K_S)] \cdot [S_O/(S_O+K_O)] \cdot X_A$
Growth of dormant cell	−1	− (1-Y_D)		Y_D	$q_D^{max} \cdot [S_S/(S_S+K_S)] \cdot [S_O/(S_O+K_O)] \cdot X_D$

overlap but remain attached, as described previously (Picioreanu et al., 2004).

(iii) *Shoving*: The growth and division processes cause overlapping of the spherical particles. To minimize these overlaps, each particle pushes its neighboring particles multiple times in the shoving algorithm (Picioreanu et al., 2004). Kagawa et al. (2014) related the number of shoves to the area of the overlap region in two-dimensional (2D) space. In the present study, the number of shoves was fixed at 200. Previous models considered bacterial decay, death and detachment processes (Xavier et al., 2005); these factors were excluded in the present model.

Movement of the particles obeys the following two boundary conditions: (a) particles cannot penetrate into the bottom surface of the system, and (b) periodic boundary conditions are imposed at the lateral boundaries.

The spatial distributions of the nutrient and oxygen concentrations were calculated by the following reaction–diffusion equations:

$$\partial_t S_S = D_S(\partial_x^2 S_S + \partial_y^2 S_S + \partial_z^2 S_S) + r_S, \qquad (3)$$

$$\partial_t S_O = D_O(\partial_x^2 S_O + \partial_y^2 S_O + \partial_z^2 S_O) + r_O, \qquad (4)$$

where D_S and D_O are the diffusion coefficients of the nutrient and oxygen, respectively. r_S and r_O are the net reaction rates of the nutrient and oxygen respectively, obtained by summing the

TABLE 3 | Stoichiometric parameters for microbial reactions.

Parameters	Symbol	Value	Unit	Reference
Yield on nutrient at active cell	Y_A	0.5	gCOD/gCOD	Xavier et al., 2005
Yield on nutrient at dormant cell	Y_D	0	gCOD/gCOD	This study

rates of all processes involving these respective growth factors. Explicitly, r_S and r_O are expressed by the following equations:

$$r_S = -q_A \cdot X_A - q_D \cdot X_D, \qquad (5)$$

$$r_O = -(1 - Y_A)q_A \cdot X_A - (1 - Y_D)q_D \cdot X_D, \qquad (6)$$

where X_A and X_D are the local biomass densities of active and dormant cells, respectively.

The 3D reaction–diffusion Equations (3–6) are numerically solved under the following three boundary conditions (the coordinate system of the cubic computational space is defined in Supplementary Figure 1B): (a) A Dirichlet boundary condition is imposed along the top boundary, i.e., the concentration remains constant at the interface between boundary layer and bulk liquid. The boundary is defined as the plane $x = (N_{bf} + N_{bl}) \Delta x$, where N_{bf} and N_{bl} are the maximum biofilm thickness and the boundary layer thickness, respectively, expressed in units of grid size (integer), defined as $\Delta x = L_x/N$. Note that this boundary moves upwards as the biofilm grows over time. This boundary

TABLE 4 | Parameter values of simulated dormant cells.

Mechanism of dormancy	Parameter	Symbol	Values used in simulation	Unit
(i) Stochastic process	Frequency of dormancy	$R_{a \to d}$	0.01, 0.02, 0.04	day^{-1}
(ii.a) Nutrient-dependent process*	Half saturation constant of nutrient	K_S	2, 20, 200	$gCOD/m^3$
(ii.b) Oxygen-dependent process*	Half saturation constant of oxygen	K_O	0.035, 0.35, 3.5	g/m^3
(iii) Time-dependent process	Threshold time to become dormancy	$T_{a \to d}$	10, 12, 14	h

*In these mechanisms, the maximum frequency of dormancy $R_{a \to d}^{max}$ was set to 0.12 day^{-1}.

condition is expressed as:

$$S_S(x, y, z) = S_S^{bulk} \text{ and } S_O(x, y, z) = S_O^{bulk},$$
$$\text{for } x \geq (N_{bf} + N_{bl})\Delta x. \quad (7)$$

(b) Neumann boundary conditions are imposed at the bottom of the system, where the nutrient and oxygen fluxes are zero:

$$\partial_x S_S(x = 0, y, z) = 0 \text{ and } \partial_x S_O(x = 0, y, z) = 0, \quad (8)$$

and (c) periodic boundary conditions are imposed at the lateral boundaries:

$$S_S(x, y = 0, z) = S_S(x, y = L_y, z),$$
$$S_O(x, y = 0, z) = S_O(x, y = L_y, z),$$
$$S_S(x, y, z = 0) = S_S(x, y, z = L_z),$$
$$\text{and } S_O(x, y, z = 0) = S_O(x, y, z = L_z). \quad (9)$$

Implementation of Dormant Cell Formation Mechanisms

Four possible mechanisms of dormant cell formation were implemented in the 3D biofilm model. These mechanisms are briefly described below.

(i) Dormant cell formation by stochastic process: Bacterial cells stochastically enter the dormant state anywhere in the biofilm at constant frequency $R_{a \to d}$ (unit = day^{-1}) (Chambless et al., 2006).

(ii) Dormant cell formation by a nutrient-dependent process or an oxygen-dependent process: Bacterial cells rarely become dormant cells at high nutrient or oxygen concentration, but readily become dormant at low nutrient or oxygen concentration. The frequency of dormant cell formation by a nutrient-dependent or an oxygen-dependent process is respectively given by:

$$R_{a \to d} = R_{a \to d}^{max} exp(-S/K). \quad (10)$$

where S (g/m^3) is the concentration of nutrient (S_S) or oxygen (S_O), K (g/m^3) is the half saturation constant of nutrient (K_S) or oxygen (K_O), and $R_{a \to d}^{max}$ (day^{-1}) is the maximum frequency of dormancy.

(iii) Dormant cell formation by a time-dependent process: Bacterial cells become dormant when the duration from the last division exceeds some threshold time $T_{a \to d}$ (h).

Each mechanism was simulated using the parameter values specified in **Table 4**. In the simulation, dormant cells are shown in red to visualize their distribution through the biofilm. Dormant cells consume a small amount of nutrient and oxygen for their maintenance without growing. We did not implement the resuscitation of dormant cells in this model because the molecular mechanisms behind the switching back to growth after dormancy are largely unknown.

Numerical Simulations

The simulation flow is detailed elsewhere (Picioreanu et al., 2004). Briefly, the initial condition of each simulation ($t = 0$) is ten particles with mass m_{min} randomly inoculated along the bottom surface ($x = 0$). In each time step, the spatial distributions of the nutrient and oxygen in the system were obtained by solving Equations (3–9) in steady state. The particles grew, divided, or entered the dormant state as described above. Biofilm formation was simulated for 1 day, or 2 days in cases of slow-growing biofilms. Three simulations with different seeds were conducted for each hypothesis.

Quantitative Analysis of the Distribution of Dormant Cells in Biofilm

The composition ratio of the dormant cells along the x direction of the biofilm, i.e., the height from the bottom surface, was derived from the simulation results. First, the particles residing at $x = 0.8H$, where H is the maximum biofilm thickness (in μm), were collected, and the positions (x) and states (dormant or active) of all particles below the collected particle were investigated. Precisely, if the collected particle was located at (x_P, y_P, z_P), all particles with centers positioned at (x_I, y_I, z_I) satisfying the following criteria were investigated;

$$(y_I - y_P)^2 + (z_I - z_P)^2 < R_I^2, \quad (11)$$

where R_I is the radius of the particle. Note that x_I can be less than $0.8H$. For each run of the simulation, fifty to two-hundred particles were collected (i.e., these particles resided at $x = 0.8H$). Position data of dormant cells below the collected particles were accumulated in three runs of simulations and calculated the abundance of dormant cells for each height level.

Results and Discussion

Representative examples of the simulated growing biofilms, assuming each hypothesis of dormant cell formation, are shown

in Movies 1–4 (Supplementary Material). **Figure 1** shows cross-sections of the biofilms 24 h after inoculation. As shown in this figure, the morphologies of the formed biofilms are very similar under the different hypotheses. Moreover, spatiotemporal dynamics of nutrient and oxygen concentrations within growing biofilms are shown in Movies 5–12 (Supplementary Material). As shown in these Movies, the spatial distributions of nutrient and oxygen are also very similar under the different hypotheses. However, nutrient was completely depleted at the bottom of the biofilm whereas oxygen remained even at the bottom of the biofilm (the value of the oxygen concentration at the bottom of the biofilm was about $2.9\,g/m^3$). Therefore, concentration gradients of nutrient and oxygen were different within growing biofilms.

On the other hand, **Figure 1** reveals prominent differences in the spatial distributions of dormant cells among the four models. For example, when the cells become dormant by a time-dependent process (**Figure 1D**), they are restricted to the bottom of the biofilm, whereas they are widely distributed throughout the biofilms in the other three models. This reflects the low growth rate of bacterial cells residing near the bottom of the biofilm, where the nutrient and/or oxygen concentrations are substantially reduced. In fact, when the threshold value $T_{a \to d}$ was decreased in the simulations, the region of dormant cells expanded toward the upper region of the biofilm (data not shown). Thus, the size of the dormant region strongly depends on the model parameter values. Moreover, when the half saturation constant of the nutrient K_S was decreased and the maximum frequency of dormant cell formation $R_{a \to d}^{max}$ was increased in the nutrient-dependent hypothesis, the dormant cell distribution was similar to that of **Figure 1D**, i.e., dormant cells distributed exclusively near the bottom of the biofilm (Supplementary

Figure 2). Thus, the shape of the dormant region is sensitive to the model parameter values.

Because the parameter values related to dormant cell formation alter the spatial distributions of dormant cells both qualitatively (the shape of the dormant region) and quantitatively (the size of the dormant region), the four hypotheses cannot be discriminated merely from the spatial distributions of dormant cells at a given time. Therefore, we simulated time evolution of the spatial distribution of dormant cells in each of the four proposed models (**Figure 2**). In this investigation, when dormancy was driven by a time-dependent process (**Figure 2D**), dormant cells emerged 20 h after inoculation. This process of dormant cell formation was very different from those of the other three models.

All three of the remaining models underwent similar processes of dormant cell formation. Dormant cells were widely distributed throughout the biofilms, and their abundances decreased with increasing height from the bottom of the biofilm (**Figures 2A–C**).

FIGURE 1 | Cross sections of biofilms 24 h post-inoculation, developed under four dormancy mechanisms. Dormancy was induced by **(A)** stochastic process ($R_{a \to d} = 0.04\,day^{-1}$), **(B)** nutrient-dependent process ($K_S = 20\,gCOD/m^3$), **(C)** oxygen-dependent process ($K_O = 3.5\,g/m^3$), and **(D)** a time-dependent process ($T_{a \to d} = 14\,h$). In the stochastic, nutrient-dependent and oxygen-dependent processes, the dormant cells are widely distributed through the biofilm. In the time-dependent process, dormant cells reside near the bottom of the biofilm. These spatial distributions of dormant cells largely depend on the parameter values related to the dormant cell formation mechanism (see Supplementary Figure 2).

FIGURE 2 | Time evolution of the spatial distribution of dormant cells. The abundances of dormant cells are calculated as described in the text and plotted against x, the height from the bottom of the biofilm. Dormancy was induced by **(A)** stochastic process, **(B)** nutrient-dependent process, **(C)** oxygen-dependent process, and **(D)** time-dependent process. Each plot was smoothed by moving-average with a $10\,\mu m$ window (3 points). Distributions obtained at 2-h intervals from 12 to 24 h post-inoculation are plotted in different colors.

Counter-intuitively, the abundance of dormant cells established a gradient along the x direction in the stochastically driven dormancy model, although the probability of dormant cell formation is position-independent in this model (**Figure 2A**). This gradient probably resulted from the cell velocity gradient along x, which was driven by the cell division process. Namely, the velocity in deeper biofilm regions was lower than in higher regions because of the low nutrient and oxygen concentrations. To test this, we conducted the additional simulation under the condition that dormant cells are formed by stochastic process as described below. In the simulation, to cancel the variation in cell velocities along the x direction, we reset K_S and K_O to be very small values. The results showed that the abundance of dormant cells did not establish a gradient along the x direction (Supplementary Figure 3). Therefore, the variation in the cell velocities along the x direction is responsible for the gradient of the abundance of dormant cells in the stochastically driven dormancy model.

To confirm the difference between these three models, we can investigate how the dormant cell distribution responds to an increase or decrease in the nutrient and oxygen concentrations in the bulk liquid. Such an investigation should be simple and straightforward. Thus, biofilm formation was simulated under three conditions of nutrient and oxygen concentrations in the bulk liquid (expressed in units of gCOD/m^3 and g/m^3, respectively): (condition I) 10 gCOD/m^3 and 0.4 g/m^3, (condition II) 10 gCOD/m^3 and 8 g/m^3, and (condition III) 200 gCOD/m^3 and 0.4 g/m^3. When dormant cell formation was induced by stochastic or time-dependent processes, the dormant cell distribution was not qualitatively affected by altering the bulk concentrations of nutrient and oxygen, i.e., there was a height gradient in the abundance of dormant cells under all three conditions (**Figures 3A,D**). Conversely, if dormancy was induced by a nutrient-dependent process, dormant cells rarely emerged in the biofilm at very high nutrient concentration (200 gCOD/m^3; condition III) (**Figure 3B**). Similarly, in the oxygen-dependent model, dormant cells rarely emerged when the oxygen concentration was high (8 g/m^3; condition II) (**Figure 3C**). Therefore, these three models can be discriminated by investigating their qualitative responses to altered bulk concentrations of nutrient and oxygen.

As shown in **Figures 1, 2**, the dormant cells resided in the deeper region of biofilms in the present four simulation models. There were several previous studies investigating the actual spatial distributions of dormant cells within biofilms. In one study, colony biofilms of *P. aeruginosa* PAO1 containing a plasmid with an isopropylthio-β-D-galactoside (IPTG) inducible GFP gene were cultured on polycarbonate membranes placed on LB agar plates. After 48 h cultivation, the membranes were transferred to an LB plate containing IPTG for an additional 4 h. The IPTG induced GFP expression in the active cells. The GFP-expressing cells were observed only in the upper region of the colony biofilm, although the nutrient was supplied through the membrane at the bottom of the colony biofilm (Kim et al., 2009). Williamson et al. (2012) also performed similar experiments using a continuous-drip flow biofilm system which nutrient enter from above and obtained similar results. This result implies that
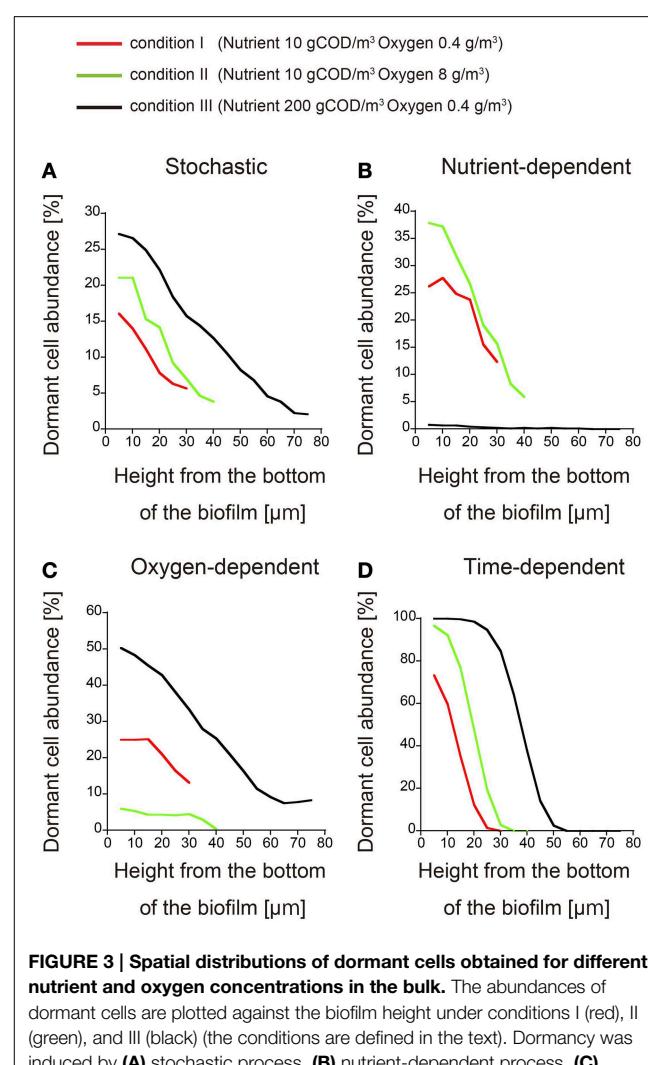
FIGURE 3 | Spatial distributions of dormant cells obtained for different nutrient and oxygen concentrations in the bulk. The abundances of dormant cells are plotted against the biofilm height under conditions I (red), II (green), and III (black) (the conditions are defined in the text). Dormancy was induced by **(A)** stochastic process, **(B)** nutrient-dependent process, **(C)** oxygen-dependent process, and **(D)** time-dependent process. Each plot was smoothed by moving-average with a 10 μm window (3 points).

dormant cells reside in the deeper regions of biofilms, which is consistent with our simulation results. The spatial distribution of dormant cells in biofilms obtained in the above reports can reject none of the four hypotheses on the mechanisms of dormant cell formation implemented into the computational models developed in the present study. As stated above, time evolution of the distribution and/or the responses to the increase/decrease of the substrate/oxygen may provide novel insights about the mechanism of dormancy.

Conclusions

To predict the formation of dormant cells in growing biofilms, we proposed four hypotheses of dormant cell formation and implemented them in a 3D biofilm model based on the IbM algorithm. In numerical simulations of the model, we found that (i) in the stochastic hypothesis of dormant cell formation, an unexpected gradient appeared in the abundance of dormant cells along the depth direction; thus (ii) investigating the

spatial distributions of dormant cells at a specific time cannot discriminate among the four suggested dormancy mechanisms; however, (iii) all four hypotheses were discriminated in spatiotemporal studies of the dormant cell distributions while varying the bulk concentrations of nutrient and/or oxygen. The proposed simulation methodology could guide experiments for efficiently elucidating the mechanisms of dormant cell formation in growing biofilms.

The following steps are the method to determine what kinds of experimental studies are effective for discriminating dormant cell formation mechanisms in biofilms. First, we will establish the biofilm into which nutrients enter from above in liquid medium, for example, a continuous-drip flow biofilm system, and should investigate the spatial distribution of the dormant cell within growing biofilm. At that time, we should use the detecting system of dormant cell, such as degradable GFP (half-life < 1 h) under the control of the ribosomal *rrn*BP1 promoter, which normally controls expression of the *rrnB* gene which codes for 16S rRNA (Shah et al., 2006; Maisonneuve et al., 2013), or TIMER[bac] fluorescence system in which both rapidly maturing green and slowly maturing orange TIMER molecules can accumulate, whereas in dividing cells, rapidly maturing green molecules dominate over orange molecules that are diluted by cell division (Claudi et al., 2014). Second, we will observe time evolution of the spatial distribution of dormant cells in growing biofilm by measurement with the use of microscopy. In this observation, if dormant cell arise near the bottom of the biofilm good long time after inoculation, a time-dependent process will be a plausible mechanism of dormancy (**Figure 2D**). Third, if dormant cells are widely distributed throughout the biofilm, we should investigate the responses to the increase/decrease of the nutrient/oxygen. If

the mechanism of dormant cell formation was caused by stochastic process, the distribution of dormant cells in biofilms was not affected qualitatively by the change in the bulk concentrations of the nutrient and oxygen (**Figure 3A**). On the other hand, if the mechanism was caused by the nutrient-dependent process, dormant cells rarely emerged in the biofilm when the concentration of the nutrient was very high (condition III) (**Figure 3B**). Similarly, if the mechanism was caused by oxygen-dependent process, dormant cells rarely emerged when the concentration of oxygen is high (condition II) (**Figure 3C**). As stated above, we could find a clue of the dynamics of dormant cell formation within growing biofilm by comparing the simulation results provided in present study with experimental results.

In summary, the simulation results of this study suggest that, by experimentally investigating the spatiotemporal dormant cell distributions while varying the nutrient and oxygen concentrations in the bulk, we could gain new insights into how dormant cell populations establish in biofilms.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb. 2015.00534/abstract

References

Amato, S., Orman, M., and Brynildsen, M. (2013). Metabolic control of persister formation in *Escherichia coli*. *Mol. Cell.* 50, 475–487. doi: 10.1016/j.molcel.2013.04.002

Balaban, N., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* 305, 1622–1625. doi: 10.1126/science.1099390

Chambless, J., Hunt, S., and Stewart, P. (2006). A three-dimensional computer model of four hypothetical mechanisms protecting biofilms from antimicrobials. *Appl. Environ. Microbiol.* 72, 2005–2013. doi: 10.1128/AEM.72.3.2005-2013.2006

Claudi, B., Sprote, P., Chirkova, A., Personnic, N., Zankl, J., Schurmann, N., et al. (2014). Phenotypic variation of *Salmonella* in host tissues delays eradication by antimicrobial chemotherapy. *Cell* 158, 722–733. doi: 10.1016/j.cell.2014. 06.045

Costerton, J., Stewart, P., and Greenberg, E. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318–1322. doi: 10.1126/science.284.5418.1318

Elowitz, M., Levine, A., Siggia, E., and Swain, P. (2002). Stochastic gene expression in a single cell. *Science* 297, 1183–1186. doi: 10.1126/science.1070919

Hall-Stoodley, L., Costerton, J., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108. doi: 10.1038/nrmicro821

Harrison, F. (2007). Microbial ecology of the cystic fibrosis lung. *Microbiology* 153, 917–923. doi: 10.1099/mic.0.2006/004077-0

Kagawa, Y., Horita, N., Taniguchi, H., and Tsuneda, S. (2014). Modeling of stem cell dynamics in human colonic crypts *in silico*. *J. Gastroenterol.* 49, 263–269. doi: 10.1007/s00535-013-0887-x

Kagawa, Y., Tahata, J., Kishida, N., Matsumoto, S., Picioreanu, C., van Loosdrecht, M., et al. (2015). Modeling the nutrient removal process in aerobic granular sludge system by coupling the reactor- and granule-scale models. *Biotechnol. Bioeng.* 112, 53–64. doi: 10.1002/bit.25331

Kim, J., Hahn, J., Franklin, M., Stewart, P., and Yoon, J. (2009). Tolerance of dormant and active cells in *Pseudomonas aeruginosa* PA01 biofilm to antimicrobial agents. *J. Antimicrob. Chemother.* 63, 129–135. doi: 10.1093/jac/dkn462

Kreft, J., Booth, G., and Wimpenny, J. (1998). BacSim, a simulator for individual-based modelling of bacterial colony growth. *Microbiology* 144, 3275–3287. doi: 10.1099/00221287-144-12-3275

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nat. Rev. Microbiol.* 5, 48–56. doi: 10.1038/nrmicro1557

Lewis, K., Gottesman, S., and Harwood, C. (2010). Persister cells. *Ann. Rev. Microbiol.* 64, 357–372. doi: 10.1146/annurev.micro.112408.134306

Maisonneuve, E., Castro-Camargo, M., and Gerdes, K. (2013). (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. *Cell* 154, 1140–1150. doi: 10.1016/j.cell.2013.07.048

Maisonneuve, E., and Gerdes, K. (2014). Molecular mechanisms underlying bacterial persisters. *Cell* 157, 539–548. doi: 10.1016/j.cell.2014. 02.050

Matsumoto, S., Katoku, M., Saeki, G., Terada, A., Aoi, Y., Tsuneda, S., et al. (2010). Microbial community structure in autotrophic nitrifying granules

characterized by experimental and simulation analyses. *Environ. Microbiol.* 12, 192–206. doi: 10.1111/j.1462-2920.2009.02060.x

Moyed, H., and Bertrand, K. (1983). HipA, A newly recognized gene of *Escherichia-coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J. Bacteriol.* 155, 768–775.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., et al. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* 334, 982–986. doi: 10.1126/science.1211037

Picioreanu, C., Kreft, J., Haagensen, J., Tolker-Nielsen, T., and Molin, S. (2007). Microbial motility involvement in biofilm structure formation—a 3D modelling study. *Water Sci. Technol.* 55, 337–343. doi: 10.2166/wst.2007.275

Picioreanu, C., Kreft, J., and van Loosdrecht, M. (2004). Particle-based multidimensional multispecies biofilm model. *Appl. Environ. Microbiol.* 70, 3024–3040. doi: 10.1128/AEM.70.5.3024-3040.2004

Picioreanu, C., van Loosdrecht, M., and Heijnen, J. (1998a). A new combined differential-discrete cellular automaton approach for biofilm modeling: application for growth in gel beads. *Biotechnol. Bioeng.* 57, 718–731.

Picioreanu, C., van Loosdrecht, M., and Heijnen, J. (1998b). Mathematical modeling of biofilm structure with a hybrid differential-discrete cellular automaton approach. *Biotechnol. Bioeng.* 58, 101–116.

Rittmann, B., Stilwell, D., and Ohashi, A. (2002). The transient-state, multiple-species biofilm model for biofiltration processes. *Water Res.* 36, 2342–2356. doi: 10.1016/S0043-1354(01)00441-9

Shah, D., Zhang, Z., Khodursky, A., Kaldalu, N., Kurg, K., and Lewis, K. (2006). Persisters: a distinct physiological state of *E-coli*. *BMC Microbiol.* 6:53. doi: 10.1186/1471-2180-6-53

Stewart, P. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol.* 292, 107–113. doi: 10.1078/1438-4221-00196

Stewart, P., and Costerton, J. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135–138. doi: 10.1016/S0140-6736(01)05321-1

Stewart, P., and Franklin, M. (2008). Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* 6, 199–210. doi: 10.1038/nrmicro1838

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., et al. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. *Science* 339, 91–95. doi: 10.1126/science.1229858

Williamson, K., Richards, L., Perez-Osorio, A., Pitts, B., McInnerney, K., Stewart, P., et al. (2012). Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. *J. Bacteriol.* 194, 2062–2073. doi: 10.1128/JB.00022-12

Xavier, J., Picioreanu, C., and van Loosdrecht, M. (2005). A general description of detachment for multidimensional modelling of biofilms. *Biotechnol. Bioeng.* 91, 651–669. doi: 10.1002/bit.20544

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Interspecies interactions are an integral determinant of microbial community dynamics

Fatma A. A. Aziz[1,2]*, Kenshi Suzuki[2], Akihiro Ohtaki[3], Keita Sagegami[3], Hidetaka Hirai[2], Jun Seno[2], Naoko Mizuno[2], Yuma Inuzuka[2], Yasuhisa Saito[4], Yosuke Tashiro[2], Akira Hiraishi[3] and Hiroyuki Futamata[2]*

[1] Laboratory of Food Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang, Malaysia, [2] Department of Applied Chemistry and Biochemical Engineering, Graduate School of Engineering, Shizuoka University, Hamamatsu, Japan, [3] Department of Environmental and Life Sciences, Toyohashi University of Technology, Toyohashi, Japan, [4] Department of Mathematics, Shimane University, Matsue, Japan

This study investigated the factors that determine the dynamics of bacterial communities in a complex system using multidisciplinary methods. Since natural and engineered microbial ecosystems are too complex to study, six types of synthetic microbial ecosystems (SMEs) were constructed under chemostat conditions with phenol as the sole carbon and energy source. Two to four phenol-degrading, phylogenetically and physiologically different bacterial strains were used in each SME. Phylogeny was based on the nucleotide sequence of 16S rRNA genes, while physiologic traits were based on kinetic and growth parameters on phenol. Two indices, J parameter and "interspecies interaction," were compared to predict which strain would become dominant in an SME. The J parameter was calculated from kinetic and growth parameters. On the other hand, "interspecies interaction," a new index proposed in this study, was evaluated by measuring the specific growth activity, which was determined on the basis of relative growth of a strain with or without the supernatant prepared from other bacterial cultures. Population densities of strains used in SMEs were enumerated by real-time quantitative PCR (qPCR) targeting the gene encoding the large subunit of phenol hydroxylase and were compared to predictions made from J parameter and interspecies interaction calculations. In 4 of 6 SEMs tested the final dominant strain shown by real-time qPCR analyses coincided with the strain predicted by both the J parameter and the interspecies interaction. However, in SMEII-2 and SMEII-3 the final dominant *Variovorax* strains coincided with prediction of the interspecies interaction but not the J parameter. These results demonstrate that the effects of interspecies interactions within microbial communities contribute to determining the dynamics of the microbial ecosystem.

Keywords: microbial ecosystem, population dynamics, interaction, self-organization, chemostat, phenol

INTRODUCTION

Microbial populations influence each other during the development of their ecosystems, while at the same time the microbial ecosystem is affected by its surrounding environments and vice versa (Fernández et al., 2000; Hashsham et al., 2000; Little et al., 2008; Klitgord and Segrè, 2010). It is predicted that the sustainability of an ecosystem will be maintained by dynamic changes of the bacterial community (dynamic equilibrium) (Ishii et al., 2012; Yamamoto et al., 2014). Comprehensive understanding of the principles of microbial ecosystems is not only central to research in microbial ecology but also important for efficient bioremediation, wastewater treatment, agriculture and human health but has been a challenging subject for microbial ecologists (Fernández et al., 1999, 2000; Futamata et al., 2005; El-Chakhtoura et al., 2015).

We previously observed a unique phenomenon of bacterial population dynamics in a chemostat soil bioreactor enriched with phenol (Futamata et al., 2005). Usually, since the chemostat culture is enriched with a low concentration of a sole substrate, bacteria exhibiting the highest affinity for this substrate eventually come to dominate the culture (Watanabe et al., 1998a). However, members of *Variovorax* exhibiting lower affinities for phenol constituted the final dominant population in this soil bioreactor (Futamata et al., 2005). This result indicated that kinetic parameters are not necessarily the sole determinant for predicting bacterial community dynamics in a chemostat culture. Therefore, further research is necessary to unveil the mechanism by which the *Variovorax* species became dominant in a complex chemostat ecosystem despite exhibiting lower affinity for phenol.

Kinetic and growth parameters have been used to predict bacterial population dynamics and the "*J* parameter" has been reported as a useful predictor for which strain will become dominant in a mixed culture (Hansen and Hubbell, 1980). Additionally, interactions between bacterial cells is also thought to be an important mechanism that plays a major role in biofilm formation through quorum sensing and other biological processes (Tashiro et al., 2013; DeSalvo et al., 2015; Inaba et al., 2015). Here, we investigated whether kinetic and growth parameters or interactions between populations of different species are more important for determining the composition of a microbial ecosystem.

The complete set of all possible interactions between many species of bacteria has to be investigated in order to fully understand a natural microbial ecosystem. However, microbial ecosystems in natural and engineered systems (including the soil bioreactor described above), are far too complex to be described by a single determinant and/or law. Currently, the factors and processes that influence the behavior and functionality of bacterial ecosystems remain largely unknown. Due to the high complexity of natural systems, an approach known as the synthetic microbial ecosystem (SME) has gained much interest of late (Narisawa et al., 2008; Mee et al., 2014; De Roy et al., 2014). Because of their reduced complexity and increased controllability, synthetic communities are often preferred over complex communities when examining ecological theories. The possible factors that influence the microbial community are reduced to a minimum, allowing the factors that affect specific community dynamics to be managed and identified.

The objective of this study was to understand the surprising bacterial community dynamics observed in the soil bioreactor. To this end, we determined the *J* parameter (based on kinetic and growth parameters) (Hansen and Hubbell, 1980) and the interspecies interactions (based on the specific growth activity) of several phenol degrading bacterial strains. These data were used to predict which strain would become dominant in different SMEs and the results were compared to real-time qPCR quantification of each strain. From this work we have determined that interactions between species, in addition to kinetic and growth parameters, are integral in determining bacterial community dynamics.

MATERIALS AND METHODS

Bacterial Strains Used in This Study

Phenol-degrading bacteria isolated from a phenol-degrading soil bioreactor and its inoculum (Futamata et al., 2001a, 2005) were used in this study (**Table 1**). *Pseudomonas putida* strains P-2, P-5, P-6, and P-8, and *Ralsotonia* sp. strain P-10 were isolated from trichloroethene (TCE) -contaminated aquifer soil (Futamata et al., 2001a). Other strains were isolated from chemostat enrichment cultures grown on phenol with pristine aquifer soil sampled from near the TCE-contaminated site. These bacteria were grown at 25°C in BSM medium supplemented with phenol at 2.0 mM (Futamata et al., 2001a) or MP medium (Watanabe et al., 1998a) supplemented with phenol at 2.0 mM.

Kinetic Analyses

Isolated strains or SMEs were grown in a chemostat reactor with BSM medium and phenol as the sole carbon and energy source. The phenol or catechol-oxygenating activity (phenol or catechol-consumption rate) was measured at various substrate concentrations using an oxygen electrode (DO METER TD-51, Toko Chemical Lab. Co., Ltd) after the respiratory oxygen consumption was suppressed by adding potassium cyanide (Watanabe et al., 1996). Kinetic parameters were calculated using the initial phenol-oxygenating velocities at more than 10 different substrate concentrations. The data were fitted to the Michaelis-Menten's equation or the Haldane's equation (Folsom et al., 1990; Watanabe et al., 1998b; Futamata et al., 2005) using JMP statistical visualization software (SAS Institute Inc.). The apparent kinetic constants, K_S (affinity constant), K_I (inhibition constant), and V_{max} (theoretical maximum activity) were determined using the nonlinear regression method as described previously (Watanabe et al., 1996, 1998a). Following Folsom et al. (1990), the term K_S was employed instead of K_m because the activity was measured using intact cells rather than purified enzymes.

Construction of Synthetic Microbial Ecosystem

Six kinds of SMEs, differing based on their strain composition and flow rate, were constructed with phenol as sole carbon

TABLE 1 | Kinetic parameters for phenol-degradation of strains isolated from the soil-bioreactor.

Isolated strains	K_S (µM)	K_I (µM)	V_{max} (µmol min^{-1} g^{-1} of dry cells)	No.[a]	Accession number
Acinetobacter sp. c1[b]	1.1 ± 0.40	4800 ± 1600	31 ± 1.4	3	AB167183
Acinetobacter sp. c26[b]	2.4 ± 1.8	80 ± 30	16 ± 2.4	30	AB167203
Acinetobacter sp. c40	11 ± 3.1	530 ± 92	50 ± 4.9	15	AB167208
Arthrobacter sp. c106	3.1 ± 0.11	110 ± 12	39 ± 1.2	29	AB167237
Pseudomonas sp. LAB-06	2.3 ± 0.31	4800 ± 800	21 ± 0.84	2	AB051693
Pseudomonas sp. LAB-08	2.2 ± 0.23	3200 ± 370	30 ± 0.73	4	AB051694
Pseudomonas sp. LAB-20	2.8 ± 0.55	1500 ± 290	28 ± 1.5	10	AB051697
Pseudomonas sp. LAB-23	2.3 ± 0.23	2700 ± 450	16 ± 0.50	6	AB051699
Pseudomonas putida P-2[c]	4.1 ± 1.2	3100 ± 1300	13 ± 1.1	5	AB038136
Pseudomonas putida P-5[c]	3.0 ± 0.66	1100 ± 260	48 ± 2.8	12	AB038140
Pseudomonas putida P-6[c]	3.9 ± 1.7	460 ± 260	16 ± 2.9	17	AB038141
Pseudomonas putida P-8[c]	5.3 ± 1.6	6900 ± 1600	23 ± 1.2	1	AB038142
Pseudomonas sp. LAB-26	2.5 ± 0.55	2300 ± 520	33 ± 1.6	7	AB051700
Ralstonia sp. P-10[c]	4.4 ± 0.89	580 ± 200	100 ± 20	14	AB016860
Ralstonia sp. c5	7.9 ± 1.6	220 ± 60	49 ± 5.3	22	AB167187
Ralstonia sp. chemo32	5.1 ± 0.60	470 ± 110	57 ± 3.4	16	LC086859
Ralstonia sp. c41	3.2 ± 1.0	200 ± 15	60 ± 10	24	AB167209
Ralstonia sp. HAB-01	5.6 ± 0.74	260 ± 46	60 ± 3.5	20	AB051680
Ralstonia sp. HAB-02	9.6 ± 1.6	180 ± 37	75 ± 7.5	25	AB051681
Ralstonia sp. HAB-11	2.2 ± 0.29	850 ± 120	14 ± 0.45	13	AB051683
Ralstonia sp. HAB-18	6.2 ± 0.93	310 ± 70	56 ± 3.7	18	AB051684
Unidentified strain TUT-005	2.9 ± 0.60	1600 ± 350	62 ± 2.8	9	NR[d]
Unidentified strain TUT-006	3.4 ± 0.50	1600 ± 200	130 ± 4.7	8	NR
Variovorax sp. c24[b]	8.2 ± 1.2	220 ± 45	93 ± 6.5	21	AB622239
Variovorax sp. c52	1.7 ± 0.4	270 ± 20	16 ± 3.1	19	AB167215
Variovorax sp. HAB-24[b]	7.6 ± 1.5	180 ± 47	106 ± 12	26	AB051688
Variovorax sp. HAB-27	7.1 ± 1.6	120 ± 47	96 ± 15	28	AB051689
Variovorax sp. HAB-29	7.4 ± 1.2	150 ± 38	108 ± 11	27	AB051690
Variovorax sp. HAB-30[b]	5.8 ± 0.94	200 ± 40	160 ± 12	23	AB051691
Variovorax sp. YN07[b]	12 ± 1.4	1200 ± 120	66 ± 2.5	11	AB622227

[a] Number links the number shown in **Figure 1**.

[b] These data were reported in *Futamata et al. (2005)*.

[c] These data were reported in *Futamata et al. (2001a)*.

[d] The strain was not registerd in Genbank.

and energy source under chemostat conditions. The SMEI series had 2–4 strains present and a high flow rate while the SMEII series had a lower flow rate and had different combinations of three strains. SMEI-1 consisted of *P. putida* P-8 (Futamata et al., 2001a) and *Variovorax* sp. HAB-24 (Futamata et al., 2001b). SMEI-2 consisted of *P. putida* strains P-8, *Variovorax* sp. HAB-24 and *Acinetobacter* sp. c26 (Futamata et al., 2005). SMEI-3 included *Ralstonia* sp. c41 in addition to the above-noted 3 strains. It was previously demonstrated that strains P-8 and HAB-24 belong to High-K_S (Group III) and Low-K_S (Group I) types according to their nucleotide sequences of the gene cording large subunit of multicomponent phenol hydroxylase (Futamata et al., 2001b). Furthermore, it was shown that the population density of Group III is approximately 10-fold higher than that of Group I. Therefore, initial population density of strain P-8 was set to be 10-fold higher than that of strain HAB-24. The same strains used in SMEI-2 were used in SMEII-1

to investigate the effect of medium flow rate on population dynamics. SMEII-2 consisted of *P. putida* LAB-06 (Futamata et al., 2001b), *Acinetobacter* sp. c26 and *Variovorax* sp. HAB24. SMEII-3 consisted of strain LAB-06, *Ralstonia* sp. chemo32, and *Variovorax* sp. HAB-30 (Futamata et al., 2001b). Prior to inoculation into the chemostat, all strains were precultured at 25°C in BSM medium supplemented with 2 mM phenol as the sole carbon source. Cultures were harvested at the early- or mid-exponential growth phase and then were transferred into 1.5 L of BSM medium containing 0.2 mM of phenol [in a chemostat reactor [(2 L in capacity)]. The initial cell density of each strain was adjusted to approximately 1.0×10^5 cells mL^{-1} by measuring the optical density at 600 nm (OD$_{600 \text{ nm}}$). An OD$_{600 \text{ nm}}$ of 0.1 corresponded to 1.0×10^9 cells mL^{-1} for strains HAB-24 and HAB-30 and to 5.0×10^8 cells mL^{-1} for the other bacteria. After the initially added phenol was almost completely degraded (start-up phase), the SMEI series cultures were supplied

continuously with BSM medium containing phenol (1500 mg L^{-1}) at a flow rate of 31.5 mL h^{-1}, corresponding to a dilution rate (D) of 0.5 d^{-1} (31.5 mL h^{-1} × 24 h/1500 mL). The hydraulic residence time (HRT), calculated as 1/D, was 2 days. The SMEII series cultures were supplied continuously with BSM medium containing phenol (1500 mg L^{-1}) at a flow rate of 10.4 mL h^{-1} (HRT was 6 days). The culture volume was maintained at 1.5 L. The culture was stirred at 150 rpm, and the temperature and pH were maintained at 25°C and 7.0, respectively. Air was filtered through 0.2 μm-pore-size membrane filters (Millipore) and supplied to the culture at 1.5 L min^{-1}. The concentration of phenol in the culture was measured using a colorimetric assay with a Phenol Test Wako kit (Wako Pure Chemicals) (Futamata et al., 2001b). The detection limit of this method was around 1.0 μM.

Monitoring of Strains in Synthetic Microbial Ecosystems

The population density of each strain was monitored using real-time qPCR targeting the gene encoding the large subunit of phenol hydroxylase (LmPH). Specific sets of primers were designed by the alignment of various LmPH genes (Supplemental Table 1). A specific PCR-product amplified with each specific primer set was used as a standard DNA fragment in a real-time qPCR analysis. For the monitoring of the *P. putida* P-8, *Variovoras* sp. HAB-24, and *Ralstonia* sp. c41, the PCR profile consisted of preheating at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 5 s, and extension at 72°C for 15 s. The annealing temperature was set to 56°C and 68°C for the monitoring of strains *Acinetobacter* sp. c26 and *Variovorax* sp. HAB30, respectively. Annealing temperature was set to 58°C for the monitoring of strains *P. putida* LAB-06 and *R.* sp. chemo32. The fluorescence signal was detected at 72°C in each cycle, and a melting curve was obtained by heating the product to 95°C and cooling to 40°C. The reaction was performed using a LightCycler FastStart DNA Master SYBR GREEN I kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and a LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The copy number of each of the amplicons was calculated using the LightCycler software version 3.52.

Simulation

We performed computer simulations about SMEI-1 (strains P-8 and HAB-24) using the Runge-Kutta method (Saito et al., 1999; Saito, 2002), with important modifications described in the equations below.

$$S' = \left(S^{(0)} - S\right)D - \mu_1 S/(K_{SI} + S)x_1/r_1 - \mu_2 S/(K_{S2} + S)x_2/r_2$$

$x_1' = x_1\{\mu_1 S/(K_{SI}+S)-D\}$ and $x_2' = x_2\{\mu_2 S/(K_{S2}+S)-D\}$ where S is the substrate (phenol in this study) concentration (mg L^{-1}), D is the dilution rate (h^{-1}), μ is the growth rate constant (h^{-1}), x is the dried cell density (mg L^{-1}), r is the cell yield (g [cell] g^{-1} [substrate]) and K_S is the half-saturation constant (μM). Here "1" and "2" mean strains P-8 and HAB-24, respectively.

Specific Growth Activity

Since it is known that supernatants of microbial culture can affect the metabolic processes of other microbes (Tanaka et al., 2005; Tashiro et al., 2013; Inaba et al., 2015), the effect of interspecies communication was investigated using a supernatant collected from a pure chemostat culture and was evaluated as specific growth activity. Growth curves were recorded to estimate the physiological changes that occurred after the addition of a supernatant. Each strain was incubated in the BSM medium under the conditions of the chemostat culture supplemented with phenol as the sole carbon source. After the culture was stable, which means that phenol was not detected and OD$_{600 nm}$ of the culture reached plateau, the culture was centrifuged at 4°C and 5800 × g. The supernatant was sterilized by filtration through a Steriflip-GP Filter ([pore size is 0.22 μm], Millipore). The supernatant was stored at −20°C after filtration. Frozen supernatant was thawed for each use. We empirically knew that the growth inhibiting activity of supernatant was kept for approximate 6 month at least. The effect of a single supernatant on growth of all strains was always assessed at the same time as a control. Cells precultured in the BSM medium supplemented with 2.0 mM phenol (BSM2.0phe) and 0.3 mL of filter-sterilized supernatant were transferred into 2.7 mL of fresh BSM2.0phe medium. The initial amount of cells was adjusted to an OD$_{600nm}$ of 0.01. As the control condition, 0.3 mL of BSM medium without phenol was added instead of the supernatant. The growth curve was automatically measured using a Bio-photorecorder (TVS062CA, ADVANTEC). Growth parameters, including a lag time (h), growth rate constant (μ [h^{-1}]) and amount of growth at a stationary phase (OD$_{max}$), were calculated using the growth curve. Here, we defined the specific growth activity as the surviving activity maintaining the cell density over 1.0×10^9 cells mL^{-1} in a chemostat culture under the condition of a dilution rate. Therefore, 1 unit (U) of specific growth activity was calculated using the following equations: 1 U = 0.021 (h^{-1}) × 10^9 (cells mL^{-1}) under the HRT condition of 2 days (in SMEI series) or 1 U = 0.0069 (h^{-1}) × 10^9 (cells mL^{-1}) under the HRT condition of 6 days (in SMEII series). As mentioned above, the cell density of *Variovorax* sp. strains HAB-24 and HAB-30 were 1.0×10^9 cells mL^{-1} at an OD$_{600nm}$ of 0.1, whereas the other strains had cell densities of 5.0×10^8 cells mL^{-1} at an OD$_{600nm}$ of 0.1. The OD$_{max}$ was then converted to cell density. Thus, one unit of specific growth activity changes according to the strain and the dilution rate of the chemostat. Units of specific growth activity for the tested strains were calculated according to the following equation:

$$U = (\mu \times cell\ density\ from\ ODmax)/(1\ U \times lag\ time).$$

J parameter

J parameter was calculated according to the following equation (Hansen and Hubbell, 1980): $J = (K_s \times D)/(\mu - D)$, where K_S is the half-saturation constant (μM), D is the dilution rate (h^{-1}), and μ is the growth rate constant (h^{-1}). Parameters of D in SMEI and SMEII series were 0.021 (h^{-1}) and 0.0069 (h^{-1}), respectively. Based on the equation, the strain exhibiting a lower J parameter should out-grow strains exhibiting higher J parameters.

RESULTS

Kinetic Parameters for Phenol of Strains

Kinetic parameters for phenol of 30 strains isolated from the soil bioreactor and its inoculum are shown in **Table 1** and plotted in **Figure 1**. Overall, *Pseudomonas* sp. strains, with the exception of *Pseudomonas* sp. P-6, exhibited lower K_S-values ($3.0 \pm 1.1 \, \mu M$) and higher K_I-values ($3200 \pm 1900 \, \mu M$) than the other strains. The *Ralstonia* sp. strains exhibited middle K_S-values ($4.9 \pm 1.9 \, \mu M$) and K_I-values ($410 \pm 240 \, \mu M$) with the exception of *Ralstonia* sp. HAB-02. The *Variovorax* sp. strains exhibited higher K_S-values ($7.2 \pm 0.86 \, \mu M$) and lower K_I-values ($170 \pm 40 \, \mu M$) with the exception of *Variovorax* sp. strains YN07 and chemo52. Almost all of the kinetic parameters for phenol of the individual strains were similar to those from the soil bioreactor as described previously (Haruta et al., 2013). The K_S-values varied within one order of magnitude (from 1 to 10 μM), whereas the K_I-values varied within two orders of magnitude (from 100 to 1000 μM). Furthermore, kinetic parameters (K_S- and K_I-values) of the strains and the soil bioreactor changed one-thirtieth fold decrease in the K_I-values with a three-fold increase in the K_S-value.

Population Dynamics in the Mixed Chemostat Reactor
SMEI-1

To understand the previous observation that high K_S-type bacteria eventually became dominant in the soil bioreactor, we attempted to reproduce the result in an SME constructed with the isolated strains *P. putida* P-8 (No. 1 shown in **Figure 1**) and *Variovorax* sp. HAB-24 (No. 26 shown in **Figure 1**). The SME was run at a flow rate of 31.5 mL h^{-1} (an HRT of 2.0 days) and the population densities were monitored by the real-time qPCR technique (**Figure 2A**). It was previously shown that *Pseudomonas* and *Variovorax* strains were the dominant genera in, respectively, the early and final stages of reactor operation (Futamata et al., 2005). The kinetic parameters of strains P-8

and HAB-24 were similar to those of the soil bioreactor. From these phylogenetic and kinetic data, it was predicted that strain HAB-24 would be the final dominant strain. However, in the SME strain P-8 overcame strain HAB-24 (**Figure 2A**); the population densities of strains P-8 and HAB-24 were stable at $3.6 \pm 1.1 \times 10^9$ cells mL^{-1} and $1.3 \pm 0.84 \times 10^7$ cells mL^{-1}, respectively. The result of a simulation showed that only strain P-8 would survive and be maintained at 9.8×10^9 cells mL^{-1} (**Figure 2B**), whereas strain HAB-24 would be eradicated (**Figure 2C**).
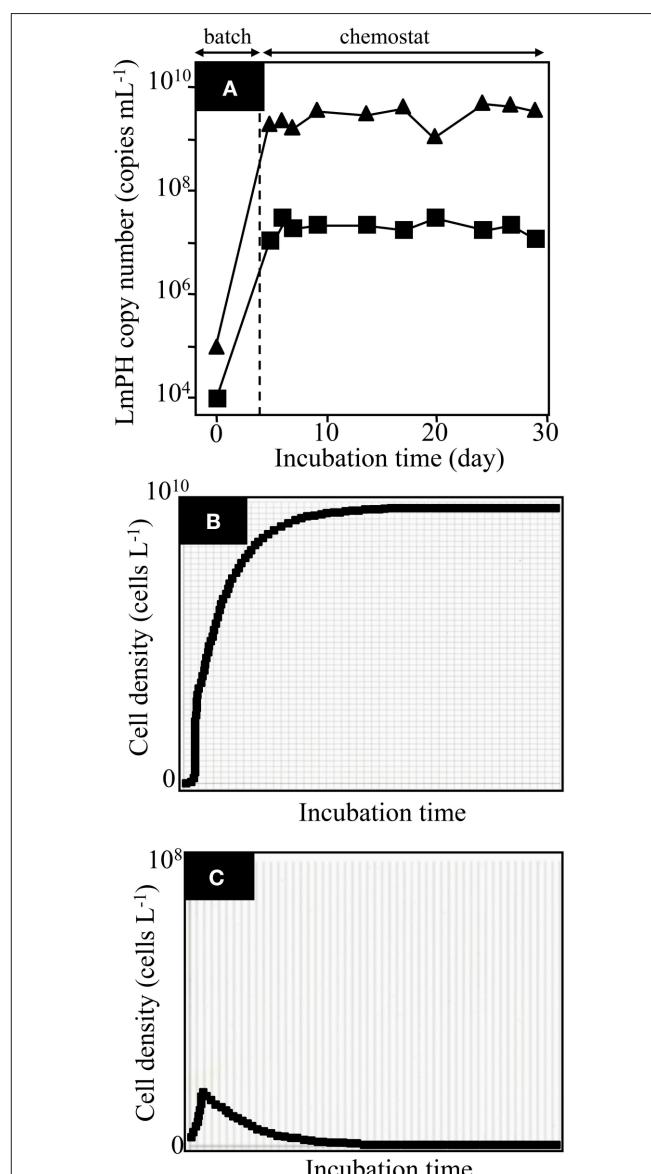
FIGURE 2 | Population dynamics in the chemostat reactor SMEI-1 run at a flow rate of 31.5 mL h^{-1} (a hydraulic residence time of 2.0 days) and a simulation. (A) The population densities of the strains in the mixed chemostat culture were monitored using real-time qPCR targeting the gene encoding LmPH. *Psuedomonas putida* P-8 (▲) and *Variovorax* sp. HAB-24 (■). Error bars are smaller than the symbol. **(B)** The result of simulation showing the population of *P. putida* P-8. **(C)** The result of simulation showing the population of *Variovorax* sp. HAB-24.

FIGURE 1 | Kinetic parameters of isolated strains and the soil bioreactor for phenol. Number indicates the strain shown in **Table 1**.

SMEI-2

SMEI-2, which consisted of strains *P. putida* P-8, *Acinetobacter* sp. c26 (No. 30 shown in **Figure 1**), and *Variovorax* sp. HAB-24, was run at a flow rate of 31.5 mL h^{-1} (an HRT of 2.0 days) and the population densities were monitored using real-time qPCR (**Figure 3A**). The genera *Pseudomonas*, *Acinetobacter*, and *Variovorax* corresponded respectively to the first, second, and third dominant genera in the soil bioreactor. According to the affinity dynamics of the soil bioreactor for phenol and catechol, the affinity parameters of strain c26 for phenol and catechol were located at an inflection point from low K_S for phenol and high K_S for catechol to high K_S for phenol and low K_S for catechol of the soil bioreactor (Supplemental Figure 1). It was thought that a strain located at this inflection point would be needed for strain HAB-24 to become dominant. Strains P-8 and c26, rather than strain HAB-24, grew quickly in the batch mode because of their higher μ-values (**Table 2**). The population density of strain c26 was maintained at around $1.1 \pm 0.073 \times 10^9$ cells mL^{-1}

FIGURE 3 | Population dynamics in the chemostat reactors SMEI-2 and SMEI-3 run at a flow rate of 31.5 mL h^{-1} (a hydraulic residence time of 2.0 days). The deduced population densities of the strains in the mixed chemostat culture were monitored using real-time qPCR targeting the gene encoding LmPH. **(A)** Monitoring of the bacterial populations in the SMEI-2 reactor. *Pseudomonas putida* P-8 (▲) *Acinetobacter* sp. c26 (●), and *Variovorax* sp. HAB-24 (■). **(B)** Monitoring of the bacterial populations in the SMEI-3 reactor. *Pseudomonas putida* P-8 (▲) *Acinetobacter* sp. c26 (●) *Ralstonia* sp. c41 (♦), and *Variovorax* sp. HAB-24 (■). Error bars are smaller than the symbol. Gray bar indicates the period when phenol was detected in the reactors.

after day 5. Although strain P-8 grew well at a similar level to strain c26 under batch mode, the population density of strain P-8 decreased gradually from 6.1×10^8 cells mL^{-1} to 4.7×10^7 cells mL^{-1}. On the other hand, the population density of strain HAB-24 remained stable at about $4.11 \pm 0.24 \times 10^5$ cells mL^{-1} after day 5. Small amounts of phenol were detected in the effluent from the SMEI-2 around day 7 as well as from day 17 to 20 (data not shown).

SMEI-3

SMEI-3, which consisted of *P. putida* P-8, *Acinetobacter* sp. c26, *Ralstonia* sp. c41 (No. 24 shown in **Figure 1**), and *Variovorax* sp. HAB-24, was run at a flow rate of 31.5 mL h^{-1} (an HRT of 2.0 days) and the population densities were again monitored (**Figure 3B**). According to the affinity dynamics of the soil bioreactor for phenol and catechol, it was predicted that strain c41 would be an intermediate between strains P-8, HAB-24, and c26 (Supplemental Figure 1). The amount of growth of the four strains corresponded to their μ-values under batch conditions. Their population densities were roughly maintained under chemostat conditions of this experiment; the population densities of strains c26, P-8, c41, and HAB-24 were $1.3 \pm 0.21 \times 10^9$ cells mL^{-1}, $2.3 \pm 0.26 \times 10^8$ cells mL^{-1}, $7.6 \pm 0.81 \times 10^6$ cells mL^{-1}, and $2.9 \pm 1.71 \times 10^5$ cells mL^{-1}, respectively. Again, small amounts of phenol were detected in the effluent from the SMEI-3 on days 7–10 and days 16–20 (data not shown).

SMEII–1

SMEII-1 consisted of *P. putida* P-8, *Acinetobacter* sp. c26, and *Variovorax* sp. HAB-24, which were the same strains as used in SMEI-2). The purpose of SMEII-1 was to investigate the effect of flow rate on population dynamics. Here the flow rate was decreased from 31.5 mL h^{-1} (HRT = 2.0 days) to 10.4 mL h^{-1} (HRT = 6.0 days). The population densities were monitored using real-time QPCR with specific sets of primers (**Figure 4A**). Strains P-8 and c26 grew faster than strain HAB-24 in the batch mode, as was the case in SMEI-2. The population density of strain P-8 was stable at $6.2 \pm 0.78 \times 10^9$ cells mL^{-1} from day 10 to day 48, after which the population density decreased below 1.0×10^7 cells mL^{-1} by the end of the experiment. Conversely, the population density of strain c26 was stable at $8.2 \pm 0.16 \times 10^7$ cells mL^{-1} from day 15 to day 30, after which the population density increased to approximately $4.2 \pm 0.32 \times 10^9$ cells mL^{-1} by the end of the experiment. The population density of strain HAB-24 was stable at around $6.2 \pm 0.76 \times 10^5$ cells mL^{-1} after day 5. The population dynamics of SMEII-1 were similar to that of SMEI-2, showing that the final dominant bacterium was *Acinetobacter* sp. c26. Phenol was never detected in the effluent during this experiment. Therefore, all subsequent SMEII series chemostats were run with a flow rate at 10.4 mL h^{-1} (HRT = 6.0 days).

SMEII-2

SMEII-2 was constructed with *Pseudomonas* sp. LAB-06 (No. 2 shown in **Figure 1**), *Acinetobacter* sp. c26 and *Variovorax* sp. HAB-24, and run with a flow rate of 10.4 mL h^{-1} (an HRT of 6.0 days) (**Figure 4B**). Strain c26, but not strains LAB-06 and HAB-24, grew quickly in the batch mode because of higher μ-value

TABLE 2 | Kinetic and growth parameters on phenol of isolates used in mixed chemostat culture.

Strains	μ (h⁻¹)	Maximum OD	J parameter (μM)[a]	J parameter (μM)[b]
Acinetobacter sp. c26	0.63 ± 0.13	0.18 ± 0.046	0.086 ± 0.025	0.027 ± 0.0077
Ralstonia sp. chemo32	0.22 ± 0.089	0.27 ± 0.11	0.59 ± 0.17	0.18 ± 0.048
Ralstonia sp. c41	0.17 ± 0.029	0.32 ± 0.043	0.46 ± 0.096	0.14 ± 0.026
Pseudomonas sp. LAB-06	0.39 ± 0.14	0.23 ± 0.0084	0.41 ± 0.20	0.13 ± 0.060
Pseudomonas sp. P-8	0.37 ± 0.054	0.24 ± 0.0093	0.33 ± 0.059	0.10 ± 0.018
Variovorax sp. HAB-24	0.11 ± 0.012	0.25 ± 0.020	1.7 ± 0.21	0.49 ± 0.052
Variovorax sp. HAB-30	0.13 ± 0.021	0.20 ± 0.084	1.2 ± 0.19	0.33 ± 0.050

[a]*Dilution rate was 0.021 h⁻¹ (HRT = 2 days). It was a chemostat condition of BRI-series.*
[b]*Dilution rate was 0.0069 h⁻¹ (HRT = 6 days). It was a chemostat condition of BRII-series.*

(**Table 2**). Strain c26 remained dominant until day 10. On day 15, the population densities of the three strains were all close to $2.8 \pm 2.5 \times 10^6$ cells mL⁻¹, after which the population density of HAB-24 increased and was stable at $2.5 \pm 1.1 \times 10^9$ cells mL⁻¹ after day 35. Conversely, the population density of LAB-06 and c26 were $2.6 \pm 1.8 \times 10^6$ cells mL⁻¹ and $2.7 \pm 1.6 \times 10^7$ cells mL⁻¹ after day 30, respectively. Bacterial community succession was observed, resulting in the dominance of HAB-24.

SMEII-3

SMEII-3 was constructed with *Pseudomonas* sp. LAB-06 (No. 2 shown in **Figure 1**), *Ralstonia* sp. chemo32 (No. 16 shown in **Figure 1**), and *Variovorax* sp. HAB-30 (No. 23 shown in **Figure 1**) and run at a flow rate of 10.4 mL h⁻¹ (an HRT of 6.0 days) (**Figure 4C**). Strains LAB-06 and chemo32 grew faster than strain HAB-30 in the batch mode because of their higher μ-values (**Table 2**). Strain LAB-06 became dominant at a cell density of $1.6 \pm 0.89 \times 10^8$ cells mL⁻¹ until day 20. The population densities of strains LAB-06 and chemo32 decreased to $9.1 \pm 8.9 \times 10^5$ cells mL⁻¹ and $8.6 \pm 8.2 \times 10^4$ cells mL⁻¹, respectively, after day 40. Conversely, the population density of HAB-30 remained stable at around $1.3 \pm 1.3 \times 10^7$ cells mL⁻¹ from days 10 to 60. Thus, SMEII-3 exhibited a bacterial community succession, where strain HAB-30 became dominant. Small amounts of phenol were detected in the effluent from the SMEII on days 65–70 (data not shown).

Interspecies Interactions

Strains Used in the SMEI Series SMEs

Interactions among the four strains used in the SMEI series SEMs, *P. putida* P-8, *Variovorax* sp. HAB-24, *Acinetobacter* sp. c26, and, *Ralstonia* sp. c41, were investigated by measuring their specific growth activities (**Table 3**). The supernatant of strain P-8 culture enhanced the growth activities of strains P-8 and c26 to approximately 140 and 180%, respectively but repressed those of strains c41 and HAB-24 to approximately 6.5 and 7.4%, respectively. The supernatant of strain c26 did not affect the growth activities of strains P-8 and c26, but repressed those of strains c41 and HAB-24 to approximately 25 and 38%, respectively. The supernatant of strain c41 enhanced the growth activities of strains P-8 and c26 to approximately 120% but did not affect the growth activities

of strains c41 and HAB-24. The supernatant of strain HAB-24 enhanced the growth activities of strains c26 and HAB-24 to approximately 160 and 150%, respectively, but repressed those of strains P-8 and c41 to approximately 40 and 11%, respectively.

Strains Used in the SMEII Series SMEs

Interactions among the three strains used in SMEII-2 (**Table 4**) and SMEII-3 (**Table 5**) were investigated by measuring their specific growth activities. The supernatant of strain LAB-06 did not affect its own growth activity but it repressed that of strain c26 to approximately 71% and enhanced that of strain HAB-24 to approximately 180% (**Table 4**). Supernatant of strain c26 did not affect the growth activity of strain LAB-06 and itself but repressed that of strain HAB-24 to approximately 38%. Supernatant of strain HAB-24 repressed the growth activity of strain LAB-06 to approximately 40% but enhanced those of strains c26 and HAB-24 to approximately 160 and 153%, respectively. The supernatant of strain LAB-06 repressed those of strains chemo32 and HAB-30 to approximately 75 and 22%, respectively (**Table 5**). The supernatant of strain chemo32 did not affect the growth activities of strains LAB-06 and chemo32 but enhanced that of strain HAB-30 to approximately 160%. The supernatant of strain HAB-30 enhanced its own growth activity to approximately 140% but repressed those of strains LAB-06 and chemo32 to approximately 87 and 48%, respectively. The mixed supernatant of strains chemo32 and HAB-30 did not affect the growth activity of LAB-06. Similarly, the mixed supernatant of strains LAB-06 and chemo32 did not affect the growth activity of HAB-30. The mixed supernatant of strain LAB-06 and HAB-30 repressed the growth activity of chemo32 to approximately 85%.

Dynamics of Kinetics Parameters in the SMEII-3 SME

The kinetic parameters (K_S- and K_I-values) of the SMEII-3 SME for phenol and catechol were monitored (**Figure 5**). The K_S- and K_I-values for phenol shifted from $2.6 \pm 0.17\ \mu M$ and $2060 \pm 200\ \mu M$ at day 10 to $0.42 \pm 0.037\ \mu M$ and $1060 \pm 28\ \mu M$ at day 30, which indicated that the affinity for phenol of the SMEII-3 reactor increased. However, these kinetic parameters changed dramatically and exhibited a dynamic equilibrium at higher K_S-values ($4.0 \pm 1.8\ \mu M$) and lower K_I-values ($275 \pm$
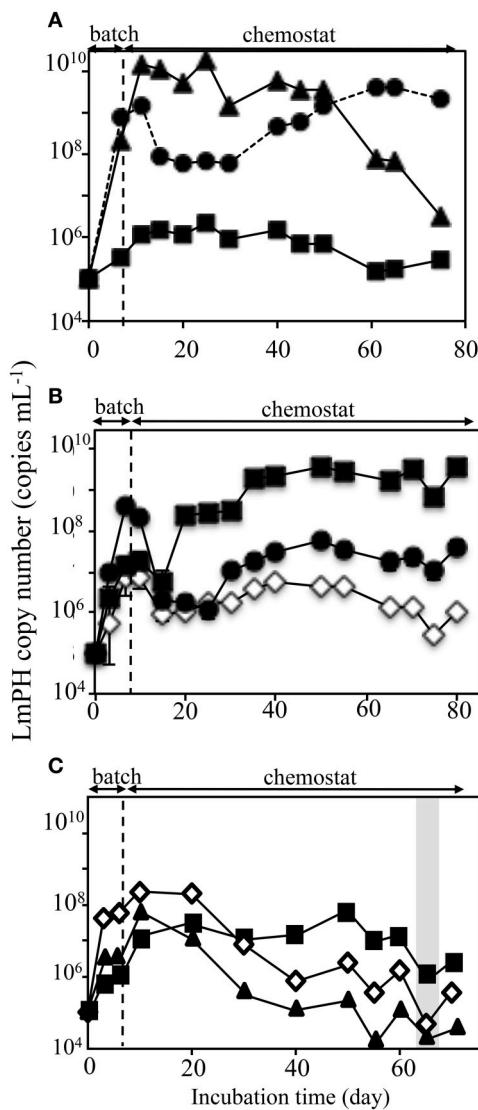
FIGURE 4 | Population dynamics in the chemostat reactors SMEII series run at a flow rate of 10.4 mL h^{-1} (a hydraulic residence time of 6.0 days). The deduced population densities of the strains in the mixed chemostat culture were monitored using real-time qPCR targeting the gene encoding LmPH. **(A)** Monitoring of the bacterial populations used in the SMEII-1 reactor. *Psuedomonas putida* P-8 (▲) *Acinetobacter* sp. c26 (●), and *Variovorax* sp. HAB-24 (■). **(B)** Monitoring of the bacterial populations used in the SMEII-2 reactor. *Pseudomonas* sp. LAB-06 (◇), *Acinetobacter* sp. c26 (●), and *Variovorax* sp. HAB-24 (■). **(C)** Monitoring of the bacterial populations used in the SMEII-3 reactor. *Psuedomonas* sp. LAB-06 (◇), *Acinetobacter* sp. chemo32 (▲), and *Variovorax* sp. HAB-24 (■). The gray bar indicates the period when phenol was detected in the reactors. The error bars indicate standard deviations and some bars were hided with symbols.

TABLE 3 | Relative specific growth among strains used in SMEI-series and SMEII-1[a].

Strains	Supernatant of strains			
	P-8	c26	chemo41	HAB-24
Pseudomonas sp. P-8	140 ± 35	105 ± 11	120 ± 16	40 ± 5.0
Acinetobacter sp. c26	180 ± 90	100 ± 3.0	120 ± 58	160 ± 90
Ralstonia sp. c41	6.5 ± 0.5	25 ± 9.3	97 ± 39	1 ± 9.1
Variovorax sp. HAB-24	7.4 ± 1.3	38 ± 8.6	90 ± 12	153 ± 8.9

[a]*The specific growth activity without supernatant (control condition) was calculated as 100%.*

TABLE 4 | Relative specific growth among strains used in SMEII-2[a].

Strains	Supernatant of strains		
	LAB-06	c26	HAB-24
Pseudomonas sp. LAB-06	93 ± 7.3	105 ± 11	77 ± 8.4
Acinetobacter sp. c26	71 ± 6.8	100 ± 3.0	160 ± 90
Variovorax sp. HAB-24	180 ± 90	38 ± 8.6	153 ± 8.9

[a]*Specific growth activity without supernatant was calculated as 100% (control condition).*

TABLE 5 | Relative specific growth among strains used in SMEII-3[a].

Strains	Supernatant of strains			
	LAB-06	chemo32	HAB-30	Mixed supernatant
Pseudomonas sp. LAB-06	93 ± 7.3	103 ± 41	87 ± 12	103 ± 8.5[b]
Ralstonia sp. chemo32	75 ± 6.3	100 ± 50	48 ± 17	85 ± 16[c]
Variovorax sp. HAB-30	22 ± 6.4	160 ± 35	140 ± 33	105 ± 4.8[d]

[a]*Specific growth activity without supernatant (control condition) was calculated as 100%.*
[b]*Supernatants obtained from strain c32 and HAB-30 were used.*
[c]*Supernatants obtained from strain LAB-06 and HAB-30 were used.*
[d]*Supernatants obtained from strain LAB-06 and c32 were used.*
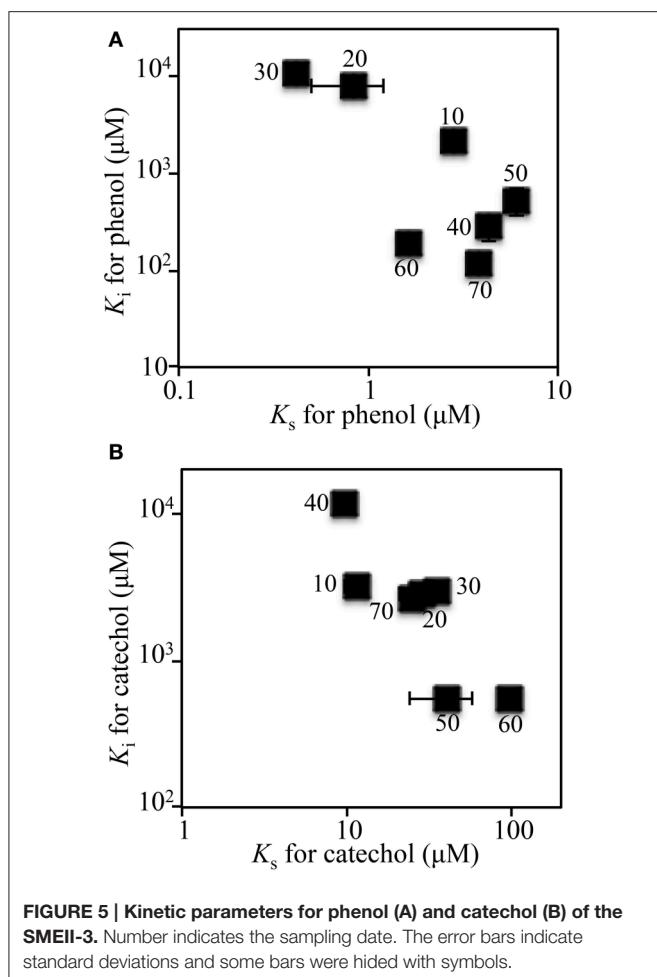
47 µM at day 60 and then returned to their initial values at day 70.

DISCUSSION

In a soil bioreactor fed with phenol we previously observed a unique phenomenon where the kinetic parameters shifted toward higher K_S and lower K_I-values (Futamata et al., 2005; Haruta et al., 2013). In the present study, to understand this unique phenomenon, SMEs were constructed using isolated strains exhibiting different phenotypic features. By observing changes in population densities we have developed a better understanding of population dynamics in SMEs and soil bioreactors.

Kinetic parameters have been thought to be one of the most important determinants for predicting the competitiveness of strains grown on a single substrate (Watanabe et al., 1996, 1998a). Since the *J* parameter is calculated using not only the K_S-value (affinity) but also µ (growth rate constant) and

180 µM) from days 40 to 70. The K_S-value for catechol shifted more dynamically than that for phenol (**Figure 5B**). The K_S- and K_I-values for catechol were stable at 25 ± 13 µM and 3000 ± 170 µM, respectively, from days 10 to 30. However, these kinetic parameters fluctuated dynamically from 9.9 ± 0.56 µM and 11500 ± 860 µM at day 40 to 100 ± 6.2 µM and 540 ±

FIGURE 5 | Kinetic parameters for phenol (A) and catechol (B) of the SMEII-3. Number indicates the sampling date. The error bars indicate standard deviations and some bars were hided with symbols.

and it was natural that *Acinetobacter* sp. strain c26 became dominant.

It remained unknown whether the *J* parameter or interspecies interactions were more important for predicting population dynamics in a chemostat culture. Therefore, SMEII-2 was constructed with *Acinetobacter* sp. strain c26, *Variovorax* sp. strain HAB-24, and *Pseudomonas* sp. strain LAB-06 instead of *P. putida* strain P-8. Based on their *J* parameters (**Table 2**), *Acinetobacter* sp. strain c26 was predicted to become dominant. However, from these interspecies interactions (**Table 4**), it was predicted that *Variovorax* sp. strain HAB-24 would become dominant as strain LAB-06 would repress strain c26 and enhance strain HAB-24. In actuality the final dominant organism was strain HAB-24 not strain c26 (**Figure 4B**), suggesting that the interspecies interactions did indeed play a more important role in bacterial community dynamics than the *J* parameter. To confirm this possibility, SMEII-3 was constructed with *Pseudomonas* sp. strain LAB-06, *Ralstonia* sp. strain chemo32, and *Variovorax* sp. strain HAB-30. Although strain LAB-06 exhibited the lowest *J* parameter (indicating it should be the most competitive) among these three strains (**Table 2**), the final dominant was strain HAB-30. While interspecies interaction data (**Table 5**) indicated that strain LAB-06 inhibited HAB-30, the addition of chemo32 was also expected to rescue this inhibition. Thus, the complexity of this three-way interaction resulted in strain HAB-30 becoming dominant after community succession and fluctuation of kinetic parameters (**Figure 5**). Importantly it was also demonstrated that this SME reproduced the unique phenomenon previously observed in the soil bioreactor (Futamata et al., 2005; Haruta et al., 2013).

Since it is recognized that interactive networks develop among diverse microbes in natural ecosystems (Gilbert et al., 2012), it was expected that understanding these complex interactions would be important for understanding how microbial ecosystems develop. Striking a balance between the enhancing and repressing relationships was considered to be essential for maintaining the stable coexistence of the five bacterial strains in a cellulose-degrading community (Kato et al., 2005). While it is possible to qualitatively understand such interspecies interactions, it remains difficult to quantify their outcome. As a physiological process with defined, quantifiable output, cell growth was expected to be an ideal indicator for understanding the effects of interspecies interactions. Here, using relative growth in the presence of supernatants of other strains, we have demonstrated that the specific growth activity was indeed useful for understanding the dynamics of microbial ecosystems and for predicting the dominant species in a mixed, SME.

In this study we characterized both kinetic, physiological traits of multiple phenol-degrading strains as well as their binary interactions with each other. Using this quantitative method for evaluating interspecies interactions we have developed a method for predicting microbial community dynamics and demonstrated that the complex interactions between species are a more significant determinant for microbial community dynamics than the *J* parameter is for predicting which species will

D (dilution rate), it is especially useful for predicting what strain will become dominant in a chemostat culture (Hansen and Hubbell, 1980). Additionally, cell-to-cell interactions are important for understanding the microbial ecosystem (Watts and Strogatz, 1998; Flagan et al., 2003; Kato et al., 2005; Narisawa et al., 2008). To the best of our knowledge this study presents the first attempt to use both the *J* parameter and interspecies interaction information simultaneously for understanding the dynamics of microbial community in a complex system.

It is reported that *Variovorax* strains are capable of utilizing or producing acyl-homoserine lactones which are quorum signaling molecules in many species of the class *Proteobacteria* (Leadbetter and Greenberg, 2000; d'Angelo-Picard et al., 2005; Yang et al., 2006; Satola et al., 2013). We hypothesized that the *Variovorax* spp. became dominant in the soil bioreactor through the use of quorum signaling. The SMEI series and SMEII-1 were expected to reproduce the unique phenomenon of the soil bioreactor, however, *Variovorax* sp. strain HAB-24 exhibiting highest *K*$_S$-value among strains used in SMEs did not become dominant (**Figures 2**, **3**, **4A**). Therefore, it was demonstrated that either the *J* parameter or interspecies interactions data were capable of predicting the experimentally observed population dynamics

become dominant. Interestingly, although bacterial community succession was observed, all strains still co-existed in all SMEs and none were eradicated, suggesting that these strains shared a role in phenol degradation. Further work still needs to be done as it was thought that these strains shared a role in degradation of phenol by changing metabolic process. A novel biomathematical theory is also required to fully understand dynamic microbial ecosystems. Research subjects that attract attention with respect to microbial community dynamics are relevant to the identification of growth-repressing compounds, mechanisms of interspecies interactions and comparison of metabolism under the conditions of pure and complex cultures.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01148

REFERENCES

d'Angelo-Picard, C., Faure, D., Penot, I., and Dessaux, Y. (2005). Diversity of *N*-acyl homoserine lactone-producing and -degrading bacteria in soil and tobacco rhizosphere. *Environ. Microbiol.* 7, 1796–1808. doi: 10.1111/j.1462-2920.2005.00886.x

De Roy, K., Marzorati, M., den Abbeele, P. V., de Wiele, T. V., and Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ. Microbiol.* 16, 1472–1481. doi: 10.1111/1462-2920.12343

DeSalvo, S. C., Liu, Y., Choudhary, G. S., Ren, D., Nangia, S., Sureshkumar, R., et al. (2015). Signaling factor interactions with polysaccharide aggregates of bacterial biofilms. *Langmuir* 31, 1958–1966. doi: 10.1021/la504721b

El-Chakhtoura, J., Prest, E., Saikaly, P., van Loosdrecht, M., Hammers, F., and Vrouwenvelder, H. (2015). Dynamics of bacterial communities before and after distribution in a full-scale drinking water network. *Water Res.* 74, 180–190. doi: 10.1016/j.watres.2015.02.015

Fernández, A., Juang, S., Seston, S., Xing, J., Hickey, R., Criddle, C., et al. (1999). How stable is stable? Function versus community composition. *Appl. Environ. Microbiol.* 65, 3697–3704.

Fernández, A. S., Hashsham, S. A., Dollhopf, S. L., Raskin, L., Glagoleva, O., Dazzo, F. B., et al. (2000). Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. *Appl. Environ. Microbiol.* 66, 4058–4067. doi: 10.1128/AEM.66.9.4058-4067.2000

Flagan, S., Chign, W. K., and Leadbetter, J. R. (2003). *Arthrobacter* strain VAI-A utilizes acyl-homoserine lactone inactivation products and stimulates quorum signal biodegradation by *Variovorax paradoxus*. *Appl. Environ. Microbiol.* 69, 909–916. doi: 10.1128/AEM.69.2.909-916.2003

Folsom, B. R., Chapman, P. J., and Pritchard, P. H. (1990). Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. *Appl. Environ. Microbiol.* 56, 1279–1285.

Futamata, H., Harayama, S., and Watanabe, K. (2001a). Diversity in kinetics of trichloroethylene-degrading activities exhibited by phenol-degrading bacteria. *Appl. Microbiol. Biotechnol.* 55, 248–253. doi: 10.1007/s002530000500

Futamata, H., Harayama, S., and Watanabe, K. (2001b). Group-specific monitoring of phenol hydroxylase genes for a functional assessment of phenol-stimulated trichloroethylene bioremediation. *Appl. Environ. Microbiol.* 67, 4671–4677. doi: 10.1128/AEM.67.10.4671-4677.2001

Futamata, H., Nagano, Y., Watanabe, K., and Hiraishi, A. (2005). Unique kinetic properties of phenol-degrading *Variovorax* strains responsible for efficient trichloroethylene degradation in a chemostat enrichment culture. *Appl. Environ. Microbiol.* 71, 904–911. doi: 10.1128/AEM.71.2.904-911.2005

Gilbert, J. A., Steele, J. A., Gaporaso, J. G., Steinbrück, L., Reeder, J., Temperton, B., et al. (2012). Defining seasonal marine microbial community dynamics. *ISME J.* 6, 298–308. doi: 10.1038/ismej.2011.107

Hansen, S. R., and Hubbell, S. P. (1980). Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. *Science* 207, 1491–1493. doi: 10.1126/science.6767274

Haruta, S., Yoshida, T., Aoi, Y., Kaneko, K., and Futamata, H. (2013). Challenges for complex microbial ecosystems: combination of experimental approaches with mathematical modeling. *Microbes Environ.* 28, 285–294. doi: 10.1264/jsme2.ME13034

Hashsham, S. A., Fernandez, A. S., Dollhopf, S. L., Dazzo, F. B., Hickey, R. F., Tiedje, J. M., et al. (2000). Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor communities perturbed by glucose. *Appl. Environ. Microbiol.* 66, 4050–4057. doi: 10.1128/AEM.66.9.4050-4057.2000

Inaba, T., Oura, H., Morinaga, K., Toyofuku, M., and Nomura, N. (2015). The *Pseudomonas* quinolone signal inhibits biofilm development of Streptococcus mutants. *Microbes Environ.* 30, 189–191. doi: 10.1264/jsme2.ME14140

Ishii, S., Suzuki, S., Norden-Krichmar, T. M., Nealson, K. H., Sekiguchi, Y., Gorby, Y. A., et al. (2012). Functionally stable and phylogenetically diverse microbial enrichments from microbial fuel cells during wastewater treatment. *PLoS ONE* 7:e30495. doi: 10.1371/journal.pone.0030495

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., and Igarashi, Y. (2005). Stable coexistence of five bacterial strains as a cellulose-degrading community. *Appl. Environ. Microbiol.* 71, 7099–7107. doi: 10.1128/AEM.71.11.7099-7106.2005

Klitgord, N., and Segrè, D. (2010). Environments that induce synthetic microbial ecosystems. *PLoS Comput. Biol.* 6:e1001002. doi: 10.1371/journal.pcbi.1001002

Leadbetter, J. R., and Greenberg, E. P. (2000). Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *J. Bacteriol.* 182, 6921–6926. doi: 10.1128/JB.182.24.6921-6926.2000

Little, A. E. F., Robinson, C. J., Peterson, S. B., Raffa, K. E., and Handelsman, J. (2008). Rules of engagement: interspecies interactions that regulate microbial communities. *Annu. Rev. Microbiol.* 62, 375–401. doi: 10.1146/annurev.micro.030608.101423

Mee, M. T., Collins, J. J., Church, G. M., and Wang, H. H. (2014). Synthrophic exchange in systhetic microbial communities. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2149–E2156. doi: 10.1073/pnas.1405641111

Narisawa, N., Haruta, S., Arai, H., Ishii, M., and Igarashi, Y. (2008). Coexistence of antibiotic-producing and antibiotic-sensitive bacteria in biofilm is mediated by resistant bacteria. *Appl. Environ. Microbial.* 74, 3887–3894. doi: 10.1128/AEM.02497-07

Saito, Y. (2002). The necessary and sufficient condition for global stability of a Lotka-Volterra cooperative or competition system with delays. *J. Math. Anal. Appl.* 268, 109–124. doi: 10.1006/jmaa.2001.7801

Saito, Y., Hara, T., and Ma, W. (1999). Necessary and sufficient conditions for permanence and global stability of a Lotka-Volterra system with two delays. *J. Math. Anal. Appl.* 236, 534–556. doi: 10.1006/jmaa.1999.6464

Satola, B., Wübbeler, J. H., and Steinbüchel, A. (2013). Metabolic characteristics of the species *Variovorax paradoxus*. *Appl. Microbiol. Biotehcnol.* 97, 541–560. doi: 10.1007/s00253-012-4585-z

Tanaka, Y., Hanada, S., Tamaki, H., Nakamura, K., and Kamagata, Y. (2005). Isolation and identification of bacterial strains producing diffusible growth factor(s) for *Catellibacterium nectariphilium* strain AST4[T]. *Microbes Environ.* 20, 110–116. doi: 10.1264/jsme2.20.110

Tashiro, Y., Yawata, Y., Toyofuku, M., Uchiyama, H., and Nomura, N. (2013). Interspecies interaction between *Pseudomonas aeruginosa* and

other microorganisms. *Microbes Envrion*. 28, 13–24. doi: 10.1264/jsme2. ME12167

Watanabe, K., Hino, S., Onodera, K., Kajie, S., and Takahashi, N. (1996). Diversity in kinetics of bacterial phenol-oxygenating activity. *J. Ferment. Bioeng*. 81, 560–563. doi: 10.1016/0922-338X(96)81481-4

Watanabe, K., Teramoto, M., Futamata, H., and Harayama, S. (1998a). Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Appl. Environ. Microbiol*. 64, 4396–4402.

Watanabe, K., Yamamoto, S., Hino, S., and Harayama, S. (1998b). Population dynamics of phenol-degrading bacteria in activated sludge determined by *gyrB*-targeted quantitative PCR. *Appl. Environ. Microbiol*. 64, 1203–1209.

Watts, D. J., and Strogatz, S. H. (1998). Collective dynamics of 'small-world' networks. *Nature* 393, 440–442. doi: 10.1038/30918

Yamamoto, S., Suzuki, K., Araki, Y., Mochihara, H., Hosokawa, T., Kubota, H., et al. (2014). Dynamics of different bacterial communities are capable of generating sustainable electricity from microbial fuel cell

with organic waste. *Microbes Envrion*. 29, 145–153. doi: 10.1264/jsme2. ME13140

Yang, W. W., Han, J. I., and Leadbetter, J. R. (2006). Utilization of homoserine lactone as a sole source of carbon and energy by soil *Arthrobacter* and *Burkholderia* species. *Arch. Microbiol*. 185, 47–54. doi: 10.1007/s00203-005-0065-5

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Estimating and mapping ecological processes influencing microbial community assembly

James C. Stegen*, Xueju Lin, Jim K. Fredrickson and Allan E. Konopka

Fundamental and Computational Sciences Directorate, Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA

Ecological community assembly is governed by a combination of (i) selection resulting from among-taxa differences in performance; (ii) dispersal resulting from organismal movement; and (iii) ecological drift resulting from stochastic changes in population sizes. The relative importance and nature of these processes can vary across environments. Selection can be homogeneous or variable, and while dispersal is a rate, we conceptualize extreme dispersal rates as two categories; dispersal limitation results from limited exchange of organisms among communities, and homogenizing dispersal results from high levels of organism exchange. To estimate the influence and spatial variation of each process we extend a recently developed statistical framework, use a simulation model to evaluate the accuracy of the extended framework, and use the framework to examine subsurface microbial communities over two geologic formations. For each subsurface community we estimate the degree to which it is influenced by homogeneous selection, variable selection, dispersal limitation, and homogenizing dispersal. Our analyses revealed that the relative influences of these ecological processes vary substantially across communities even within a geologic formation. We further identify environmental and spatial features associated with each ecological process, which allowed mapping of spatial variation in ecological-process-influences. The resulting maps provide a new lens through which ecological systems can be understood; in the subsurface system investigated here they revealed that the influence of variable selection was associated with the rate at which redox conditions change with subsurface depth.

Keywords: ecological niche theory, ecological neutral theory, Hanford Site 300 Area, microbial biogeography, null modeling, phylogenetic beta-diversity, phylogenetic signal, Raup–Crick

Introduction

In his conceptual synthesis, Vellend (2010) proposed that ecological communities are influenced by a combination of ecological selection, organismal dispersal, ecological drift, and speciation (see also Hanson et al., 2012). This is a useful perspective that places all ecological communities within the same conceptual framework, thereby facilitating cross-system comparisons. While there is some knowledge of what causes shifts in the relative influences of governing processes (e.g., Horner-Devine and Bohannan, 2006; Chase, 2007, 2010; Stegen et al., 2012), turning Vellend's conceptual framework into an operational framework is a significant challenge. As an initial

attempt, Stegen et al. (2013) proposed a null modeling approach that estimates the relative influences of ecological components within Vellend's framework at the scale of a metacommunity. To provide context, we next introduce the concepts synthesized in Vellend (2010).

Ecological selection results from different organisms having different levels of fitness for a given set of environmental conditions; here we consider 'environmental conditions' to include both abiotic variables (e.g., temperature) and biotic factors related to organismal interactions. If environmental conditions are homogeneous through space, the selective environment will also be homogeneous (Vellend, 2010). The scenario in which a consistent selective pressure—that results from consistent environment conditions—is the primary cause of low compositional turnover is referred to as 'homogeneous selection' (**Figure 1**), as in Dini-Andreote et al. (2015). On the other hand, if environmental conditions change through space, among-taxa fitness differences can change (Vellend, 2010). The scenario in which a shift in selective pressure—that results from a shift in environment conditions—is the primary cause of high compositional turnover is referred to as 'variable selection' (**Figure 1**).

Organismal dispersal refers to the movement of organisms through space (Vellend, 2010). High dispersal rates have the potential to homogenize community composition such that there is low turnover in composition (Leibold et al., 2004). High dispersal rates can, in principle, overwhelm selection-based influences, a scenario at the population level known as source-sink dynamics (Dias, 1996). The scenario in which a high dispersal rate—between a given pair of communities—is the primary cause of low compositional turnover is referred to as 'homogenizing dispersal' (Stegen et al., 2013).

On the other hand, when selection is relatively weak and organisms rarely move between communities, ecological drift (i.e., stochastic changes in population sizes) can lead to marked differences in community composition (Hubbell, 2001; Vellend, 2010). The scenario in which high turnover in composition is primarily due to a low rate of dispersal enabling community composition to drift apart is referred to here as 'dispersal limitation.' To be clear, our use of the term 'dispersal limitation' does not directly refer to a low rate of dispersal between a given pair of communities. We use 'dispersal limitation' to indicate a situation in which a low dispersal rate is the *primary cause* of high compositional turnover; differences in selective environments may be the primary cause of high compositional turnover even when dispersal rates are low, which would fall within the conceptualization of 'variable selection.'

It is important to consider that the rate of dispersal and strength of selection are continuous variables with context-dependent magnitudes and that these processes simultaneously influence ecological communities. The above-summarized scenarios assume relatively extreme levels of dispersal and/or selection, but it is likely that some natural systems (or parts of systems) are characterized by moderate levels of dispersal and selection. In this case, neither process may dominate, leading to compositional differences between communities that are due to a mixture of stochastic organismal movements and stochastic birth–death events. Stegen et al. (2013) used the term 'ecological drift' to refer

to this scenario, but here we use the term 'undominated' to refer more directly to the scenario in which neither dispersal nor selection is the primary cause of between-community compositional differences.

Here we first develop and test an extended version of the Stegen et al. (2013) framework and, in turn, apply the extended framework to subsurface microbial communities distributed across two geologic formations within the 300 Area of the U.S. Department of Energy's Hanford Site in southcentral Washington State. Our framework uses null models to estimate the degree to which compositional turnover between a single focal community and all other sampled communities is governed by different ecological processes (homogeneous selection, variable selection, homogenizing dispersal, and dispersal limitation). The framework also estimates the portion of compositional turnover that is not dominated by a single process. To evaluate this framework we simulated community assembly under different scenarios; the analytical framework was applied to simulated communities to ask how robust the framework was in detecting the dominance of specific ecological processes. In turn, process estimates were generated for the field-sampled subsurface communities; these estimates were combined with environmental data and community spatial locations to identify environmental drivers and to characterize spatial variation in the relative influence of each ecological process.
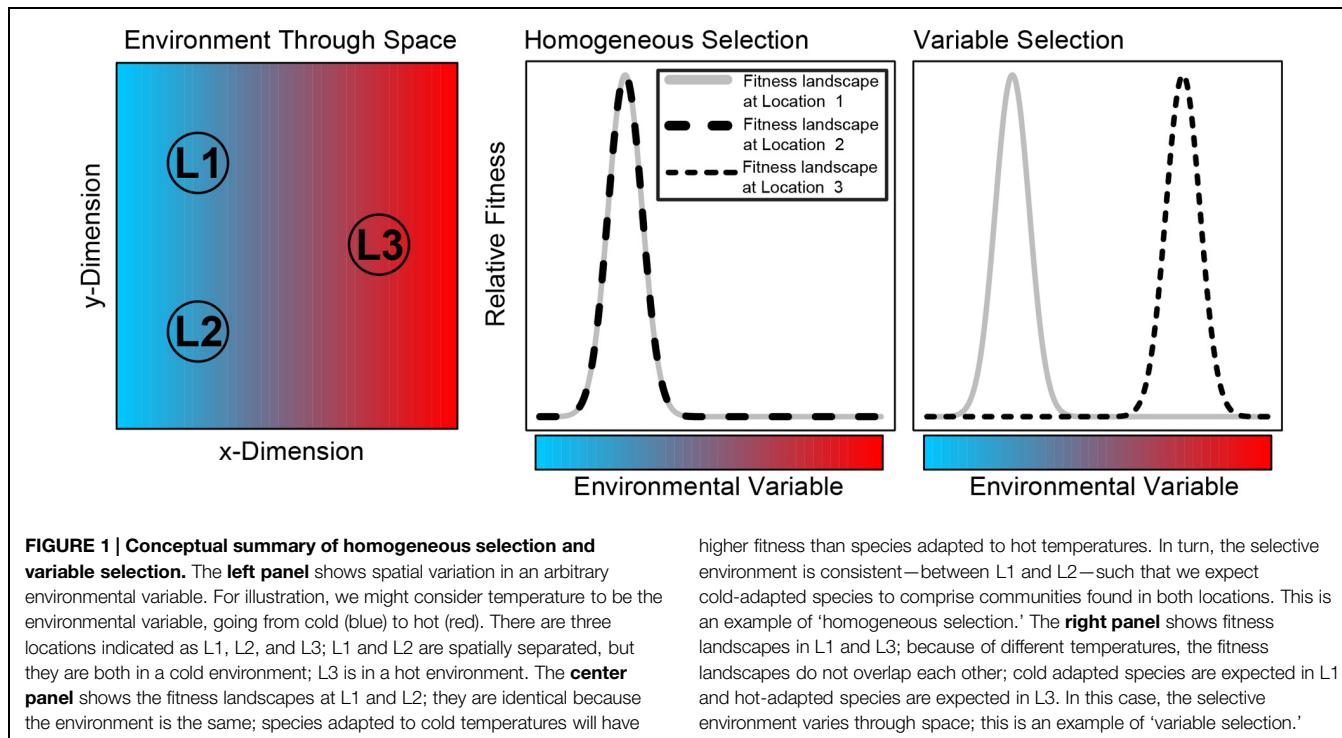
Materials and Methods

Subsurface Sampling

The dataset used here is the same as in Stegen et al. (2013) such that here we provide a brief summary of methods used to generate those data. We analyzed sediment-associated bacterial communities found within two subsurface geologic formations roughly 250 m from the Columbia River in Richland, WA, USA. The sampled communities were within the Hanford Integrated Field Research Challenge (IFRC) site[1], and were sampled during the drilling of 26 IFRC wells (Bjornstad et al., 2009); supplemental material in Stegen et al. (2013) provides details on sample locations. The sampled formations are structurally and hydrologically distinct, are separated vertically, but are in the same horizontal location; 16 communities were sampled within the deeper-lying Ringold Formation, which has restricted water flow and finer-grain sediments; 28 communities were sampled within the shallower Hanford formation, which has less restricted water flow and coarser-grain sediments (Bjornstad et al., 2009). In addition, the Ringold formation changes with depth from oxidized to reduced conditions (Bjornstad et al., 2009). Microbial communities were characterized through pyrosequencing of the 16S rRNA gene (see Lin et al., 2012a,b), DNA sequences were processed using QIIME (Caporaso et al., 2010), and statistical analyses were done using R[2] (for molecular and bioinformatics methods see Stegen et al., 2013). Each microbial community was associated with environmental metadata including its spatial position, its

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[1]http://ifchanford.pnnl.gov/

[2]http://cran.r-project.org/

FIGURE 1 | Conceptual summary of homogeneous selection and variable selection. The **left panel** shows spatial variation in an arbitrary environmental variable. For illustration, we might consider temperature to be the environmental variable, going from cold (blue) to hot (red). There are three locations indicated as L1, L2, and L3; L1 and L2 are spatially separated, but they are both in a cold environment; L3 is in a hot environment. The **center panel** shows the fitness landscapes at L1 and L2; they are identical because the environment is the same; species adapted to cold temperatures will have

higher fitness than species adapted to hot temperatures. In turn, the selective environment is consistent—between L1 and L2—such that we expect cold-adapted species to comprise communities found in both locations. This is an example of 'homogeneous selection.' The **right panel** shows fitness landscapes in L1 and L3; because of different temperatures, the fitness landscapes do not overlap each other; cold adapted species are expected in L1 and hot-adapted species are expected in L3. In this case, the selective environment varies through space; this is an example of 'variable selection.'

horizontal distance from the Columbia River, the elevations of reduced and oxidized portions of the Ringold Formation, and the vertical thickness of the oxidized portion of the Ringold. Vertically structured features were obtained from Bjornstad et al. (2009).

Statistical Approach

Our statistical framework uses phylogenetic turnover between communities to infer ecological selection (Stegen et al., 2013). This approach requires significant 'phylogenetic signal' (Losos, 2008) whereby there is a relationship between phylogenetic relatedness and ecological similarity (Kraft et al., 2007; Cavender-Bares et al., 2009; Fine and Kembel, 2011); as in other microbial systems (e.g., Wang et al., 2013) this was found to be true such that more closely related taxa were more similar ecologically (see Figure 2 in Stegen et al., 2013).

To quantify phylogenetic turnover between communities we used the between-community mean-nearest-taxon-distance (βMNTD) metric (Fine and Kembel, 2011; Webb et al., 2011), which quantifies the phylogenetic distance between each species in one community (k) and its closest relative in a second community (m):

$$\beta MNTD = 0.5 \left[\Sigma_{ik=1}^{n_k} f_{ik} \min\left(\Delta_{i_k j_m}\right) + \Sigma_{i_m=1}^{n_m} f_{i_m} \min\left(\Delta_{i_m j_k}\right) \right],$$

where f_{ik} is the relative abundance of species i in community k, n_k is the number of species in k, and $\min\left(\Delta_{i_k j_m}\right)$ is the minimum phylogenetic distance between species i in community k and all species j in community m. βMNTD was calculated using the R function 'comdistnt' (abundance.weighted = TRUE; package 'picante').

For each pair of communities within each geologic formation we generated the value of βMNTD expected if ecological selection was *not* the primary factor governing compositional differences by randomly shuffling species across the tips of the phylogeny and, recalculating βMNTD. This procedure was repeated 999 times to provide a distribution of null values. If the observed βMNTD value was significantly (a) less or (b) greater than the null expectation, we inferred that (a) homogeneous selection or (b) variable selection was responsible for the similarity or differences, respectively, between the pair of communities. Significance was evaluated via the β-Nearest Taxon Index (βNTI), which expresses the difference between observed βMNTD and the mean of the null distribution in units of SDs; βNTI values < -2 or $> +2$ indicate significance (Stegen et al., 2012). These interpretations of the βNTI metric were evaluated and supported via simulation modeling, as described below.

If observed βMNTD does not significantly deviate from the null expectation it indicates that the observed compositional difference is not due to selection (Hardy, 2008), and should therefore be due to very low rates of dispersal (i.e., dispersal limitation), very high rates of dispersal (i.e., homogenizing dispersal), or a lack of dominance between selection and dispersal (i.e., the 'undominated' scenario described in the Introduction). To distinguish among these possibilities, we used the Raup–Crick metric (Chase et al., 2011) extended to incorporate species' relative abundances (as in Stegen et al., 2013); referred to as RC_{bray}. Like βNTI, RC_{bray} was based on a comparison between observed and expected levels of turnover, but without using phylogenetic information.

To generate an expected degree of turnover—for use in quantifying RC_{bray}—we drew species into each local community until

empirically observed local species richness was reached. The probability of drawing a given species was proportional to the number of local sites occupied by that species (as in Chase et al., 2011). In turn, we drew individuals into each selected species until total abundance—summed across species—was equal to observed total abundance. The probability of drawing an individual into a given species was proportional to the relative abundance of that species across all local communities (as in Stegen et al., 2013). This procedure represented stochastic assembly of local communities under the assumptions of weak selection and random dispersal. Compositional differences were quantified with Bray–Curtis (Bray and Curtis, 1957). Repeating stochastic assembly 999 times for each pair of communities provided a null distribution of Bray–Curtis dissimilarities.

To compare observed Bray–Curtis to the null distribution we followed Chase et al. (2011). The number of comparisons between randomly assembled communities that had a Bray–Curtis value greater than the empirical Bray–Curtis was added to half the number of ties. This sum was standardized to range from -1 to $+1$ by subtracting 0.5 and multiplying by 2; the resulting value is RC_{bray}; values between -0.95 and $+0.95$ indicate that compositional turnover between a given pair of communities is 'undominated.' In turn, we infer that RC_{bray} values <-0.95 or $>+0.95$ indicate that selection, dispersal limitation, or homogenizing dispersal govern observed compositional differences. Furthermore, dispersal limitation is expected to increase differences in community composition and should therefore result in RC_{bray} values greater than $+0.95$. Homogenizing dispersal, in contrast, increases compositional similarity and should therefore result in RC_{bray} values less than -0.95 (Stegen et al., 2013). These interpretations of the RC_{bray} metric were evaluated and supported via simulation modeling, as described below.

Quantifying Influences of Ecological Processes

The composition of each community was compared to the composition of all other sampled communities within the same geologic formation. For a given community we estimated the relative influence of variable selection or homogeneous selection as the fraction of its comparisons with $\beta NTI > +2$ or $\beta NTI < -2$, respectively. Selection is excluded as the dominant process when $|\beta NTI| < 2$; in these cases $RC_{bray} > +0.95$ or < -0.95 were taken as evidence that dispersal limitation or homogenizing dispersal, respectively, was the dominant process. For a given local community the relative influence of dispersal limitation was therefore estimated as the fraction of its between-community comparisons with $|\beta NTI| < 2$ and $RC_{bray} > +0.95$. Similarly, the relative influence of homogenizing dispersal was estimated as the fraction of comparisons with $|\beta NTI| < 2$ and $RC_{bray} < -0.95$. The scenario where $|\beta NTI| < 2$ and $|RC_{bray}| < 0.95$ indicates that neither selection nor dispersal strongly drive compositional turnover; this is the 'undominated' scenario and its relative contribution was estimated as the fraction of comparisons characterized by $|\beta NTI| < 2$ and $|RC_{bray}| < 0.95$. These interpretations of the βNTI and RC_{bray} metrics were

evaluated and supported via simulation modeling, as described below.

Statistical Models of Ecological-Process-Influences

In addition to estimating ecological-process-influences we aimed to map spatial variation in those influences. In the subsurface system studied here there were spatial locations where environmental features were characterized but where microbial communities were not. To predict ecological-process-influences across the entire system we first characterized explanatory variables using spatial and environmental data from all sampled locations.

The spatial positions of sampled locations were used with Principal Coordinates of Neighbor Matrices' (PCNM, now referred to as 'Moran's Eigenvector Maps') to describe spatial eigenvectors (function 'pcnm' in R package 'vegan'; Borcard and Legendre, 2002; Borcard et al., 2011). The horizontal positions of locations at which microbial communities were sampled were unique to each formation such that spatial eigenvectors (referred to as PCNM axes) were generated independently for each formation. We also examined horizontally structured environmental features that included horizontal distance from the Columbia River, the elevations of the reduced and oxidized portions of the Ringold Formation, and the vertical thickness of the oxidized Ringold. Spatial eigenvectors and the four environmental features were used to construct statistical models for each ecological process in each formation; in all cases the statistical models were linear, multiple regression models.

For each ecological process we used environmental features and formation-specific PCNM axes as explanatory variables to construct all possible models with up to seven independent variables. Explanatory variables were described using data from all locations for which there were environmental data (**Figure 2**), but ecological-process estimates were only available from locations where microbial communities were sampled (**Figures 3** and **4**). While three of the environmental variables characterize the Ringold Formation, they were used as potential explanatory variables in models for both formations. Doing so evaluated the hypothesis that features of the Ringold Formation influence ecological processes in one or both formations.

Microbial communities were characterized at 16 and 28 locations in the Ringold and Hanford, respectively. We therefore chose the statistical (i.e., the linear, multiple regression) model with the lowest small-sample-size-corrected Akaike Information Criterion (AICc; Burnham and Anderson, 2002). Prior to the construction of statistical models some explanatory variables were removed to avoid strong co-variation. The set of retained explanatory variables was unique to each ecological process in each formation, and was determined as follows. First, explanatory variables were prioritized; a primary goal was to determine the influence of measured environmental features over spatial patterns in ecological processes; measured environmental features had higher priority than the spatial PCNM axes. In addition, ranking within each class of variable—environmental or
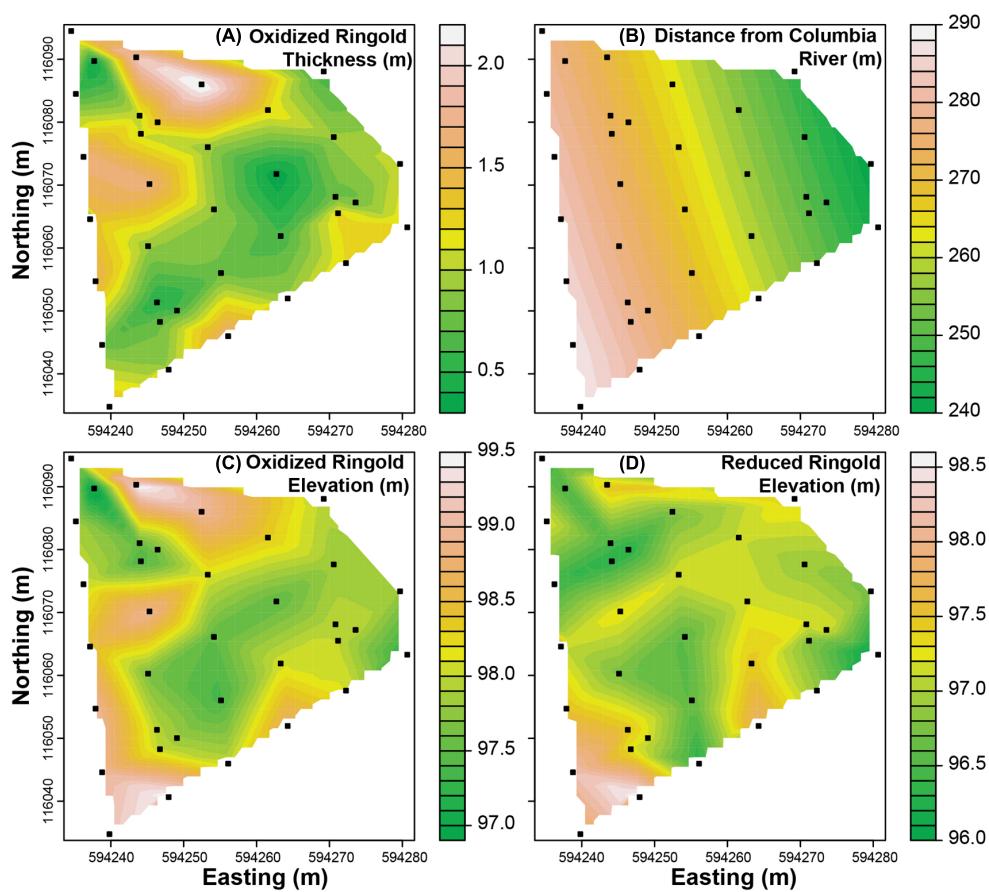
FIGURE 2 | Maps of measured environmental features evaluated as predictors of estimates of ecological-process-influences. (A) The vertical thickness of the oxidized portion of the Ringold formation, **(B)** the horizontal distance from the Columbia River, **(C)** the elevation at the top of the oxidized Ringold formation, and **(D)** the elevation at the top of the reduced portion of the Ringold formation. Solid symbols indicate sampling locations and colors indicate interpolations with magnitudes corresponding to scale bars associated with each panel.

PCNM—was based on the strength of correlation to the ecological process being evaluated. If two explanatory variables were significantly correlated (R function 'cor.test'), only the explanatory variable with higher priority was retained. Using this approach, lower priority variables that were strongly correlated with higher priority variables were removed prior to model selection.

Models with the lowest AICc for each ecological process in each formation were used to construct spatial maps of ecological-process-influences. For each ecological process in each formation, estimates of explanatory variables from each sampled location were fed into the selected models to generate predictions of ecological-process-influences. This was done for all sampled locations, which included locations where microbial communities were characterized and locations where microbial communities were not characterized. The statistical models were therefore used to extrapolate to locations without microbial data. In turn, the predicted ecological-process-influences were spatially interpolated (function 'interp' in R library 'akima') and visualized (function 'filled.contour' in R package 'graphics').

To help determine which environmental features are most likely to have a causal effect on ecological-process-influences, environmental features were related to ecological-process-influences using univariate linear regression. This was only done when a measured environmental feature was the most important component within the multiple regression model of a given ecological process; the most important variable had the largest absolute standardized regression parameter.

Simulation Model

Overview

The preceding two subsections contain a number of assertions regarding the interpretation of βNTI and RC_{bray} that need to be evaluated to refine interpretations and better understand limitations. For an initial evaluation we developed a purposely simple simulation model that uses idealized cases of specific ecological scenarios to generate a set of ecological communities that are used to evaluate our proposed interpretations.

The simulation model has two components; the first evolves a regional pool of species, tracking species' evolutionary relationships and evolution in species' optimal environments; the
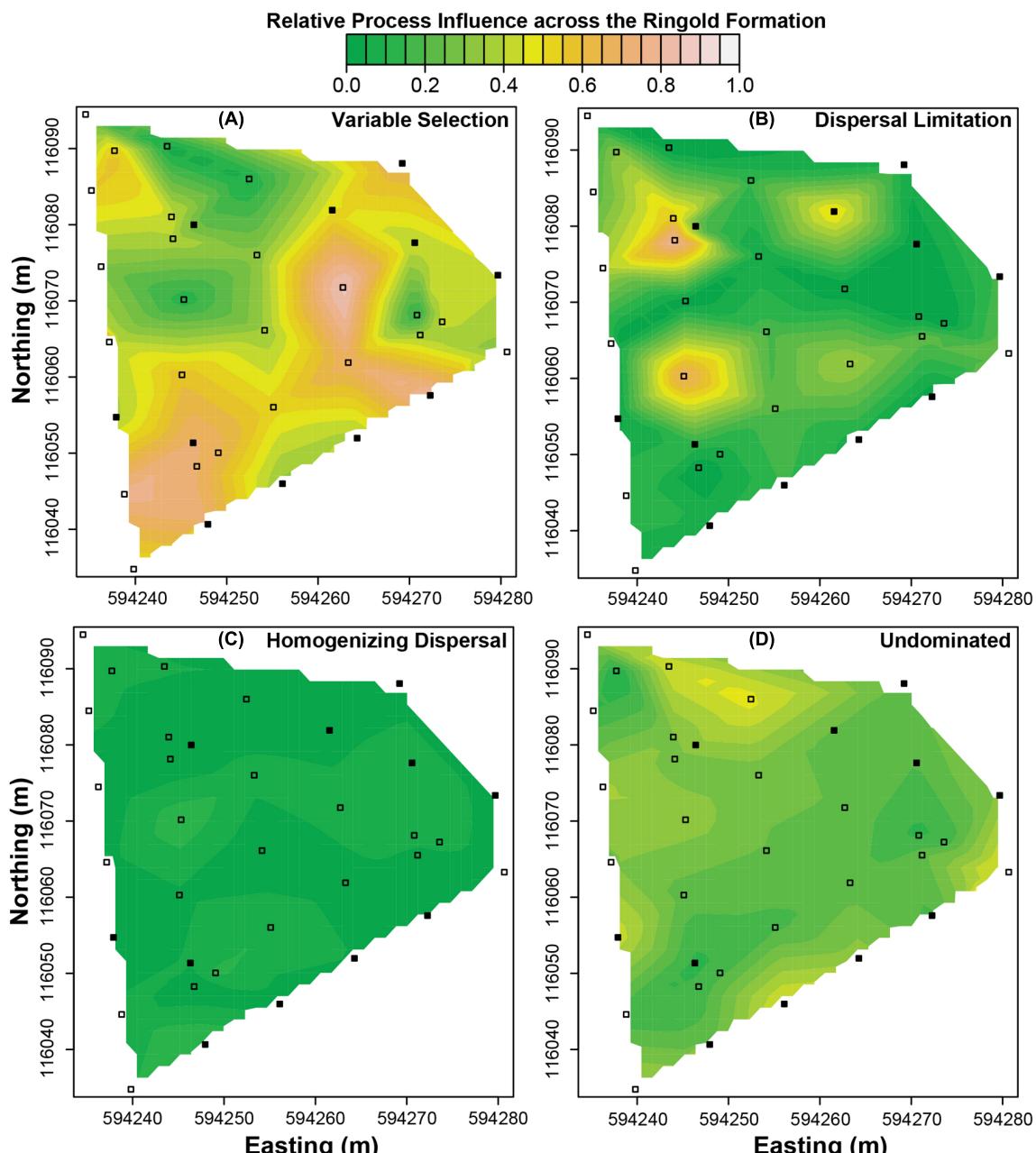
FIGURE 3 | Predicted spatial variation in the relative influences of ecological processes across the Ringold formation. (A) Variable selection, **(B)** dispersal limitation, **(C)** homogenizing dispersal, and **(D)** the undominated fraction. Squares indicate spatial locations where field samples were used to estimate environmental features. Filled squares indicate where field samples were also used to characterize microbial communities. The scale bar applies to all panels.

second uses this regional pool to assemble local communities based on scenarios that reflect our conceptualization of how each ecological process influences community composition (**Figure 5**).

The first component of the model simulates diversification in which new species arise asexually through mutations in the environmental optima of ancestral species. Environmental optima evolve and diversify along an arbitrary environmental axis that takes on values from 0 to 1. Evolution is effectively Brownian

due to no variation in fitness across the environmental axis. The number of species in the regional pool reaches equilibrium due to the following constraints, which are similar to those imposed in Hurlbert and Stegen (2014): there is a maximum total number of individuals (2 million) summed across all species such that population sizes (equal across species) decline with increasing number of species, and the probability of species extinction increases with decreasing population size following a
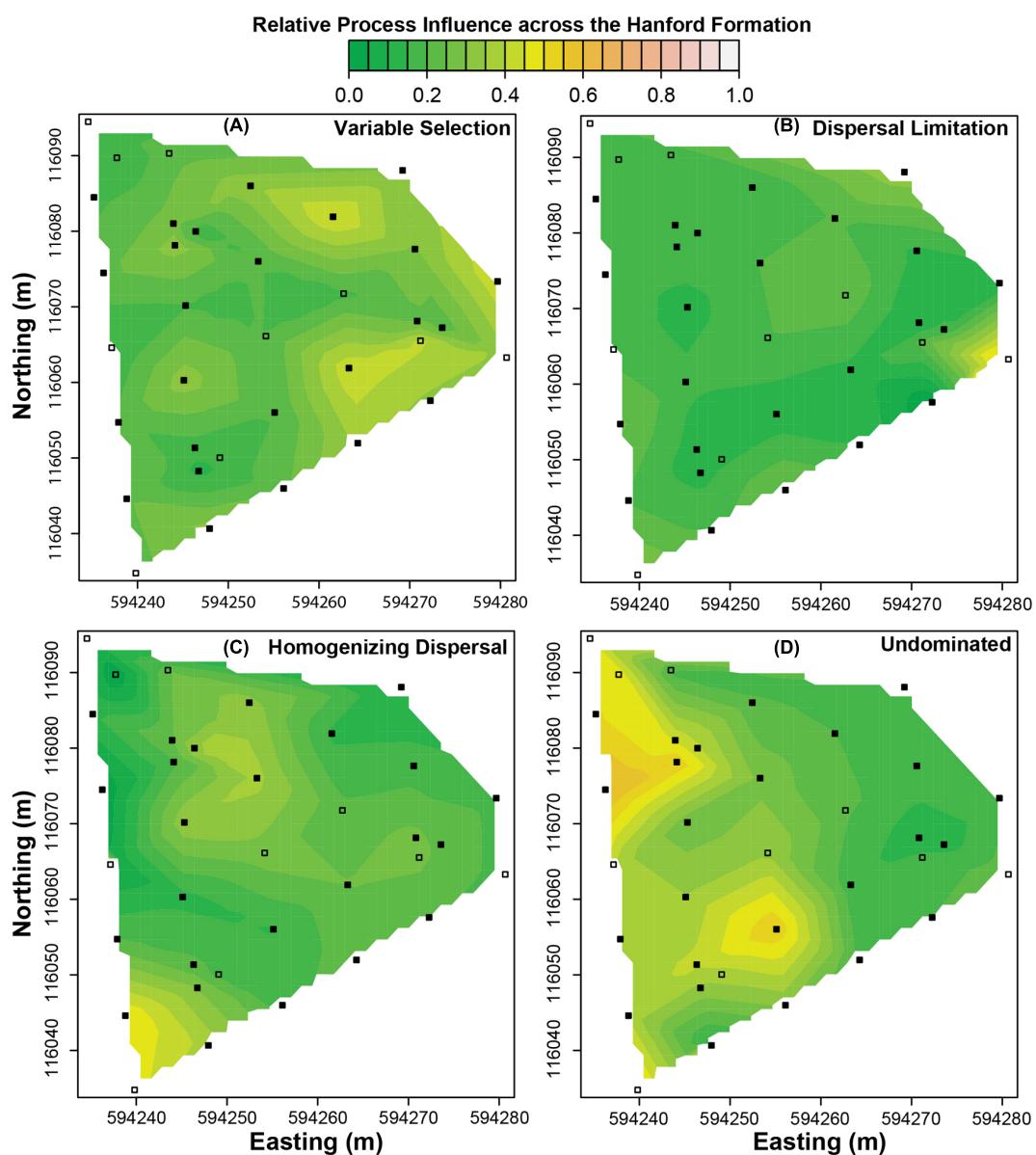
FIGURE 4 | Predicted spatial variation in the relative influences of ecological processes across the Hanford formation. (A) Variable selection, **(B)** dispersal limitation, **(C)** homogenizing dispersal, and **(D)** the undominated fraction. Squares indicate spatial locations where field samples were used to estimate environmental features. Filled squares indicate where field samples were also used to characterize microbial communities. The scale bar applies to all panels.

negative exponential function [population extinction probability $\propto \exp(-0.001{*}\text{population size})$].

The system was initiated with one ancestor that had a randomly chosen environmental optimum. The maximum number of individuals in the system was always achieved such that the ancestor had an initial population size of 2 million. The probability of mutation increased with population size, and a descendant's environmental optimum deviated from its ancestor's by a quantity selected from a Gaussian distribution with mean of 0 and SD of 0.15. Following mutation, population sizes were adjusted so that the total number of individuals was 2 million. Within a time step, mutation and extinction occurred probabilistically and population sizes were adjusted. The simulation was run for 250 time steps, which was sufficient to reach equilibrium species richness. This simulation procedure, which included tracking of evolutionary relationships among species, provided significant phylogenetic signal (Supplementary Figure S1) for a regional species pool comprised of 1140 species with environmental optima that spanned the environmental axis (Supplementary Figure S2).

In the ecological component of our model, species were drawn from the simulated regional pool to assemble four communities under relatively weak ecological selection (blue environment,
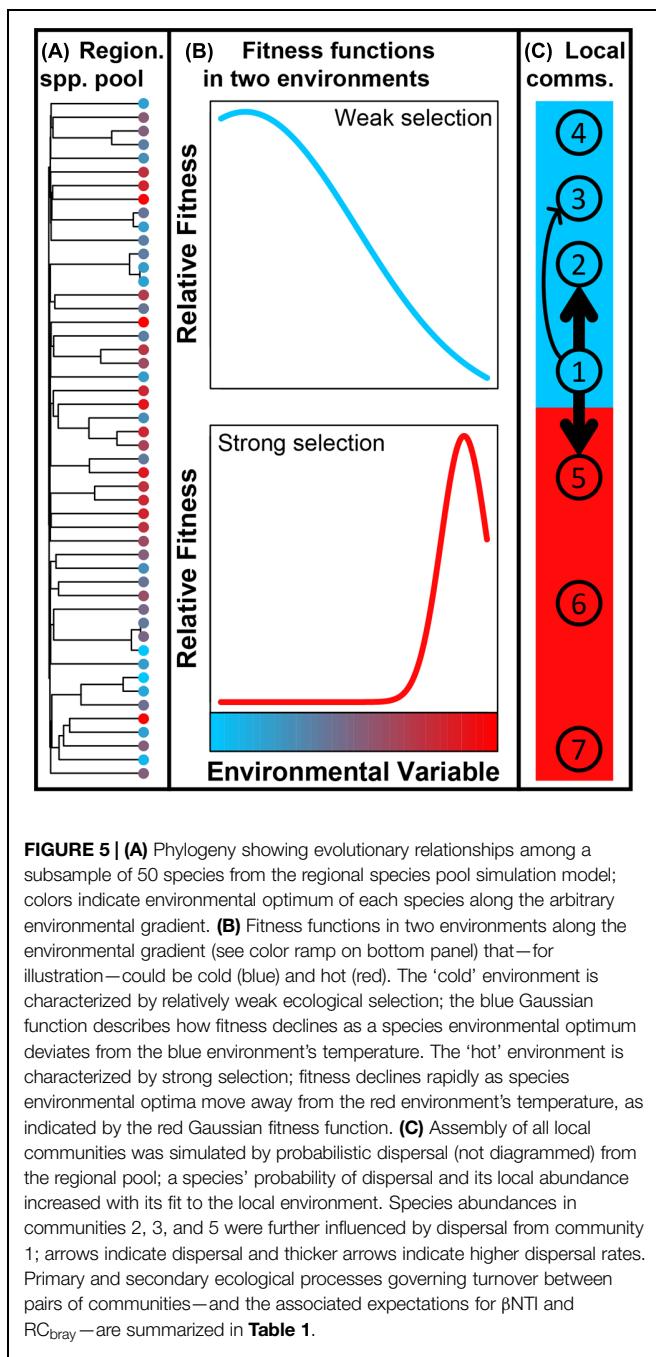
FIGURE 5 | (A) Phylogeny showing evolutionary relationships among a subsample of 50 species from the regional species pool simulation model; colors indicate environmental optimum of each species along the arbitrary environmental gradient. **(B)** Fitness functions in two environments along the environmental gradient (see color ramp on bottom panel) that—for illustration—could be cold (blue) and hot (red). The 'cold' environment is characterized by relatively weak ecological selection; the blue Gaussian function describes how fitness declines as a species environmental optimum deviates from the blue environment's temperature. The 'hot' environment is characterized by strong selection; fitness declines rapidly as species environmental optima move away from the red environment's temperature, as indicated by the red Gaussian fitness function. **(C)** Assembly of all local communities was simulated by probabilistic dispersal (not diagrammed) from the regional pool; a species' probability of dispersal and its local abundance increased with its fit to the local environment. Species abundances in communities 2, 3, and 5 were further influenced by dispersal from community 1; arrows indicate dispersal and thicker arrows indicate higher dispersal rates. Primary and secondary ecological processes governing turnover between pairs of communities—and the associated expectations for βNTI and RC$_{bray}$—are summarized in **Table 1**.

Figure 5) and three communities under stronger ecological selection (red environment, **Figure 5**). All communities had 100 species and 10,000 individuals, drawn probabilistically from the regional species pool using ecological rules summarized below. The ecological rules enabled development of *a priori* expectations (summarized in **Table 1**) for the magnitudes of βNTI and RC$_{bray}$, under the assumption that greater fitness translates into higher relative abundance. Following community assembly these expectations were evaluated by comparing one focal community to five other simulated communities, with one exception discussed below.

To characterize the degree of support for our proposed interpretations of βNTI and RC$_{bray}$, the ecological assembly model was run 1000 times. Each iteration used the same regional pool, which was evolved once, and βNTI and RC$_{bray}$ were quantified following community assembly. This approach generated a distribution of both metrics for each pairwise community comparison. These distributions were used to characterize the degree of support for our *a priori* expectations, which were tied directly to our proposed conceptual interpretations of βNTI and RC$_{bray}$. We next describe the different ecological rules imposed within the assembly model.

The Focal Community (Community 1)

Hundred species were drawn without replacement from the regional species pool with probabilities proportional to their fitness in an environment of 0.05; fit was quantified with a Gaussian function centered on 0.05 and with variance of 0.175 (blue curve, **Figure 5**); the arbitrary environmental variable used in the simulations took on values between 0 and 1. Individuals were probabilistically drawn into the 100 selected species until reaching 10,000 individuals. Note that the strength of selection is relatively weak in the blue environment as indicated by the relatively broad fitness function; for comparison, note that selection is relatively strong in the red environment as indicated by the narrower fitness function (**Figure 5**).

Homogenizing Dispersal (Community 2)

Species and individuals were probabilistically drawn as for community 1, but the probabilities were altered to reflect a high rate of dispersal from community 1. More specifically, species' probabilities based on the Gaussian fitness function (within the blue environment) were modified by adding a quantity equal to 0.05*(species abundance in the focal community $^{1.1}$); the exponent controls the rate of dispersal and was selected through preliminary exploration of parameter-space. Given a high rate of dispersal between communities 1 and 2, compositional turnover between communities 1 and 2 will be primarily governed by homogenizing dispersal; the expectations are |βNTI| < 2 and RC$_{bray}$ < −0.95.

Undominated (Community 3)

In our conceptualization, an 'undominated' scenario arises when there is a moderate rate of dispersal and the strength of selection is relatively weak; high dispersal leads to homogenizing dispersal, low dispersal leads to dispersal limitation, and strong selection will constrain community composition. To generate this scenario, species were drawn into community 3 as for community 2, but with a lower dispersal rate; species' probabilities based on the Gaussian fitness function were modified by adding a quantity equal to 0.05*(species abundance in the focal community$^{0.8}$); this smaller exponent caused a lower rate of dispersal. Compositional turnover between communities 2 and 3 should be undominated such that |βNTI| < 2 and | RC$_{bray}$| < 0.95 are expected.

Dispersal Limitation (Community 4)

Species were drawn into community 4 using only probabilities based on fit to the environment (as for community 1 assembly).

TABLE 1 | Ecological processes primarily, and secondarily, responsible for turnover between indicated pairs of communities within the simulation model (Figure 1 depicts relationships among communities).

Communities	Primary factor	Primary expectation	Primary supported	Secondary factor	Secondary expectation	Secondary supported	Fraction unexpected
1 and 2	Homogenizing dispersal	$\|\beta NTI\| < 2$ $RC_{bray} < -0.95$	91%	Homogeneous selection	$\beta NTI < -2$	8%	1%
1 and 3	Undominated	$\|\beta NTI\| < 2$ $\|RC_{bray}\| < 0.95$	87%	Homogeneous selection	$\beta NTI < -2$	11%	2%
1 and 4	Dispersal limitation	$\|\beta NTI\| < 2$ $RC_{bray} > +0.95$	88%	Homogeneous selection	$\beta NTI < -2$	12%	1%
1 and 5	Homogenizing dispersal	$\|\beta NTI\| < 2$ $RC_{bray} < -0.95$	83%	Variable selection	$\beta NTI > +2$	16%	1%
1 and (6 or 7)	Variable selection	$\beta NTI > +2$	91%	Dispersal limitation	$\|\beta NTI\| < 2$ $RC_{bray} > +0.95$	9%	0%
6 and 7	Homogeneous selection	$\beta NTI < -2$	90%	Dispersal limitation	$\|\beta NTI\| < 2$ $RC_{bray} > +0.95$	0%	10%

Primary and secondary ecological processes are inputs to the simulation model and the associated expectations are patterns that our statistical framework should reveal if it is properly parsing the influences of those ecological processes. The degree of support is summarized as the percentage of replicate simulations for which the statistical framework generates the expected magnitudes of βNTI and RC_{bray}. The frequency of observing combinations of these two metrics that depart from both the primary and secondary expectations is the scenario-specific error (Fraction unexpected).

This reflects dispersal limitation in the sense that the abundance of a given species in community 1 had no influence on its abundance in community 4 because there is no direct dispersal between the communities. Given weak selection in the blue environment and a low dispersal rate between communities 1 and 4, compositional turnover between communities 1 and 4 should be dominated by dispersal limitation; $\|\beta NTI\| < 2$ and $RC_{bray} > +0.95$ are expected.

Homogenizing Dispersal Overwhelms Variable Selection (Community 5)

To generate this scenario a second selective environment was required, and was represented as the red environment in **Figure 5**, (environmental value of 0.95). Community 5 was assembled in the red environment such that species' probabilities of occurrence were influenced by their fitness as determined by a Gaussian fitness function centered on 0.95 and with a variance of 0.0075 (red curve, **Figure 5**). The red Gaussian fitness function had lower variance than the blue Gaussian fitness function, which reflected stronger selection. To draw species and individuals into community 5, the fitness-based probabilities were modified as for community 2 assembly such that there was a high rate of dispersal from community 1 to community 5; species' probabilities based on the Gaussian fitness function (within the red environment) were modified by adding a quantity equal to 0.05*(species abundance in the focal community[1.1]). Given a high rate of dispersal between communities 1 and 5, compositional turnover between communities 1 and 5 should be governed by homogenizing dispersal even though the selective environments are different; $\|\beta NTI\| < 2$ and $RC_{bray} < -0.95$ are expected.

Variable Selection (Community 6)

Community 6 was assembled as for community 1, but instead using species' probabilities based on fitness within the red environment. In this case, large compositional differences should

arise between communities 1 and 6 due to these communities being assembled in different selective environments; variable selection should dominate such that $\beta NTI > +2$ is expected.

Homogeneous Selection (Community 7)

Homogeneous selection can dominate if communities occur in the same selective environment and if selection is relatively strong. Ecological selection in the blue environment is relatively weak such that homogeneous selection is unlikely to arise; as selection becomes weaker, species become demographically equivalent so that selection does not govern community composition (as in Hubbell, 2001). Selection in the red environment is relatively strong, however, such that homogeneous selection will emerge when assembly is governed principally by environmentally determined fitness. Community 7 was therefore assembled as for community 6; $\beta NTI < -2$ is expected.

Secondary Expectations

As summarized above, our proposed conceptual interpretations of βNTI and RC_{bray} provide *a priori* expectations for patterns of these metrics in each simulated scenario (**Table 1**). Ecological systems are, however, inherently probabilistic (as is our simulation model). While there is a high probability that species in community (1) disperse to community (2), for example, this is not guaranteed to occur—in the model or in natural systems—and it is therefore expected that homogenizing dispersal will not always dominate. Given that communities (1) and (2) are assembled under the same selective environment, we suggest that when homogenizing dispersal fails to dominate it is most likely because homogeneous selection has constrained community composition; on average, selection is relatively weak in the blue environment (**Figure 5**), but occasionally selection will strongly constrain community composition. The comparison between communities (1) and (2) in a relatively small fraction of replicate simulations should therefore be characterized by $\beta NTI < -2$; we consider this to be a 'secondary expectation.' For each pairwise community comparison we derived secondary expectations, which

are summarized in **Table 1**. The percentage of replicate simulations showing patterns of βNTI and RC_{bray} consistent with those expectations are summarized in **Table 1**.

Results

Analysis of simulation model outputs showed strong correspondence between expected and observed patterns of βNTI and RC_{bray} (**Table 1**). Five of the six scenarios showing error rates of 2% or less. The scenario with the highest error rate (10%) invoked strong homogeneous selection and was associated with the comparison between communities (6) and (7). The statistical framework was therefore applied to the data on microbial communities across the Hanford and Ringold formations.

In both geologic formations there was substantial variation across local communities in the relative influences of the ecological processes (Supplementary Figure S3). Using environmental features and PCNM axes to describe spatial variation in ecological-process-influences showed that each process within each formation was associated with a distinct set of features/axes (**Tables 2** and **3**). In most cases the best models were highly significant, contained at least one environmental feature, and explained up to 83% of spatial variation in ecological-process-influences (**Tables 2** and **3**).

Within the Ringold Formation, the model for homogeneous selection was not significant ($p = 0.11$), but the thickness of the oxidized Ringold was the most important feature retained in the model for variable selection (**Table 2**). A significant relationship

TABLE 2 | Chosen models for each ecological process within the Ringold Formation.

Variable	Coefficient	SE	t-value	p-value
Ringold homogeneous selection: model $R^2 = 0.17$, $p = 0.11$				
PCNM22	0.023	0.014	1.694	0.112
Ringold variable selection: model $R^2 = 0.81$, $p = 0.0007$				
Oxidized Ringold Thick	−0.164	0.035	−4.637	0.001
PCNM21	0.103	0.026	3.955	0.002
PCNM2	0.065	0.029	2.241	0.047
PCNM8	0.093	0.034	2.710	0.020
Ringold undominated: model $R^2 = 0.3847$, $p = 0.04257$				
Oxidized Ringold Thick	0.088	0.039	2.258	0.042
PCNM17	0.063	0.033	1.893	0.081
Ringold dispersal limitation: model $R^2 = 0.8298$, $p = 0.001333$				
Oxidized Ringold Elevation	−0.196	0.038	−5.228	0.0004
PCNM22	−0.176	0.026	−6.802	0.0000
PCNM1	0.125	0.025	4.944	0.0006
PCNM13	−0.039	0.015	−2.669	0.0235
PCNM8	0.171	0.036	4.790	0.0007
Ringold homogenizing dispersal: model $R^2 = 0.7029$, $p = 0.001742$				
Reduced Ring. Elevation	0.044	0.009	4.911	0.0004
PCNM8	−0.034	0.010	−3.378	0.0055
PCNM16	−0.033	0.010	−3.449	0.0048

All explanatory variables were standardized as standard normal deviates prior to analysis such that coefficient magnitudes are directly comparable.

TABLE 3 | Chosen models for each ecological process within the Hanford formation.

Variable	Coefficient	SE	t-value	p-value
Hanford homogeneous selection: model $R^2 = 0.65$, $p = 0.0002$				
Distance to River	−0.013	0.007	−1.787	0.0877
Oxidized Ringold Thick.	−0.015	0.008	−1.957	0.0632
PCNM22	0.019	0.006	3.023	0.0063
PCNM6	0.019	0.009	2.138	0.0439
PCNM21	0.013	0.007	1.903	0.0702
Hanford variable selection: model $R^2 = 0.29$, $p = 0.04$				
Distance to River	−0.061	0.027	−2.252	0.0337
PCNM22	−0.058	0.025	−2.269	0.0325
PCNM8	0.049	0.031	1.611	0.1204
Hanford undominated: model $R^2 = 0.5271$, $p = 0.0003783$				
Distance to River	0.114	0.025	4.558	0.0001
Reduced Ring. Elevation	−0.075	0.028	−2.686	0.0129
PCNM11	−0.070	0.027	−2.572	0.0167
Hanford dispersal limitation: model $R^2 = 0.3908$, $p = 0.00696$				
PCNM17	0.067	0.028	2.425	0.023
PCNM7	−0.042	0.023	−1.801	0.084
PCNM13	−0.044	0.024	−1.844	0.078
Hanford homogenizing dispersal: model $R^2 = 0.6128$, $p = 3.664\text{e-}05$				
Oxidized Ringold Elevation	0.042	0.026	1.625	0.1172
PCNM10	−0.075	0.018	−4.103	0.0004
PCNM4	0.069	0.021	3.275	0.0032

All explanatory variables were standardized as standard normal deviates prior to analysis such that coefficient magnitudes are directly comparable.

between the influence of variable selection and the thickness of the oxidized Ringold was also observed by univariate regression ($p = 0.01$, $R^2 = 0.38$, **Figure 6A**). Oxidized Ringold thickness was also the most important feature retained in the model of the undominated fraction (**Table 2**), but this relationship was not significant when evaluated by univariate regression ($p = 0.07$). The upper elevation of the oxidized Ringold was the most important feature retained in the model of dispersal limitation (**Table 2**), but this relationship was not significant when evaluated by univariate regression ($p = 0.75$). The elevation at the top of the reduced Ringold was the most important feature in the homogenizing dispersal model (**Table 2**), but this relationship was not significant when evaluated with univariate regression ($p = 0.09$).

Within the Hanford formation, homogeneous selection was most strongly related to PCNM axes and variable selection was most strongly related to distance from the Columbia River (**Table 3**). Variable selection in the Hanford was not, however, significantly related to distance from the Columbia River, by univariate regression ($p = 0.12$). Distance from the Columbia River was the most important feature in the model of the undominated fraction (**Table 3**), and this relationship was also observed by univariate regression ($p = 0.005$, $R^2 = 0.27$, **Figure 6B**). No environmental features were retained in the dispersal limitation model (**Table 3**). Homogenizing dispersal was most strongly related to PCNM axes (**Table 3**).

In the Ringold, process-influence-maps revealed spatial patterns of variable selection and in the undominated fraction (**Figure 3**) that showed similarities to spatial patterns of oxidized
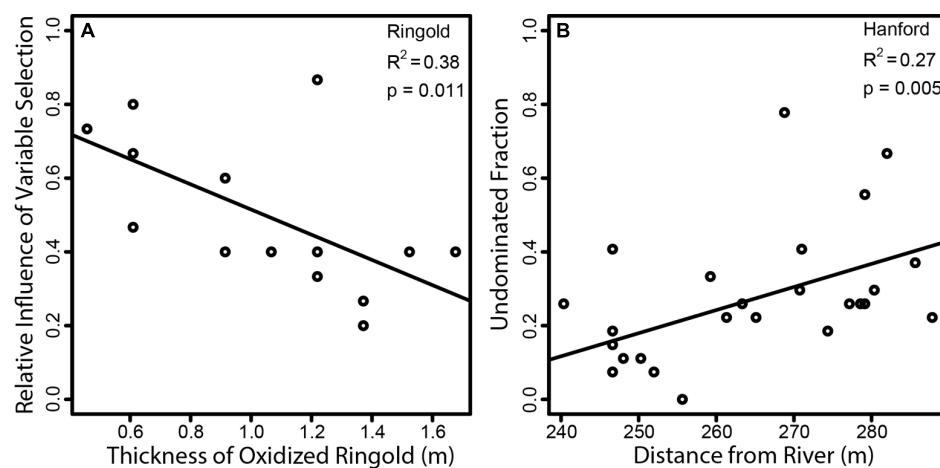
FIGURE 6 | Regressions of ecological-process-influences against environmental features that were significant in both multiple regression and univariate regression models. (A) The relationship-within the Ringold formation-between the relative influence of variable selection and the vertical thickness of the oxidized portion of the Ringold formation, and **(B)** the relationship-within the Hanford formation-between the relative contribution of the undominated fraction and horizontal distance from the Columbia River. Solid lines indicate the ordinary least squares regression model, and the geological formation (Ringold or Hanford) and model statistics are provided on each panel.

Ringold thickness (cf. **Figures 2A** and **3A,D**). Dispersal limitation also showed marked spatial variation across the Ringold Formation, with some congruence with spatial patterns in the elevation of the oxidized Ringold (cf. **Figures 2C** and **3B**). In contrast, homogenizing dispersal was characterized by relatively little spatial variation across the Ringold Formation (**Figure 3C**). The model for homogeneous selection was not significant within the Ringold Formation (see above) such that a map was not generated and, in turn, spatial variation was not evaluated.

Process-influence-maps in the Hanford revealed increases in variable selection and decreases in the undominated fraction in regions closest to the Columbia River and complex patterns across regions further from the river (cf. **Figures 2B** and **4A,D**). Homogeneous selection was relatively consistent through space (Supplementary Figure S4). The influence of dispersal limitation in the Hanford appears to be greatest near the eastern corner of the investigated spatial domain (**Figure 4B**), with no obvious correspondence to environmental features, which is consistent with no environmental variables being retained in the associated multiple regression model (**Table 3**). Homogenizing dispersal across the Hanford was characterized by a complex spatial pattern without any obvious correspondence to environmental features.

Discussion

Here we worked to improve understanding of the ecological processes that influence microbial community composition. A major component of our approach was extending the statistical framework developed in Stegen et al. (2013), which generated process estimates at the scale of a metacommunity and lumped variable and homogeneous selection into a single estimate. Our extension of their framework distinguished homogeneous selection from variable selection and estimated the influences of ecological processes for local communities. This extended framework revealed a dominant influence of variable selection—relative to homogenous selection—and enabled an evaluation of subsurface environmental features related to ecological-process-influences. In turn, we generated process-influence-maps across two geologic formations. Also distinct from Stegen et al. (2013), we evaluated the statistical framework via simulation, which showed close correspondence between expected and observed patterns of βNTI and RC$_{bray}$ (**Table 1**). This provides confidence that our statistical framework generates reasonable estimates of ecological-process-influences.

The comparison between communities 6 and 7—in which homogeneous selection was invoked—resulted in the highest error rate (10%). We hypothesize that increasing the strength of selection would reduce this error rate. Simulation studies that continuously vary the strength of selection could be used to evaluate this hypothesis. Such studies could also be used to go beyond the discrete ecological scenarios studied here. These scenarios were used to enable a tractable evaluation of our approach to inferring ecological processes from null modeling results, but selection strength and dispersal rate are continuous variables (e.g., Stegen and Hurlbert, 2011). If our approach is robust, null modeling results should change continuously with the strength of selection and rate of dispersal. Follow-on simulation studies will be needed to fully understand this coupling.

Direct comparison between our results and those from other systems is not currently feasible; we are not aware of previous work that parses homogeneous selection, variable selection, dispersal limitation, and homogenizing dispersal. We note, however, that our results are consistent with dispersal limitation having an important influence over microbial community composition. This aligns with the emerging perspective that microbes have

biogeography (Green et al., 2004, 2008; Martiny et al., 2006) and suggests that in the subsurface all microbes are not everywhere, in contrast to the classic perspective (De Wit and Bouvier, 2006; Martiny et al., 2006).

Conceptual Inferences

In both formations variable selection and the undominated fraction showed opposing, spatially structured patterns while dispersal limitation and homogenizing dispersal showed more idiosyncratic patterns. To a first approximation variable selection in the Ringold was maximized along a Southwest to Northeast axis, but was maximized in the Eastern corner of the Hanford formation. Spatial patterns of dispersal limitation and homogenizing dispersal were also different between formations. Environmental features governing the relative influences of ecological processes therefore appear to be formation-specific.

The influence of variable selection in the Ringold was most strongly related to oxidized Ringold thickness; variable selection became increasingly weak with increasing thickness (**Figure 6A**). If one defines the rate at which redox conditions change with depth as the vertical distance between oxidized and reduced conditions, redox conditions must change more rapidly with depth in locations with a thinner oxidized Ringold layer. In turn, our results suggest that variable selection increases as the vertical redox gradient becomes steeper (i.e., when redox conditions change more rapidly with depth).

The influence of variable selection may increase with steeper redox gradients if a small number of microbial taxa take advantage of rapidly changing redox conditions. This hypothesis could be directly tested by evaluating the response of microbial communities to experimentally manipulated redox patterns within laboratory flow cells. Such an approach would leverage the strengths of comparative and experimental techniques to provide a deeper level of understanding than otherwise possible (Weber and Agrawal, 2012).

In the Hanford formation the undominated fraction decreased toward the Columbia River (**Figure 4D**; **Table 3**), and there was a modest increase in the influence of variable selection toward the Columbia River (**Figure 4A**; **Table 3**). These patterns suggest an important environmental shift as one moves toward the river, which may be related to spatially structured river-water intrusion.

Elevation of the Columbia River increases annually in the spring, causing river-water intrusion into the studied subsurface system (Peterson et al., 2008; Lin et al., 2012b). To a first approximation, regions of the system that are closer to the river (**Figure 2B**) receive more river-water (Lin et al., 2012b). It may be that an annually repeated pattern of more river-water in the eastern portion of the Hanford formation has resulted in a shift in environmental conditions that cause a stronger influence of variable selection in that region. As a first test of this hypothesis it would be useful to sample a broader spatial domain that contains regions of the subsurface closer to and further from the river, relative to what has been sampled here. Coupling this expanded sampling with laboratory flow cell experiments would provide powerful hypothesis tests.

The undominated fraction should increase with decreases in the strength of selection and/or with a shift toward moderate dispersal rates. In both formations the undominated fraction generally showed patterns opposite those of variable selection. This suggests that a shift toward undominated compositional turnover is due primarily to weaker selection, as opposed to a change in dispersal rates. It is also interesting to note that both formations were characterized by relatively weak influences of homogeneous selection. This result is expected as selection is unlikely to be consistent across spatially heterogeneous systems such as the subsurface system studied here.

Dispersal limitation and homogenizing dispersal both have complex spatial structure, and it is difficult to discern which environmental features (if any) drive these spatial patterns. Dispersal limitation across both formations was unrelated to environmental features when evaluated with univariate regression, suggesting that the environmental features used in our analyses do not strongly determine the influence of dispersal limitation. Similarly, homogenizing dispersal in the Hanford was more strongly related to PCNM axes than to environmental features (**Table 3**). Instead of being driven by relatively simple environmental features, the influences of dispersal limitation and homogenizing dispersal may be governed more by complex and spatially inconsistent features that influence hydrologic transport.

Comparison to Variation Partitioning

While it may appear that variation partitioning (Legendre and Legendre, 1998) was used here to estimate ecological-process-influences, there are substantial differences between variation partitioning and our approach. In variation partitioning, variation in community composition is explained using features deemed *a priori* to reflect spatial relationships or environmental differences among communities (e.g., Tuomisto et al., 2003; Cottenie, 2005; Legendre et al., 2009; Heino et al., 2011). Intuitively, the more variation in community composition explained by spatial or environmental features, the greater the influence of dispersal limitation or selection, respectively (Stegen and Hurlbert, 2011). At least three studies have shown that this intuitive expectation is not valid and that variation partitioning cannot be used to infer the influences of ecological processes (Gilbert and Bennett, 2010; Smith and Lundholm, 2010; Stegen and Hurlbert, 2011). Variation partitioning can, at best, determine whether dispersal limitation has more or less influence than selection (Stegen and Hurlbert, 2011).

With variation partitioning it is tempting to infer that variation in community composition not explained by spatial or environmental features results from ecological drift. Unexplained variation will, however, increase artificially if any of the following occur: (*i*) important environmental features have not been measured; (*ii*) spatial axes fail to capture idiosyncratic patterns in spatial isolation among communities; or (*iii*) community composition is non-linearly related to explanatory variables (see discussions in Laliberte et al., 2009; Legendre et al., 2009; Anderson et al., 2011).

In contrast to variation partitioning we used null models to estimate the influences of ecological processes; our approach

does not relate community composition to explanatory variables. Our approach therefore solves a primary shortcoming of variation partitioning, but like all statistical frameworks has its own limitations.

Limitations, Caveats, and the Path Forward

With respect to the framework used here, two important considerations are sample size and error from sampling, DNA sequencing and data processing. Ecological process estimates likely approach their true values as sample size increases and this should be considered in cross-system comparisons.

It is not obvious that sources of error will systematically increase or decrease any particular process estimate such that we assume errors contribute equally to estimates of all ecological process. Nonetheless, future simulation studies need to evaluate the potential for bias related to sample size and sources of error.

On the conceptual side, we note that our framework does not account for influences of *in situ* diversification, which is the evolutionary component in Vellend's (2010) synthesis (see also Hanson et al., 2012). This may be relevant as high rates of *in situ* diversification could increase compositional turnover in terms of OTU presence/absence; if dispersal rates are low, new OTUs that evolve *in situ* may only be found in one community. On the other hand, among-community dispersal will minimize influences of *in situ* diversification; if newly evolved OTUs disperse away from their community-of-origin, the influence of *in situ* diversification on compositional turnover will be minimal. Outcomes are therefore influenced by the balance between dispersal and diversification rates.

Extending our framework to characterize the influence of *in situ* diversification on patterns of compositional turnover represents an important challenge. Pattern-oriented simulation modeling (e.g., Grimm et al., 2005; Rangel et al., 2007; Stegen and Hurlbert, 2011; McClain et al., 2012) is one approach that could be leveraged to meet this challenge. In the context of species richness gradients, for example, Hurlbert and Stegen (2014) recently developed a pattern-oriented simulation model that provides 'multi-metric fingerprints' that indicate the operation of specific underlying processes. A similar approach could be added to our framework to identify multi-metric fingerprints indicating the influence of *in situ* diversification on patterns of compositional turnover.

We further suggest that a pattern-oriented simulation modeling approach could be used to draw additional inferences from the undominated fraction provided by our current framework. The undominated fraction is tied to a conceptual inference—selection is too weak and dispersal rates are not extreme enough for either process to drive compositional turnover. It would be useful, however, to parse the relative influences of ecological processes within the undominated fraction. To this end, pattern-oriented simulations similar to those in Stegen and Hurlbert (2011) could be used to study compositional turnover patterns expected across a two-dimensional process-space defined by the strength of selection and the rate of dispersal. Observed turnover patterns within the undominated fraction could then be related back to simulated turnover patterns and, in turn, the balance between selection and dispersal (similar to McClain et al., 2012).

In summary, we have provided spatial projections for the relative influences of ecological processes on subsurface microbial community composition. Doing so has revealed key features of our system unrecognized through application of existing statistical frameworks. We suggest that for many systems a similar outcome is likely and that novel insights can be gained through broad application of a framework that couples the approach used here with that of Stegen et al. (2013). We look forward to these statistical frameworks being improved through additional simulation-based evaluations, to experimental tests of the hypotheses they generate, and to a coupling between the information they provide and process-based models aimed at predicting community composition across environmental conditions.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00370/abstract

References

Anderson, M. J., Crist, T. O., Chase, J. M., Vellend, M., Inouye, B. D., Freestone, A. L., et al. (2011). Navigating the multiple meanings of (diversity: a roadmap for the practicing ecologist. *Ecol. Lett.* 14, 19–28. doi: 10.1111/j.1461-0248.2010.01552.x

Bjornstad, B. N., Horner, J. A., Vermeul, V. R., Lanigan, D. C., and Thorne, P. D. (2009). *Borehole Completion and Conceptual Hydrogeologic Model for the IFRC Well Field, 300 Area, Hanford Site,* Richland, WA: Pacific Northwest National Laboratory.

Borcard, D., Gillet, F., and Legendre, L. (2011). *Numerical Ecology with R,* New York, NY: Springer.

Borcard, D., and Legendre, P. (2002). All-scale spatial analysis of ecological data by means of principal coordinates of neighbour matrices. *Ecol. Model.* 153, 51–68. doi: 10.1016/S0304-3800(01)00501-4

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of southern wisconsin. *Ecol. Monogr.* 27, 326–349. doi: 10.2307/1942268

Burnham, K. P., and Anderson, D. (2002). *Model Selection and Multi-Model Inference*. New York, NY: Springer-Verlag.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/Nmeth.F.303

Cavender-Bares, J., Kozak, K. H., Fine, P. V. A., and Kembel, S. W. (2009). The merging of community ecology and phylogenetic biology. *Ecol. Lett.* 12, 693–715. doi: 10.1111/j.1461-0248.2009.01314.x

Chase, J. M. (2007). Drought mediates the importance of stochastic community assembly. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17430–17434. doi: 10.1073/pnas.0704350104

Chase, J. M. (2010). Stochastic community assembly causes higher biodiversity in more productive environments. *Science* 328, 1388–1391. doi: 10.1126/science.1187820

Chase, J. M., Kraft, N. J. B., Smith, K. G., Vellend, M., and Inouye, B. D. (2011). Using null models to disentangle variation in community dissimilarity from variation in α-diversity. *Ecosphere* 2:art24. doi: 10.1890/es10-00117.1

Cottenie, K. (2005). Integrating environmental and spatial processes in ecological community dynamics. *Ecol. Lett.* 8, 1175–1182. doi: 10.1111/j.1461-0248.2005.00820.x

De Wit, R., and Bouvier, T. (2006). Everything is everywhere, but, the environment selects; what did Baas Becking and Beijerinck really say? *Environ. Microbiol.* 8, 755–758. doi: 10.1111/j.1462-2920.2006.01017.x

Dias, P. C. (1996). Sources and sinks in population biology. *Trends Ecol. Evol.* 11, 326–330. doi: 10.1016/0169-5347(96)10037-9

Dini-Andreote, F., Stegen, J. C., Van Elsas, J. D., and Salles, J. F. (2015). Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. *Proc. Natl. Acad. Sci. U.S.A.* 112, E1326–E1332. doi: 10.1073/pnas.1414261112

Fine, P. V. A., and Kembel, S. W. (2011). Phylogenetic community structure and phylogenetic turnover across space and edaphic gradients in western Amazonian tree communities. *Ecography* 34, 552–565. doi: 10.1111/j.1600-0587.2010.06548.x

Gilbert, B., and Bennett, J. R. (2010). Partitioning variation in ecological communities: do the numbers add up? *J. Appl. Ecol.* 47, 1071–1082. doi: 10.1111/j.1365-2664.2010.01861.x

Green, J. L., Bohannan, B. J. M., and Whitaker, R. J. (2008). Microbial biogeography: from taxonomy to traits. *Science* 320, 1039–1043. doi: 10.1126/science.1153475

Green, J. L., Holmes, A. J., Westoby, M., Oliver, I., Briscoe, D., Dangerfield, M., et al. (2004). Spatial scaling of microbial eukaryote diversity. *Nature* 432, 747–750. doi: 10.1038/Nature03034

Grimm, V., Revilla, E., Berger, U., Jeltsch, F., Mooij, W. M., Railsback, S. F., et al. (2005). Pattern-oriented modeling of agent-based complex systems: lessons from ecology. *Science* 310, 987–991. doi: 10.1126/science.1116681

Hanson, C. A., Fuhrman, J. A., Horner-Devine, M. C., and Martiny, J. B. H. (2012). Beyond biogeographic patterns: processes shaping the microbial landscape. *Nat. Rev. Microbiol.* 10, 497–506. doi: 10.1038/Nrmicro2795

Hardy, O. J. (2008). Testing the spatial phylogenetic structure of local communities: statistical performances of different null models and test statistics on a locally neutral community. *J. Ecol.* 96, 914–926. doi: 10.1111/j.1365-2745.2008.01421.x

Heino, J., Grönroos, M., Soininen, J., Virtanen, R., and Muotka, T. (2011). Context dependency and metacommunity structuring in boreal headwater streams. *Oikos* 121, 537–544. doi: 10.1111/j.1600-0706.2011.19715.x

Horner-Devine, M. C., and Bohannan, B. J. M. (2006). Phylogenetic clustering and overdispersion in bacterial communities. *Ecology* 87, 100–108. doi: 10.1890/0012-9658(2006)87[100:PCAOIB]2.0.CO;2

Hubbell, S. P. (2001). *The Unified neutral Theory of Biodiversity and Biogeography*. Princeton, NJ: Princeton University Press.

Hurlbert, A. H., and Stegen, J. C. (2014). When should species richness be energy limited, and how would we know? *Ecol. Lett.* 17, 401–413. doi: 10.1111/ele.12240

Kraft, N. J. B., Cornwell, W. K., Webb, C. O., and Ackerly, D. D. (2007). Trait evolution, community assembly, and the phylogenetic structure of ecological communities. *Am. Nat.* 170, 271–283. doi: 10.1086/519400

Laliberte, E., Paquette, A., Legendre, P., and Bouchard, A. (2009). Assessing the scale-specific importance of niches and other spatial processes on beta diversity: a case study from a temperate forest. *Oecologia* 159, 377–388. doi: 10.1007/s00442-008-1214-1218

Legendre, P., and Legendre, L. (1998). *Numerical Ecology. Amsterdam*, The Netherlands: Elsevier Science.

Legendre, P., Mi, X. C., Ren, H. B., Ma, K. P., Yu, M. J., Sun, I. F., et al. (2009). Partitioning beta diversity in a subtropical broad-leaved forest of China. *Ecology* 90, 663–674. doi: 10.1890/07-1880.1

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., et al. (2004). The metacommunity concept: a framework for multi-scale community ecology. *Ecol. Lett.* 7, 601–613. doi: 10.1111/j.1461-0248.2004.00608.x

Lin, X., Kennedy, D., Peacock, A., McKinley, J., Resch, C. T., Fredrickson, J., et al. (2012a). Distribution of microbial biomass and potential for anaerobic respiration in Hanford Site 300 Area subsurface sediment. *Appl. Environ. Microbiol.* 78, 759–767. doi: 10.1128/aem.07404–7411

Lin, X., McKinley, J., Resch, C. T., Lauber, C., Fredrickson, J., and Konopka, A. E. (2012b). Spatial and temporal dynamics of microbial community in the Hanford unconfined aquifer. *ISME J.* 6, 1665–1676. doi: 10.1038/ismej.2012.26

Losos, J. B. (2008). Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity among species. *Ecol. Lett.* 11, 995–1003. doi: 10.1111/j.1461-0248.2008.01229.x

Martiny, J. B. H., Bohannan, B. J. M., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., et al. (2006). Microbial biogeography: putting microorganisms on the map. *Nat. Rev. Micro.* 4, 102–112. doi: 10.1038/nrmicro1341

McClain, C. R., Stegen, J. C., and Hurlbert, A. H. (2012). Dispersal, environmental niches and oceanic-scale turnover in deep-sea bivalves. *Proc. R Soc. Biol. Sci.U.S.A.* 279, 1993–2002. doi: 10.1098/rspb.2011.2166

Peterson, R. E., Rockhold, M. L., Serne, R. J., Thorne, P. D., and Williams, M. D. (2008). *Uranium Contamination in the Subsurface Beneath the 300 Area, Hanford site, Washington*. Richland, WA: Pacific Northwest National Laboratory. doi: 10.2172/925719

Rangel, T. F. L. V. B., Diniz-Filho, J. A. F., and Colwell, R. K. (2007). Species richness and evolutionary niche dynamics: a spatial pattern-oriented simulation experiment. *Am. Nat.* 170, 602–616. doi: 10.1086/521315

Smith, T. W., and Lundholm, J. T. (2010). Variation partitioning as a tool to distinguish between niche and neutral processes. *Ecography* 33, 648–655. doi: 10.1111/j.1600-0587.2009.06105.x

Stegen, J. C., and Hurlbert, A. H. (2011). Inferring ecological processes from taxonomic, phylogenetic and functional trait β-diversity. *PLoS ONE* 6:e20906. doi: 10.1371/journal.pone.0020906

Stegen, J. C., Lin, X., Fredrickson, J. K., Chen, X., Kennedy, D. W., Murray, C. J., et al. (2013). Quantifying community assembly processes and identifying features that impose them. *ISME J.* 7, 2069–2079. doi: 10.1038/ismej.2013.93

Stegen, J. C., Lin, X., Konopka, A. E., and Fredrickson, J. K. (2012). Stochastic and deterministic assembly processes in subsurface microbial communities. *ISME J.* 6, 1653–1664. doi: 10.1038/ismej.2012.22

Tuomisto, H., Ruokolainen, K., and Yli-Halla, M. (2003). Dispersal, environment, and floristic variation of western amazonian forests. *Science* 299, 241–244. doi: 10.1126/science.1078037

Vellend, M. (2010). Conceptual synthesis in community ecology. *Q. Rev. Biol.* 85, 183–206. doi: 10.1086/652373

Wang, J., Shen, J., Wu, Y., Tu, C., Soininen, J., Stegen, J. C., et al. (2013). Phylogenetic beta diversity in bacterial assemblages across ecosystems: deterministic versus stochastic processes. *ISME J.* 7, 1310–1321. doi: 10.1038/ismej.2013.30

Webb, C. O., Ackerly, D. D., and Kembel, S. (2011). *Phylocom: Software for the Analysis of Phylogenetic Community Structure and Character Evolution (with Phylomatic and Ecoevolve). User's Manual, Version 4.2.* Available at: http://www.phylodiversity.net/phylocom/

Weber, M. G., and Agrawal, A. A. (2012). Phylogeny, ecology, and the coupling of comparative and experimental approaches. *Trends Ecol. Evol.* 27, 394–403. doi: 10.1016/j.tree.2012.04.010

What difference does it make if viruses are strain-, rather than species-specific?

T. Frede Thingstad *, Bernadette Pree, Jarl Giske and Selina Våge

Department of Biology, Hjort Centre for Marine Ecosystem Dynamics, University of Bergen, Bergen, Norway

Theoretical work has suggested an important role of lytic viruses in controlling the diversity of their prokaryotic hosts. Yet, providing strong experimental or observational support (or refutation) for this has proven evasive. Such models have usually assumed "host groups" to correspond to the "species" level, typically delimited by 16S rRNA gene sequence data. Recent model developments take into account the resolution of species into strains with differences in their susceptibility to viral attack. With strains as the host groups, the models will have explicit viral control of abundance at strain level, combined with explicit predator or resource control at community level, but the direct viral control at species level then disappears. Abundance of a species therefore emerges as the combination of how many strains, and at what abundance, this species can establish in competition with other species from a seeding community. We here discuss how species diversification and strain diversification may introduce competitors and defenders, respectively, and that the balance between the two may be a factor in the control of species diversity in mature natural communities. These models can also give a dominance of individuals from strains with high cost of resistance; suggesting that the high proportion of "dormant" cells among pelagic heterotrophic prokaryotes may reflect their need for expensive defense rather than the lack of suitable growth substrates in their environment.

Keywords: killing-the-winner model, biodiversity control, biodiversity–ecosystem functioning relationships, Weinbauer's Paradox, fractal similarity, microevolution

Introduction

Classical discussions of diversity-maintaining mechanisms in microbiology had a tendency to focus on competition, with the works of Hutchinson (1961) and Tilman (1977) in phytoplankton ecology as influential examples. With competitive exclusion as the theoretical outcome for species competing for the same limiting substrate, this created a need for additional hypotheses to explain observed microbial biodiversity. With the huge microbial diversity revealed by modern metagenomics (Venter et al., 2004), this gap between observation and theory is now perhaps wider than ever.

The list of mechanisms thought to maintain diversity includes, among others, substrate diversity (Tilman, 1977), environmental variability in time and/or space (Connell, 1979; Beninca et al., 2008; Dini-Andreote et al., 2014; Jankowski et al., 2014; Staley et al., 2014; Uksa et al., 2014), chaotic dynamics (Beninca et al., 2008), and selective loss (Thingstad and Lignell, 1997; Thingstad, 2000), presumably working together in a more or less additive manner in

complex natural environments. In the prokaryotic world, the issue of species diversity is further complicated not only by the problem of defining what constitutes a species (Fraser et al., 2009), but also by the fact that strains from the same species contain non-conserved, non-core genes that are sporadically distributed among members of the population (Avrani et al., 2011).

Quite different from a picture of natural communities as assemblages of clonal species populations, this suggests that a prokaryote "species" consists of a set of strains where some traits characterize the set, while other traits vary between strains. Any comparison between model and data then needs to take into account whether the experimental data resolves the genetic information at species or at strain level.

As a kind of minimum model to describe the effect of top-down control, one can combine size-selective predators with host specific viruses. This creates a two-level model where the same basic "killing-the winner" (KtW) principle (**Figure 1A**) is applied at both levels: Grazing control of the community size of heterotrophic prokaryotes allows for phytoplankton coexistence with heterotrophic bacteria (**Figure 1B**), even when bacteria are superior competitors for a shared limiting mineral nutrient (Pengerud et al., 1987). Applying the KtW principle inside the community (**Figure 1C**) allows competitive host-groups with high growth rate to coexist with defensive host-groups with a low loss to viral lysis, even if this defense comes at the expense of a slower growth rate (Bohannan and Lenski, 2000).

A lot of the experimental and observational work in the field has tried to relate their findings to this KtW model, but clear support (or rebuttal) of the theory has so far been evasive (see e.g., Winter et al., 2010). We recently suggested (Thingstad et al., 2014) that part of this problem might be rooted in the often implicitly made assumption that this model's "host groups" correspond to observational information contained in 16S rRNA gene sequence data. Since such data are more relevant to the species than to a strain level, it means that the KtW model in this case is interpreted with host groups representing species.

Models that incorporate the possibility for viral strain specificity have recently been introduced (Jover et al., 2013; Thingstad et al., 2014). In these, the explicit top-down control on species abundances disappears as illustrated in **Figure 1D**. This obviously is important for discussions of community "species" composition such as e.g., recent attempts to combine the high abundance of the SAR11 clade with its susceptibility to viral infection (Giovannoni et al., 2013; Våge et al., 2013; Zhao et al., 2013).

In the model of **Figure 1D**, the abundance of individuals belonging to a species is the sum of individuals belonging to each of its strains, with no other explicit control on species size than the maximum value given by the community size. As a consequence, abundance at the species level becomes a combination of its ability to establish many strains, and the virus-controlled abundance of individuals within each of these strains. Since establishment of a strain and abundance within this strain are functions of its competitive and its defensive abilities, respectively, abundance at species level becomes a combined function

of a species' competitive and defensive abilities (Thingstad et al., 2014).

To illustrate the ecological consequences of these models we have arranged the subsequent discussion in sections devoted to a set of questions picked from the list of themes announced for this special issue.

How Does a Foreign Microbe Become a Member of the Ecosystem?

In a system at steady state, specific growth rate (μ) must equal specific loss rate (δ) for all populations and all subpopulations, so that their net growth rate μ-δ is zero. This system can then be invaded by any new organism type x, be it a mutant or an immigrant species, for which $\mu_x - \delta_x > 0$ when it enters the established community (Symbols summarized in **Table 1**). The invasion changes the growth conditions in the established community. How numerous the invader will become in the new steady state (if this exists) depends on a new set of equilibrium conditions. This set includes the new $\mu_x - \delta_x = 0$ condition, while the equivalent condition for now excluded members in the previous state of the community has disappeared. Although, both invasion and final community size for the invader depend on how μ and δ vary with the state of the community, the conditions for invasion and population size are thus different.

Two cases are of particular interest for our subsequent discussion, (1) a mutant that can use some unexploited resource in the system and therefore has a large μ_x; and (2) an organism that has few/no enemies in the system or is good in defending itself against those already present, and therefore has a low δ_x.

How Do Microbes in Ecosystems Evolve?

The importance of the somewhat abstract reasoning above can be illustrated by considering the idealized environment of a chemostat, frequently used to study simplified virus-host systems (e.g., Bohannan and Lenski, 1997, 2000; Middelboe et al., 2009; Marston et al., 2012). This simple environment is characterized by two parameters: the dilution rate D, and the reservoir concentration S_R of limiting substrate (for simplicity assumed here to be a non-respired substance like e.g., phosphate).

In the traditional theoretical case of a clonal bacterial population, the chemostat will have a resource-controlled population size proportional to S_R and a growth rate equal to the dilution rate: $\mu(S_C) = D$. The steady-state culture concentration S_C is thus linked to dilution rate through the growth characteristics of the organism. This clonal community is invadable only by a better competitor, i.e., one that has a higher growth rate at the given S_C. If, however, the established population is infected with a lytic virus, the original susceptible clone becomes virus-controlled and is reduced to a low population level. Part of the available reservoir substrate S_R will then remain unused in the culture, i.e., leading to a high S_C. With a high S_C, this one host-one virus community will be easily invadable by a resistant host mutant, even if the mutation comes at a price (a cost of resistance, COR). With higher S_C, the original parent strain will now grow faster than D, it can
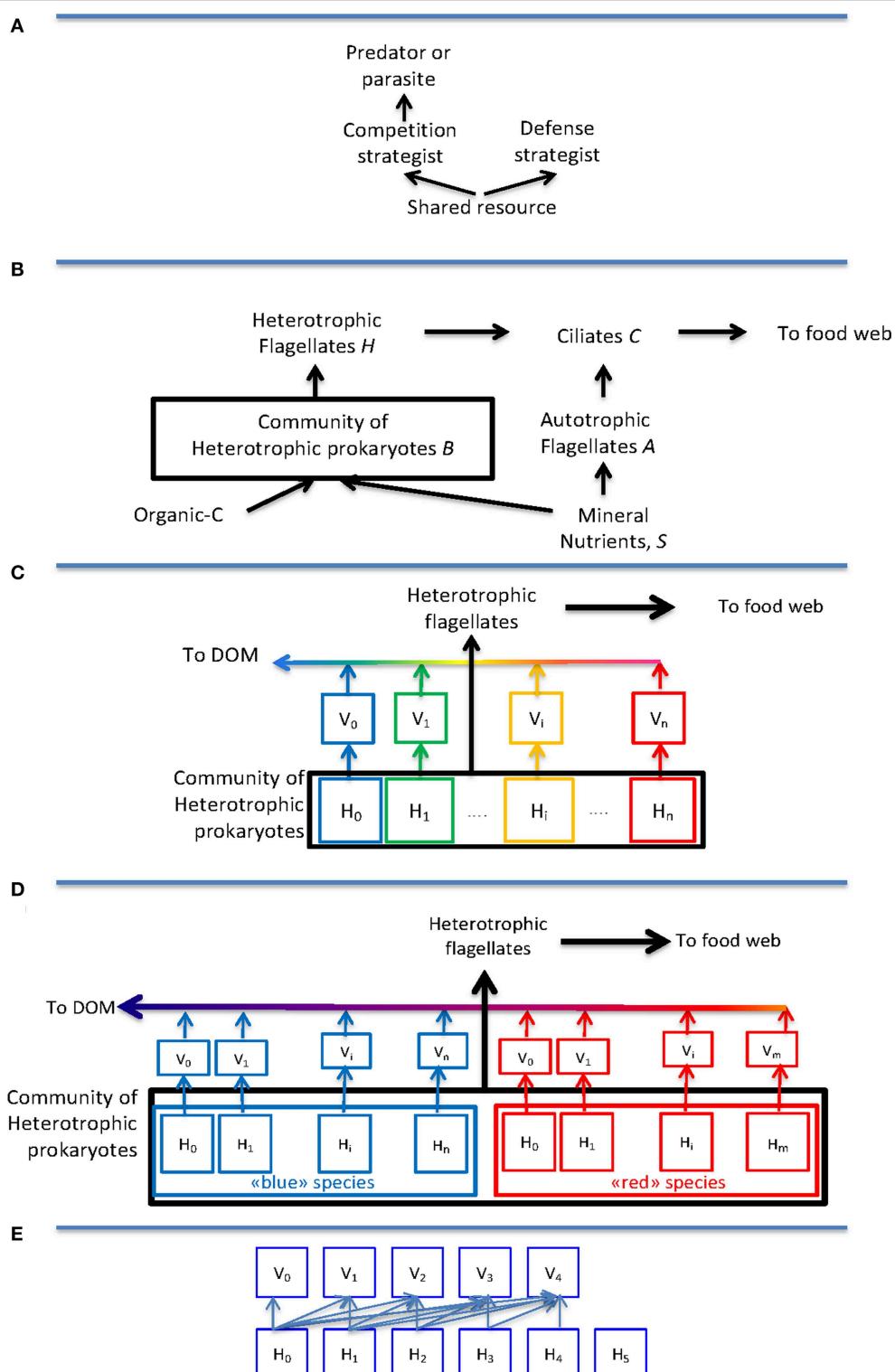
FIGURE 1 | Idealized models for trophic interactions discussed: (A) The "Killing-the-Winner" structure where abundance of the competition strategist is top-down controlled by a predator or parasite, thereby leaving resources for a resource-limited defense strategist. (B) An idealized model of the microbial food web based on the principles from **(A)**, illustrating how ciliates influence both biomass and growth conditions of heterotrophic flagellates through their grazing on heterotrophic and autotrophic flagellates, respectively. **(C)** The original one host–one virus model interpreted as host-groups corresponding to species

(Continued)

FIGURE 1 | Continued

("blue," "green," "yellow," "red") and species abundance therefore being top-down controlled. In this model, the application of the KtW principle at the predator-prey creates a transport "up" the food chain, while applying the same principle to viruses sends material "down" to dissolved organic material (DOM). **(D)** Modification of **(C)** by the assumption that host groups correspond to strains belonging to either a "blue" or a "red" species, illustrating how the direct top-down control of abundance disappears at the intermediate level of species. **(E)** Modification of the host-virus interaction from the one host–one virus relationship in **(D)** to a nested structure. This is the structure used in drawing **Figure 2C**.

TABLE 1 | List of symbols.

DESCRIPTION OF HOST SPECIES x	
$_xH_i$	Abundance of host strain i belonging to species x
$_x\mu_i$	Specific growth rate for host strain i of species x. For the graphs in **Figure 2** this is calculated from the Michaelis-Menten relationship: $_x\mu i\,(S) = \dfrac{_x\alpha_S}{1+\frac{_x\alpha_S}{_x\mu^{max}\,_x\nu^i}}$
$_x\delta$	Specific loss rate host x
Competitive abilities of host species x influencing the number of strains it can establish	
$_x\alpha$	Nutrient affinity
$_x\mu^{max}$	Maximum specific growth rate
$_x\nu$	Fractional reduction in $_x\mu^{max}$ for each mutational step in the host
Defensive properties of host species x influencing the abundance of individuals within strains	
$_x\beta$	Adsorbtion coefficient
$_x\rho$	Parameter $0 < \rho < 1$ representing the loss in effective adsorption coefficient for each new host strain added to the arms race
$_x\sigma$	Parameter $0 < \sigma < 1$ representing the fractional loss in effective adsorption coefficient to previous hosts for each new virus strain added to the arms race
VIRUS PROPERTIES	
δ_V	Specific loss rate virus
m	Burst size virus
PHYSICAL, CHEMICAL, AND BIOLOGICAL PARAMETERS DEFINING THE ENVIRONMENT AND THE FOOD WEB EFFECTS	
D	Chemostat dilution rate
S_R	Concentration of limiting element in the chemostat reservoir
S_C	Concentration of limiting substrate in the culture
A	Population size autotrophic flagellates
C	Population size ciliates
H	Population size heterotrophic flagellates
α_A	Affinity for uptake of limiting nutrient, autotrophic flagellates
α_C	Clearance rate for ciliate grazing on flagellates
α_H	Clearance rate for heterotrophic flagellates grazing on bacteria
δ_B	Non-viral bacterial loss rate
Y_H	Yield of heterotrophic flagellates on bacterial prey

therefore compensate for both dilution and viral loss and can therefore remain in the culture at a low, virus-controlled, density in coexistence with the now abundant, resource-controlled, immune mutant. The abundant mutant population represents a resource exploitable for a mutant of the virus. Establishment of such a mutant virus gives a new situation with two low-abundant host strains and high S_C, again representing a resource for a new host mutant, and so on. This evolutionary sequence is thus characterized by a "remaining resource" that alternates in form between free limiting nutrients facilitating a successful mutation in the host, and an immune host strain facilitating successful mutation in the virus. This is a model of the "Red Queen" dynamics observed in chemostat experiments of this type (Little, 2002; Martiny et al., 2014). Importantly, the remaining free resource is diminishing for each turn of the arms race until the final resistant host strain has a population size too small to carry a new virus mutant. In a more generalized analysis, Härter et al. (2014) describe such maturation processes as "narrowing staircase(s) of coexistence." Since the size of the "free resource" would affect the probability that a random mutation is successful, this could explain why antagonistic evolution is fast, but would also suggest that the rate diminishes as the process approaches maturation. The mature state in this arms race model is characterized by many virus-susceptible strains, while the initial stages are dominated by a resistant strain and have a small virus population. Interestingly, these are the two situations forming what has been termed Weinbauer's Paradox (Weinbauer, 2004), where the question is why the few virus—many resistant hosts situation is observed when trying to construct simple host-virus systems in the laboratory, while natural systems are characterized by high viral numbers and many susceptible hosts. In the arms race model above, this apparent paradox is explained simply as the differences between an early and a late stage in the maturation process of strain diversification (Thingstad et al., 2014). How long time is needed for

such strain diversifications to mature, and to what extent the speed of the arms race diminishes with the decrease in remaining resource, remain as open questions.

In their analysis of phage-bacteria infection networks, Weitz et al. (2013) defined four basic types: random, one-to-one, nested, and modular. The arms race model outlined for the chemostat example above leads to the nested type with communities that are connected as illustrated in **Figure 1E**, corresponding to an upper-triangular interaction matrix (Weitz et al., 2013; Thingstad et al., 2014). Such upper-triangular interaction matrices have been found in analysis of published host-virus networks (Flores et al., 2011; Beckett and Williams, 2013), providing some evidence that the highly idealized chemostat analysis used here may bear some relevance to natural systems. The validity of such an extrapolation to natural systems is not self-evident since each species does not evolve independently in a complex community: There, the free resource will be available to all competing species and a high-abundant immune host strain would be available to mutants from many different viruses, opening for models that could lead to more complex structures. With the assumption that no viruses infect hosts belonging to more than one species, the interaction matrix for the community will consist of upper-triangular sub-matrices, one for each species, arranged along the diagonal (Thingstad et al., 2014), very similar to the modularity found in an existing dataset (Beckett and Williams, 2013). The potentially more complex interactions of multispecies communities would be represented by non-zero elements in the void spaces of such a community interaction matrix. A search for the consequences of such complications would require a systematic approach to how to fill in these void spaces and has not been attempted here.

How Is Microbial Community Composition Determined?

The Richness Component

In the chemostat example discussed above, the mechanism generating strain richness in the mature community can be visualized by the set of growth curves for the strains shown in **Figure 2A**, where a cost of resistance (COR) is assumed in the form of a reduction in maximum growth rate for each successive mutation. The community will consist of all strains for which $\mu_i(S_C) \geq D$ where D and S_C are represented by the horizontal and the vertical lines in **Figure 2A**, respectively. As discussed above, the culture concentration S_C will increase as the arms race proceeds, illustrated by a shifting of the vertical line in **Figure 2A** one step to the right for each turn of the arms race, adding subsequently less competitive strains to the established host community. This will continue until either (1) the COR has become too large for a new mutant to compensate for dilution, or (2) the community size reaches the carrying capacity defined by the reservoir concentration S_R. In this chemostat model, viral loss is the only mechanism compensating for growth that exceeds dilution loss. The amount of viruses in the mature situation is thus the amount needed to give each strain i a loss rate by viral lysis equal to $\mu_i(S_C) - D$.

The main viral loss is thus associated with the fastest growing strains, which, as we will see later, are not the same as the dominant strains.

This graphical representation is easily extrapolated to a multi-species situation with grazing control of community size. This is illustrated for a 3-species case in **Figure 2B**, where the horizontal line now represents a non-selective grazing loss.

Application of the KtW principle at the food web level allows for models that contain a shift between mineral nutrient limited and carbon limited growth of the heterotrophic prokaryotes (Thingstad and Pengerud, 1985). The nature of the limiting element represented on the x-axis of **Figure 2B** may therefore change with the environmental conditions with at least organic carbon, nitrogen, phosphorous and iron as expected possibilities. If trade-offs at the species level are mainly between the three parameters defining the topology of **Figure 2B** (μ^{max}, α, and ν) one could conceive a situation where the topology of **Figure 2B**, and therefore the community structure, is invariant to such qualitative shifts in limitation. If, however, there is trade-off between specializations so that e.g., a good competitor under C-limitation is a poor competitor under P-limitation, the growth curves would reshuffle as the type of limitation changes, thereby inducing shifts in species/strain composition.

The Evenness Component

Figure 2B does not provide an answer to the evenness aspect of strain diversity since it does not constrain the abundance of individuals within each established strain. This abundance is controlled by the virus host-interactions, i.e., by the structure of the interaction matrix. For the nested structure in **Figure 1E**, the abundance of the undefended parent strain of species x is given as Thingstad et al. (2014):

$$_xH_0 = \frac{\delta_V + D}{(m-1)\beta}. \tag{1a}$$

While abundance of subsequent mutant strains are given by:

$$_xH_i = {_xH_0}\frac{1 - \sigma\rho}{\rho^i}, \; i \geqq 1. \tag{1b}$$

Equation 1a arises from the equilibrium condition for the first virus which only can infect the parent strain. Abundance of the parent strain thus depends on the decay rate of the virus (δ_V), the burst size (m), and the effective adsorption rate (β) between the undefended parent strain and the original virus. For the subsequent strains, this is modified by the two factors ρ, that represents a fractional loss in β for each new host mutant, and σ which represents a fractional loss in infectivity for previous hosts for each mutational step in the virus. The model thus contains mechanism where there is a cost in the form of reduced infectivity for viruses having a broad host range (Symbols summarized in **Table 1**, see Thingstad et al., 2014 for further details). Abundance within strains is thus related to the species' defensive properties, in particular the parameter $0 < \rho < 1$, where values of ρ $<< 1$ give a very rapid increase in strain size for large i (Equation 1b). Since all the parameters β, m, δ_V, ρ, and σ may vary between host species and between viruses, both the abundance of
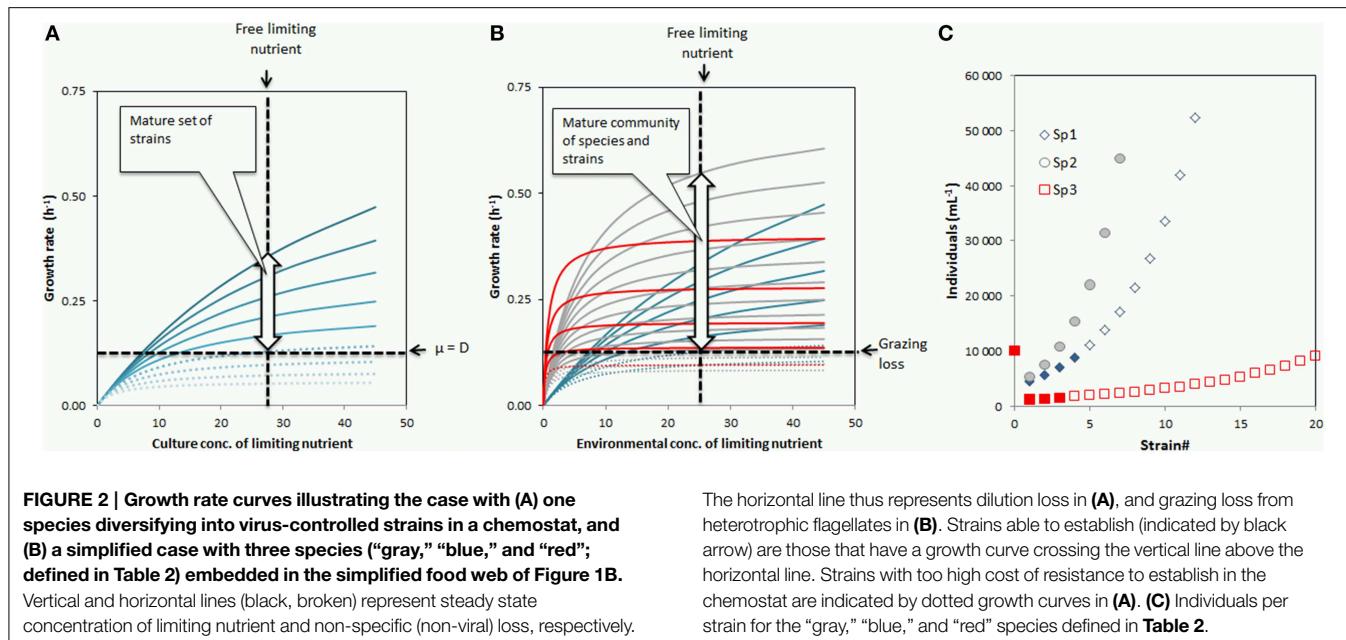
FIGURE 2 | Growth rate curves illustrating the case with (A) one species diversifying into virus-controlled strains in a chemostat, and (B) a simplified case with three species ("gray," "blue," and "red"; defined in Table 2) embedded in the simplified food web of Figure 1B. Vertical and horizontal lines (black, broken) represent steady state concentration of limiting nutrient and non-specific (non-viral) loss, respectively. The horizontal line thus represents dilution loss in **(A)**, and grazing loss from heterotrophic flagellates in **(B)**. Strains able to establish (indicated by black arrow) are those that have a growth curve crossing the vertical line above the horizontal line. Strains with too high cost of resistance to establish in the chemostat are indicated by dotted growth curves in **(A)**. **(C)** Individuals per strain for the "gray," "blue," and "red" species defined in **Table 2**.

the parent host strain (Equation 1a) and the modification of this with subsequent strains (Equation 1b) will vary between species. The set of parameters defining a species can thus be divided into two sets: one (α, μ^{max}, and ν, see **Table 1**) that describes the species' competitive properties and determining how many strains it can establish, and another (β, ρ, and σ) that defines its defensive properties and, together with the properties of its virus (m, δ_V), determines the abundance within strains. Since abundance at species level is the sum over established strains, this will depend on both the competitive and the defensive properties of the species.

Such a combination of high competitive with high defensive abilities requires a low trade-off between the two. With this insight, the question of SAR11 abundance should probably not be focused on whether this is a competition or a defense strategist, but rather whether there is something particular in the lifestyle of this organism that leads to a low trade-off between the two traits (Thingstad et al., 2014). This leads to the intriguing question of whether there is any link between the minimalistic genome design of SAR11 (Giovannoni et al., 2013, 2014) and the potential for a low trade-off between competition and defense. The answer to this is not immediately obvious from the analysis done here.

In the arms race scenario above, a host mutant needs to develop resistance to a virus community that already has co-evolved with the established host community, and it has to do so without losing too much in competitive ability. This could seem like a comparably more difficult task than the "rabbits in Australia" analogy with invasion of a foreign species not recognized by the established virus community and therefore initially not necessarily handicapped by high COR. Although viral attack on invading organisms has been demonstrated experimentally (Sano et al., 2004), such mature communities could thus theoretically seem prone to invasion. Including invasion in the models would create a new maturation process where species richness increases at a rate driven by a combination of the invasion rate and the

distribution of competitive abilities within the seeding community. The result would be an interaction between processes occurring at, at least, five characteristic time scales: the population dynamics (linked to the μ and δ functions), the time scale of the host-virus arms races driving strain diversification, the time scale of the species invasion process, and the time scale of environmental disturbances disrupting these maturation processes. On top of this comes the presumably longer time scale of evolution of new species, changing the properties of the seeding community. An important question is whether there is a theoretical balance between these processes, leading to a steady state combination of species and strains somewhere between the two extremes of (1) a single species with many strains on one side and (2) many species, each with only one strain, on the other. The problem is easiest to illustrate in the chemostat example (**Figure 2A**) where the position of the horizontal line is fixed by the value of D. As discussed above, the maturation in strain diversification that establishes new defense specialists will drive the vertical line representing S to the right. A successful invasion (or evolution) of a new species will establish a new competition strategist competing better than the last established strain in the existing community. This would exchange previously established strains with low competitive ability (high COR) for a strain from the more competitive newcomer, and thereby drive the vertical line to the left. With species and strain diversification introducing new competition and new defense strategists as assumed here, one therefore gets two opposing processes, suggesting that there is a theoretical balancing point, and therefore a theoretical steady state somewhere between the two extremes outlined above.

This gets a bit more complicated in **Figure 2B** where the position of the horizontal line is a dynamic variable coupled to grazing loss. Using the simple food web model in **Figure 1B**, the state with mineral nutrient limited bacteria is, however, still relatively transparent: Assuming steady state in the mineral nutrients (S)—autotrophic flagellates (A)—ciliates (C)—food chain, the

TABLE 2 | Numerical values used to draw the growth curves and the abundance per strain for the three species in Figures 2B,C.

	Competitive traits			Defensive traits					
	α nM-P^{-1}h^{-1}	μ^{max} h^{-1}	v	H_0 mL^{-1}	σ	ρ	Trade-off index	Species abundance mL^{-1}	% of community abundance
Sp#1 «blue»	0.2	1	0.7	1 10^4	0.8	0.8	1.5	3.6 10^4	10
Sp#2 «gray»	0.1	0.7	0.85	1 10^4	0.9	0.7	0.35	3.0 10^5	86
Sp#3 «red»	0.5	0.4	0.7	1 10^4	0.99	0.9	1.2	1.4 10^4	4
							Community abundance:	3.5 10^5	

Trade-off index calculated as described in Thingstad et al. (2014).

autotrophic flagellates must grow as fast as they are grazed. With the simplifying assumption that food intake is proportional to food concentration, the growth = loss condition for autotrophic flagellates becomes $\alpha_A SA = \alpha_C AC$, or:

$$S = \frac{\alpha_C}{\alpha_A} C. \tag{2a}$$

The position of the vertical line (S) is therefore now constrained by ciliate abundance (C) and not directly controlled by introduction of new species or strains as in the chemostat example above. Instead, it is now the position of the horizontal line that becomes a function of strains and species diversity. Using a similar steady state argument for heterotrophic flagellates, the size of the bacterial community becomes proportional to ciliate abundance:

$$B = \frac{\alpha_C}{Y_H \alpha_H} C. \tag{2b}$$

in a non-mature community, the strain diversification process will thus fill the community with successively less competitive strains, thereby driving the horizontal line downwards until the community size given by Equation (2b) is reached. A new species, sufficiently competitive to invade and initially without viral loss, will outcompete existing strains and thereby drive the horizontal line upwards. Since the horizontal line represents the bacterial loss rate to grazing:

$$\delta_B = \alpha_H H, \tag{2c}$$

its position reflects the amount (H) of bacterial grazers. The strain diversification process thus drives the system toward a smaller population of heterotrophic flagellates and reduced food chain efficiency, thus favoring the viral shunt of bacterial production back to dissolved material. The invasion (or mutation) of new, more competitive species drives the system in the other direction toward less viruses, a smaller virus-bacteria ratio and a more efficient food chain. As the two diversification processes drive the system in opposite directions, this raises the question of whether there is an equilibrium point where the two processes balance?

Equations (2b) and (2c) link the abundance of heterotrophic flagellates (H) to the abundance of ciliates (C), through the internal population structure of the bacterial community. Stated differently, they suggest a theory where the internal diversity of the bacterial community and the structure of the predator food chain are intimately connected. The analysis above thus suggests

a mechanistic basis for a biodiversity—ecosystem functioning (BEF) theory for the pelagic microbial community. It is noteworthy that these relationships were derived without any assumption of host diversity being linked to substrate specialization in the hosts, and are therefore quite different from arguments where a high biodiversity is needed to perform a large set of required biochemical reactions. The food web framework used for this analysis (**Figure 1B**) may appear unrealistically simple, but its ability to capture essential aspects of mesocosm perturbation experiments recently been demonstrated (Larsen et al., 2015). This includes the demonstration of a central controlling role for ciliates as assumed in the arguments for Equations (2b) and (2c).

The fundamental set of trophic interactions driving the arms races discussed here is summarized in the idealized structure of the KtW principle (**Figure 1A**). With this basic mechanism being the same at the predator-prey as at the host-parasite level, similar lines of argument can be applied for food web evolution as those used in the above discussion of community composition. The difference in time scale is, however, vast: Whereas the modern pelagic food web can be seen as the present (mature?) state of a 3.5 10^9 year old arms race with a characteristic time scale in the order of 0.5 10^9 years (Thingstad et al., 2010) between major new inventions in weapon technology; Red Queen dynamics of prokaryote hosts and viruses seem to occur more over time scale of days to weeks (Martiny et al., 2014).

In such a geological perspective, there may have been strong couplings between these arms races and the global climate systems as for example the rapid evolution of eukaryotic forms in the Neoproterozoic has been suggested to affect the biological pump and therefore may have been a driving force for rather than a response to extreme climatic fluctuations in this period (Lenton et al., 2014).

How Is the Physiological State of Microbes Affected by the Environment and Other Microbes?

The viral top-down control in this model does not only allow competitively inferior microbes to become members of the ecosystem, it rather suggests that the prokaryote community should be dominated by slow-growing, high-defensive strains. This opens for a possible "sieged city" re-interpretation of the old "dormant or dead" (Zweifel and Hagstrom, 1995; Jones and Lennon, 2010) debate in marine microbiology: The dominance of low-active cells found in aquatic environments is perhaps not

a sign of starvation caused by a lack of suitable substrates in their environment; it may be more because they have "shut the gates to keep the enemy out."

How Do (Apparently) Inferior Microbes Persist in the Ecosystem?

The KtW structure (**Figure 1A**) was originally designed to provide an answer to this question at the food web level, where the discovery of systems with apparent persistent phosphorous-limitation of bacteria (Pengerud et al., 1987; Vadstein et al., 1988; Cotner et al., 1997) raised the question of why their supposedly inferior phytoplankton competitors were not outcompeted to a level where bacteria would become C-limited. The same basic theory has also been applied to the fish-jellyfish dichotomy at higher levels in the pelagic food chain (Haraldsson et al., 2012). It is also a basic mechanism in size-structured plankton models where the intense grazing and rapid response of flagellates explains the (relatively) constant 10^6 mL^{-1} abundance of prokaryotes as predator (top-down) control (Azam et al., 1983). Adding nutrients to the system allows large-celled phytoplankton species to establish that are less competitive but also less grazed (resource controlled). Such models thus produce a gradient from small-celled, top-down controlled, "competition strategist" species dominating in oligotrophic regions to large-celled, resource controlled, "defense strategist" phytoplankton dominating nutrient rich upwelling areas (e.g., Ward et al., 2012). As a principle recognizable at many (all?) trophic levels, the KtW structure of **Figure 1A** generates self-similarity in pelagic ecosystems (Thingstad et al., 2010). This provides at least an analogy to fractal systems where complexity can be generated through the repetition of a simple, scale-independent, rule (Mandelbrot, 1982).

How Are Microbes Distributed in Their Ecosystems?

The models discussed above use the simplifying assumptions of a stable steady-state in an environment homogenous in time and space. This is an approximation that should be treated with caution since it means that some important aspects of host-virus interactions are lost. There are at least two relevant examples of microbial competitive and defensive behavior that seem to work only if an evolutionary adaptation to micro scale patchiness in aquatic environments is invoked: motility and altruistic suicide.

Motility is usually argued to have little effect on bacterial nutrient acquisition in homogenous environments because of the high efficiency of molecular diffusion at the 1 μm—scale (Jumars et al., 1993). Motility is thus believed only to be of any advantage in patchy environments. Since the diffusivity of viruses is about 2 orders of magnitude lower (0.2–3.10^{-7} cm^{2}s^{-1}) (Murray and Jackson, 1992) than that of small nutrient molecules (~10^{-5} cm^{2}s^{-1}), one can turn this around to argue that the collision frequency with viruses should increase significantly with motility. One should thus expect to find motile bacteria only in patchy environments (Blackburn et al., 1997). One can speculate whether this means that the fraction of motile bacteria may be

used as a proxy for environmental patchiness, and also whether this is linked to the COR since the foraging gain from swimming must outweigh the COR associated with the increased defense needed by motile hosts.

A somewhat similar argument is linked to the enigma of altruistic suicide, where some species induce apoptosis when infected (Ackermann et al., 2008; Blower et al., 2012). If this kills the host before new viruses are released, it would protect the neighbors from infection. From an evolutionary perspective, this poses the problem that altruistic suicide may not seem like an evolutionary stable strategy since the genes of the altruist are obviously not passed on to any offspring. If, however, the neighbors are close relatives, the patch becomes a kind of super-organism consisting of cells having the same genes, and altruistic suicide becomes evolutionarily stable (Smith et al., 2010), as has also been shown experimentally (Berngruber et al., 2013). With lytic events creating centers of release for both viruses and DOM, viral lysis is in itself a patchiness-generating mechanism with an interesting geometry consisting of two concentric spheres of food and danger expanding at different rates.

Concluding Comments

The consequences of applying the KtW principles at strain rather than species level are both practical and conceptual. The practical part is that the models then no longer constrain species diversity in the same manner as in the case of pure top-down control of species size, with direct consequences for model comparison with e.g., 16S rRNA gene sequence data. A main conceptual consequence, at least in the model of Thingstad et al. (2014), is the separation of the two aspects of abundance control: The number of strains being controlled by the competitive properties of the host, and the number of individuals per strain by the host's defensive abilities and the host-virus interactions. This separation may seem like a relatively robust feature transferable to models with more complicated interaction matrices. The structure of the interaction matrix would then primarily affect the abundance within strains feature of the system.

The Thingstad et al. (2014) model also involves a seeding community. While this can be approached from a descriptive side with a laborious description of the competitive and defensive properties of "all" existing host species; the intriguing challenge is to try to understand the trade-offs between traits in this population. One can visualize this problem in an n-dimensional strategy space formed by the n relevant life-history traits of hosts and viruses. Trade-offs represent correlations in this n-dimensional space and would confine the subset of feasible strategies. With a slight rephrasing of Baas Becking and Beijerinck's famous statement (de Wit and Bouvier, 2006), this would give models where "The feasible is everywhere, the environment selects."

Acknowledgments

References

Ackermann, M., Stecher, B., Freed, N. E., Songhet, P., Hardt, W.-D., and Doebeli, M. (2008). Self-destructive cooperation mediated by phenotypic noise. *Nature* 454, 987–990. doi: 10.1038/nature07067

Avrani, S., Wurtzel, O., Sharon, I., Sorek, R., and Lindell, D. (2011). Genomic island variability facilitates *Prochlorococcus*-virus coexistence. *Nature* 474, 604–608. doi: 10.1038/nature10172

Azam, F., Fenchel, T., Field, J. G., Gray, J. S., Meyer-Reil, L. A., and Thingstad, T. F. (1983). The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* 10, 257–263. doi: 10.3354/meps010257

Beckett, S. J., and Williams, H. T. P. (2013). Coevolutionary diversification creates nested-modular structure in phage-bacteria interaction networks. *Interface Focus* 3:20130033. doi: 10.1098/rsfs.2013.0033

Beninca, E., Huisman, J., Heerkloss, R., Johnk, K. D., Branco, P., Van Nes, E. H., et al. (2008). Chaos in a long-term experiment with a plankton community. *Nature* 451, 822–827. doi: 10.1038/nature06512

Berngruber, T. W., Lion, S., and Gandon, S. (2013). Evolution of suicide as a defence strategy against pathogens in a spatially structured environment. *Ecol. Lett.* 16, 446–453. doi: 10.1111/ele.12064

Blackburn, N., Azam, F., and Hagstrom, A. (1997). Spatially explicit simulations of a microbial food web. *Limnol. Oceanogr.* 42, 613–622. doi: 10.4319/lo.1997.42.4.0613

Blower, T. R., Evans, T. J., Przybilski, R., Fineran, P. C., and Salmond, G. P. C. (2012). Viral evasion of a bacterial suicide system by RNA-based molecular mimicry enables infectious altruism. *PLoS Genet.* 8:e1003023. doi: 10.1371/journal.pgen.1003023

Bohannan, B. J. M., and Lenski, R. E. (1997). Effect of resource enrichment on a chemostat community of bacteria and bacteriophage. *Ecology* 78, 2303–2315. doi: 10.1890/0012-9658(1997)078[2303:EOREOA]2.0.CO;2

Bohannan, B. J. M., and Lenski, R. E. (2000). The relative importance of competition and predation varies with productivity in a model community. *Am. Nat.* 156, 329–340. doi: 10.1086/303393

Connell, J. H. (1979). IIntermediate-disturbance hypothesis. *Science* 204, 1345–1345. doi: 10.1126/science.204.4399.1345

Cotner, J. B., Ammerman, J. W., Peele, E. R., and Bentzen, E. (1997). Phosphorus-limited bacterioplankton growth in the Sargasso Sea. *Aquat. Microb. Ecol.* 13, 141–149. doi: 10.3354/ame013141

de Wit, R., and Bouvier, T. (2006). 'Everything is everywhere, but, the environment selects'; what did Baas Becking and Beijerinck really say? *Environ. Microbiol.* 8, 755–758. doi: 10.1111/j.1462-2920.2006.01017.x

Dini-Andreote, F., de Cássia Pereira e Silva, M., Triado-Margarit, X., Casamayor, E. O., van Elsas, J. D., and Salles, J. F. (2014). Dynamics of bacterial community succession in a salt marsh chronosequence: evidences for temporal niche partitioning. *ISME J.* 8, 1989–2001. doi: 10.1038/ismej.2014.54

Flores, C. O., Meyer, J. R., Valverde, S., Farr, L., and Weitz, J. S. (2011). Statistical structure of host-phage interactions. *Proc. Natl. Acad. Sci. U.S.A.* 108, E288–E297. doi: 10.1073/pnas.1101595108

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G., and Hanage, W. P. (2009). The bacterial species challenge: making sense of genetic and ecological diversity. *Science* 323, 741–746. doi: 10.1126/science.1159388

Giovannoni, S., Temperton, B., and Zhao, Y. (2013). SAR11 viruses and defensive host strains reply. *Nature* 499, E4–E5. doi: 10.1038/nature12388

Giovannoni, S. J., Thrash, J. C., and Temperton, B. (2014). Implications of streamlining theory for microbial ecology. *ISME J.* 8, 1553–1565. doi: 10.1038/ismej.2014.60

Haraldsson, M., Tonnesson, K., Tiselius, P., Thingstad, T. F., and Aksnes, D. L. (2012). Relationship between fish and jellyfish as a function of eutrophication and water clarity. *Mar. Ecol. Prog. Ser.* 471, 73–85. doi: 10.3354/meps10036

Härter, J. O., Mitarai, N., and Sneppen, K. (2014). Phage and bacteria support mutual diversity in a narrowing staircase of coexistence. *ISME J.* 8, 2317–2326. doi: 10.1038/ismej.2014.80

Hutchinson, G. E. (1961). The paradox of the plankton. *Am. Nat.* 95, 137–145. doi: 10.1086/282171

Jankowski, K., Schindler, D. E., and Horner-Devine, M. C. (2014). Resource availability and spatial heterogeneity control bacterial community response to nutrient enrichment in lakes. *PLoS ONE* 9:e8699. doi: 10.1371/journal.pone.0086991

Jones, S. E., and Lennon, J. T. (2010). Dormancy contributes to the maintenance of microbial diversity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5881–5886. doi: 10.1073/pnas.0912765107

Jover, L. F., Cortez, M. H., and Weitz, J. S. (2013). Mechanisms of multi-strain coexistence in host-phage systems with nested infection networks. *J. Theor. Biol.* 332, 65–77. doi: 10.1016/j.jtbi.2013.04.011

Jumars, P., Deming, J., Hill, P., Karp-Boss, L., Yager, P., and Dade, W. (1993). Physical constraints on marine osmotrophy in an optimal foraging context. *Mar. Microb. Food Webs* 7, 121–159.

Larsen, A., Egge, J. K., Nejstgaard, J. C., Di Capua, I., Thyrhaug, R., Bratbak, G., et al. (2015). Contrasting response to nutrient manipulation in Arctic mesocosms are reproduced by a minimum microbial food web model. *Limnol. Oceanogr.* 60, 360–374. doi: 10.1002/lno.10025

Lenton, T. M., Boyle, R. A., Poulton, S. W., Shields-Zhou, G. A., and Butterfield, N. J. (2014). Co-evolution of eukaryotes and ocean oxygenation in the Neoproterozoic era. *Nat. Geosci.* 7, 257–265. doi: 10.1038/ngeo2108

Little, T. J. (2002). The evolutionary significance of parasitism: do parasite-driven genetic dynamics occur *ex silico*? *J. Evolut. Biol.* 15, 1–9. doi: 10.1046/j.1420-9101.2002.00366.x

Mandelbrot, B. B. (1982). *The Fractal Geometry of Nature.* New York, NY: W.H. Freeman and Company.

Marston, M. F., Pierciey, F. J. Jr., Shepard, A., Gearin, G., Qi, J., Yandava, C., et al. (2012). Rapid diversification of coevolving marine Synechococcus and a virus. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4544–4549. doi: 10.1073/pnas.1120310109

Martiny, J. B. H., Riemann, L., Marston, M. F., and Middelboe, M. (2014). Antagonistic coevolution of marine planktonic viruses and their hosts. *Ann. Rev. Mar. Sci.* 6, 393–414. doi: 10.1146/annurev-marine-010213-135108

Middelboe, M., Holmfeldt, K., Riemann, L., Nybroe, O., and Haaber, J. (2009). Bacteriophages drive strain diversification in a marine Flavobacterium: implications for phage resistance and physiological properties. *Environ. Microbiol.* 11, 1971–1982. doi: 10.1111/j.1462-2920.2009.01920.x

Murray, A. G., and Jackson, G. A. (1992). Viral dynamics - A model of the effects of size, shape, motion and abundance of single-celled planktonic organisms and other particles. *Mar. Ecol. Prog. Ser.* 89, 103–116. doi: 10.3354/meps089103

Pengerud, B., Skjoldal, E. F., and Thingstad, T. F. (1987). The reciprocal interaction between degradation of glucose and ecosystem structure - studies in mixed chemostat cultures of marine bacteria, algae, and bacterivorous nanoflagellates. *Mar. Ecol. Prog. Ser.* 35, 111–117. doi: 10.3354/meps035111

Sano, E., Carlson, S., Wegley, L., and Rohwer, F. (2004). Movement of viruses between biomes. *Appl. Environ. Microbiol.* 70, 5842–5846. doi: 10.1128/AEM.70.10.5842-5846.2004

Smith, J., Van Dyken, J. D., and Zee, P. C. (2010). A generalization of Hamilton's rule for the evolution of microbial cooperation. *Science* 328, 1700–1703. doi: 10.1126/science.1189945

Staley, C., Gould, T. J., Wang, P., Phillips, J., Cotner, J. B., and Sadowsky, M. J. (2014). Bacterial community structure is indicative of chemical inputs in the Upper Mississippi River. *Front. Microbiol.* 5:524. doi: 10.3389/fmicb.2014.00524

Thingstad, T. F. (2000). Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. *Limnol. Oceanogr.* 45, 1320–1328. doi: 10.4319/lo.2000.45.6.1320

Thingstad, T. F., and Lignell, R. (1997). Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. *Aquat. Microb. Ecol.* 13, 19–27. doi: 10.3354/ame013019

Thingstad, T. F., and Pengerud, B. (1985). Fate and effect of allochthonous organic material in aquatic microbial ecosystems - an analysis based on chemostat theory. *Mar. Ecol. Prog. Ser.* 21, 47–62. doi: 10.3354/meps021047

Thingstad, T. F., Strand, E., and Larsen, A. (2010). Stepwise building of plankton functional type (PFT) models: a feasible route to complex models? *Prog. Oceanogr.* 84, 6–15. doi: 10.1016/j.pocean.2009.09.001

Thingstad, T. F., Våge, S., Storesund, J. E., Sandaa, R.-A., and Giske, J. (2014). A theoretical analysis of how strain-specific viruses can control microbial species diversity. *Proc. Natl. Acad. Sci. U.S.A.* 111, 7813–7818. doi: 10.1073/pnas.1400909111

Tilman, D. (1977). Resource competition between planktonic algae - Experimental and theoretical approach. *Ecology* 58, 338–348. doi: 10.2307/1935608

Uksa, M., Fischer, D., Welzl, G., Kautz, T., Koepke, U., and Schloter, M. (2014). Community structure of prokaryotes and their functional potential in subsoils

is more affected by spatial heterogeneity than by temporal variations. *Soil Biol. Biochem.* 75, 197–201. doi: 10.1016/j.soilbio.2014.04.018

Vadstein, O., Jensen, A., Olsen, Y., and Reinertsen, H. (1988). Growth and phosporus status of limnetic phytoplankton and bacteria. *Limnol. Oceanogr.* 33, 489–503. doi: 10.4319/lo.1988.33.4.0489

Våge, S., Storesund, J. E., and Thingstad, T. F. (2013). SAR11 viruses and defensive host strains. *Nature* 499, E3–E4. doi: 10.1038/nature12387

Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., et al. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304, 66–74. doi: 10.1126/science.1093857

Ward, B. A., Dutkiewicz, S., Jahn, O., and Follows, M. J. (2012). A size-structured food-web model for the global ocean. *Limnol. Oceanogr.* 57, 1877–1891. doi: 10.4319/lo.2012.57.6.1877

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS. Microbiol. Ecol.* 28, 127–181. doi: 10.1016/j.femsre.2003.08.001

Weitz, J. S., Poisot, T., Meyer, J. R., Flores, C. O., Valverde, S., Sullivan, M. B., et al. (2013). Phage-bacteria infection networks. *Trends Microbiol.* 21, 82–91. doi: 10.1016/j.tim.2012.11.003

Winter, C., Bouvier, T., Weinbauer, M. G., and Thingstad, T. F. (2010). Trade-Offs between competition and defense specialists among unicellular planktonic organisms: the "killing the winner" hypothesis revisited. *Microbiol. Mol. Biol. Rev.* 74, 42–57. doi: 10.1128/MMBR.00034-09

Zhao, Y., Temperton, B., Thrash, J. C., Schwalbach, M. S., Vergin, K. L., Landry, Z. C., et al. (2013). Abundant SAR11 viruses in the ocean. *Nature* 494, 357–360. doi: 10.1038/nature11921

Zweifel, U. L., and Hagstrom, A. (1995). Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). *Appl. Environ. Microbiol.* 61, 2180–2185.

A Generalized Spatial Measure for Resilience of Microbial Systems

Ryan S. Renslow[1], Stephen R. Lindemann[2] and Hyun-Seob Song[2]*

[1] *Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA, USA,* [2] *Biological Sciences Division, Earth and Biological Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA, USA*

The emergent property of resilience is the ability of a system to return to an original state after a disturbance. Resilience may be used as an early warning system for significant or irreversible community transition; that is, a community with diminishing or low resilience may be close to catastrophic shift in function or an irreversible collapse. Typically, resilience is quantified using recovery time, which may be difficult or impossible to directly measure in microbial systems. A recent study in the literature showed that under certain conditions, a set of spatial-based metrics termed *recovery length*, can be correlated to recovery time, and thus may be a reasonable alternative measure of resilience. However, this spatial metric of resilience is limited to use for step-change perturbations. Building upon the concept of recovery length, we propose a more general form of the spatial metric of resilience that can be applied to any shape of perturbation profiles (for example, either sharp or smooth gradients). We termed this new spatial measure "perturbation-adjusted spatial metric of resilience" (PASMORE). We demonstrate the applicability of the proposed metric using a mathematical model of a microbial mat.

Keywords: resilience, microbial communities, recovery, ecology, emergent properties

INTRODUCTION

Complex networks of interacting components produce outcomes that cannot be easily predicted, even when the state of the network components and the inputs to the network are known. These difficult-to-determine outcomes have come to be known as emergent phenomena or higher-order properties, which emerge from the functioning of the whole network, rather than as a simple sum of the individual states of the parts (De La Fuente et al., 2008; Schubert, 2014). Emergent properties arise in complex networks that impact our lives, such as in the communities of microbes and higher organisms that compose ecosystems (Tilman et al., 2001), social networks (Lusseau, 2003; Mitrovic and Tadic, 2010), military-political structures (Porter et al., 2005), commercial systems (Carey and Carville, 2003; Cimellaro et al., 2010), and climate and weather systems (Higgins et al., 2002; Easterling and Kok, 2003). Consequently, developing the ability to understand and predict emergent properties from complex networks is of great importance.

Microorganisms are commonly found physically associated with one another in spatially structured communities such as biofilms or microbial mats (Curtis and Sloan, 2004; Wagner et al., 2006; Fuhrman, 2009; Robinson et al., 2010; Renslow et al., 2011). These communities may range from monocultures to highly diverse assemblages of species; however, in either case, individual members operate and interact in ways governed by their individual functional

responses and local microenvironmental conditions (Schramm et al., 1996; Hibiya et al., 2003; Bernstein et al., 2013, 2014). Formation of a biofilm matrix composed of extracellular polymeric substances confers significant fitness advantages on the microbes it shelters, including physical protection, such as from predation or shearing forces, reduction of environmental stresses, such as from rapid changes in environmental conditions or exposure to antibiotics, facilitation of beneficial interspecies relationships, and rapid exchange of genetic material (Flemming and Wingender, 2001; Laspidou and Rittmann, 2002; Czaczyk and Myszka, 2007; Cao et al., 2011). It is likely that emergent properties arise from the spatial organization of microbes, forming microenvironments and promoting interconnectedness of a multi-species metabolic network through resource exchange and intercellular communication (Xavier and Foster, 2007; Konopka, 2009; Wintermute and Silver, 2010).

Resilience is a higher-order property in microbial communities, characterized by the ability to recover from a perturbation or disturbance (Allison and Martiny, 2008; Shade et al., 2012; Griffiths and Philippot, 2013; Hawkes and Keitt, 2015). While resilience is not yet conclusively defined in microbial communities, we use this term to imply the rate of recovery of a given *function* in a community (or, more generally, their *functional* relationship with environmental variables) after perturbation. The concept of functional resilience in both engineered and ecological microbial systems, and the relationship between state and functional properties is examined in detail in our companion paper (Song et al., 2015). Attempts at quantifying resilience have primarily been done by monitoring functional recovery over time, with resilience being negatively correlated to recovery time (i.e., the time required for function to recover a defined percent of original function), or being correlated to recovery speed (i.e., the rate of recovery of function with units of slope). Faster recovery of function is therefore an indicator of higher resilience. These ideas have been explored theoretically and experimentally (Wissel, 1984). For example, recovery rate was monitored for cyanobacterial cultures exposed to a dilution perturbation by flushing out 10% of the population volume; the recovery rate decreased as the population lost resilience and approached a tipping point where function became irrecoverable (Veraart et al., 2012). In this experiment where the recovery over time was readily observable over a relevant timescale, assessing the system's resilience is straightforward. Such quantification of recovery time is important because it is known that systems nearing the verge of collapse or those trending toward unstable dynamics frequently exhibit reduced rates of recovery, or decreasing resilience (van Nes and Scheffer, 2007). This is known as "critical slowing down," meaning that, as a system nears a tipping point, recovery of function after a perturbation slackens (Scheffer et al., 2009, 2012). Thus quantitative measures of resilience are essential for early warning of impending system collapse, from which function cannot be regained.

Although recovery rate provides a direct measurement of resilience, in some systems it is not possible or practical to measure recovery time on time scales relevant to the perturbation. Additionally, some systems do not allow for measurement of resilience because the experiments needed for quantify recovery time are impossible, unethical (e.g., when imposing a perturbation and monitoring function would endanger ecosystems or humans), or otherwise detrimental to the function of neighboring systems. In such cases not amenable to temporal analysis, resilience can alternatively be quantified in terms of *spatial* recovery as a proxy for temporal recovery (Dai et al., 2013). This means that resilience may still be predicted by observing spatial, rather than temporal, features of the system in cases where temporal and spatial recovery are reasonably correlated. The ability to monitor microbial function over relevant spatial scales has become increasingly possible due to advances in imaging and sensor capabilities, enabling a link between function, structure, and microbial identity (Behrens et al., 2008; Li et al., 2008; Pett-Ridge and Weber, 2012; Babauta et al., 2014; Vanwonterghem et al., 2014).
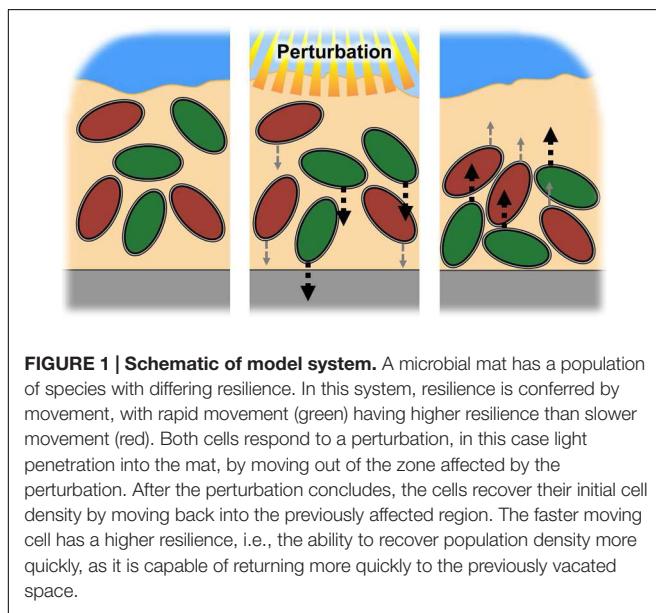
Recently, Dai et al. (2013) put forward recovery length as a measure for resilience. Recovery length is characterized by the distance required for function to recover from a spatial perturbation, and it was initially based on the observation that recovery length, which was correlated to recovery time, increased when a yeast system was on the verge of collapse. In their experiment, populations of *Saccharomyces cerevisiae* were connected spatially along a one-dimensional array through discrete dispersal events. Population stability was measured as the dilution factor was increased, imposing a perturbation. Recovery length was quantified by measuring the population density across distance, which provided a warning signal of imminent population collapse as the dilution factor approached unsustainable levels. Furthermore, these indicators increased with gradual increases in dilution factor, revealing deterioration of resilience in the system in real time. The recovery length concept proposed by Dai and coworkers faithfully quantified resilience to perturbations with sharp boundaries (such as step-change perturbations). As the authors pointed out, however, the same may not be effective for more realistic forms of perturbations that follow gradients or which are blurred across the spatial dimension. In reality, perturbation may come as gradients across both time and space. For example, spatial perturbations that form gradients include the intrusion of substrate or toxins into soil and its subsequent removal (Cao et al., 2012; Moran et al., 2014) or sunlight penetrating into a microbial mat (Bhaya et al., 2001; Häder and Lebert, 2001; Lindemann et al., 2013).

In this study we propose a perturbation-independent resilience metric, termed perturbation-adjusted spatial metric of resilience (PASMORE), to be applicable to perturbations that may be either step-change or gradient-based, and thus extending the recovery length work by Dai et al. (2013). To test this new metric, we developed a simple model of a microbial mat containing species with differing resilience. We discuss cases where the previously proposed metric fails to faithfully measure resilience under gradient perturbations, and then demonstrate how PASMORE provides an appropriate assessment of resilience for the same and other types of perturbation profiles. We also explored the impact of limited knowledge about the shape of

perturbation profiles on the effectiveness of PASMORE. Finally, we investigated a test application of PASMORE to systems of two interacting species, and observed how resilience is affected by interspecies competition.

MODEL SYSTEM

We considered a simple mathematical model, which simulates a microbial mat with populations of microbial species: one with faster motility, and another with slower motility (see **Figure 1** for illustration). Each species responds to a perturbation (e.g., light) with movement proportional and opposite to the perturbation; during perturbation, both species move away from the perturbation toward a region where the perturbation is not present. The key aspect of this simulation is that the motility allows the microbial species to recover their initial population density after the perturbation occurs, with the more motile species recovering more quickly, and thus displaying higher resilience. While resilience is bestowed by motility rate in our model system, there are many other properties of a microbial species that can confer resilience beyond motility, and these features may present themselves within spatial constructions besides microbial mats. Furthermore, our proposed resilience measure has been formulated to be agnostic to the type of function being monitored. As discussed in Song et al. (2015), it is the researcher's job to identify the function of interest that is measured in a spatially defined community. Function may be defined as any trait or variable of interest, and resilience of the chosen function must be relative to a specific perturbation (Felix and Wagner, 2008). This is the cardinal "What to What?" question discussed by Carpenter et al. (2001) and Lesne (2008). For an in-depth discussion on the biological features that contribute to microbial resilience see the review by Shade et al. (2012). For the sake of evaluating our proposed resilience

measure, motility suffices to provide a good working model, with population density as the example function of interest (or as a proxy for community functions that are closely linked to it).

The simulation was built using Comsol Multiphysics (v. 5.0.1.276) finite element analysis software with the chemical reaction engineering module. The microbial mat model was implemented using the diffusion application mode, with a one-dimensional geometry, similar to models previously described by our group (Renslow et al., 2013a,b,c).

THE CONCEPT OF THE RECOVERY LENGTH AND PROPOSED EXTENSION

Dai et al. (2013) proposed the half-point recovery length definition of spatial recovery, which increases in proportion to the loss of resilience. The half-point recovery length is defined as L^{half} in Equation 1:

$$f(L^{\text{half}}) = \frac{1}{2}[f(x^{\text{b}}) + f(x^{\text{eq}})] \qquad (1)$$

where f is the function of interest, x^{b} is the spatial distance at the boundary of the perturbation, $f(x^{\text{eq}})$ is the functional value at the corresponding equilibrium (e.g., unperturbed) condition (a diagram of these terms is provided in Dai et al. (2013) supplementary information). For our simulation, we define $f(x^{\text{eq}})$ as the functional value when it has reached 99% of its true equilibrium value, similar to how we calculate temporal recovery, described below. The equilibrium condition may also be considered to be $f(x^{\text{eq}})$ where the function is fully perturbed, especially in the case where recovery length is measured at a boundary or where a full recovery profile (i.e., one that includes both perturbed and unperturbed equilibrium regions) may not be available (see Dai et al., Supplementary Figures 6 and 8 for a more detailed discussion on this special case). In cases where the perturbation boundary is not well defined or unknown, such as during a gradient perturbation, we assign x^{b} to be the spatial distance at the start of the perturbation. This does not alter the recovery length–resilience correlation for step-change perturbations in our model, but simply allows for quantifying L^{half} in gradient perturbation cases. Also note that, as described in Dai et al., function profiles are normalized by $f(x^{\text{eq}})$, and all results are shown on normalized scales for clarity and ease of comparison.

A new measure proposed in this work, PASMORE takes into account the shape of the perturbation, whether it follows a sharp step-change or a gradient. PASMORE is defined as a weighted integral, where the perturbation profile is the weighting function:

$$\text{PASMORE} = \int_{\text{s}} p(x)f(x)dx \qquad (2)$$

where f is the function of interest, p is the perturbation profile, and PASMORE is the integral of pf over the defined system space, S. In our simulation, the defined system space is the microbial mat where the perturbation has effect.

FIGURE 1 | Schematic of model system. A microbial mat has a population of species with differing resilience. In this system, resilience is conferred by movement, with rapid movement (green) having higher resilience than slower movement (red). Both cells respond to a perturbation, in this case light penetration into the mat, by moving out of the zone affected by the perturbation. After the perturbation concludes, the cells recover their initial cell density by moving back into the previously affected region. The faster moving cell has a higher resilience, i.e., the ability to recover population density more quickly, as it is capable of returning more quickly to the previously vacated space.

COMPARISON OF RECOVERY LENGTH AND PASMORE IN MICROBIAL MATS WITH NON-INTERACTING SPECIES
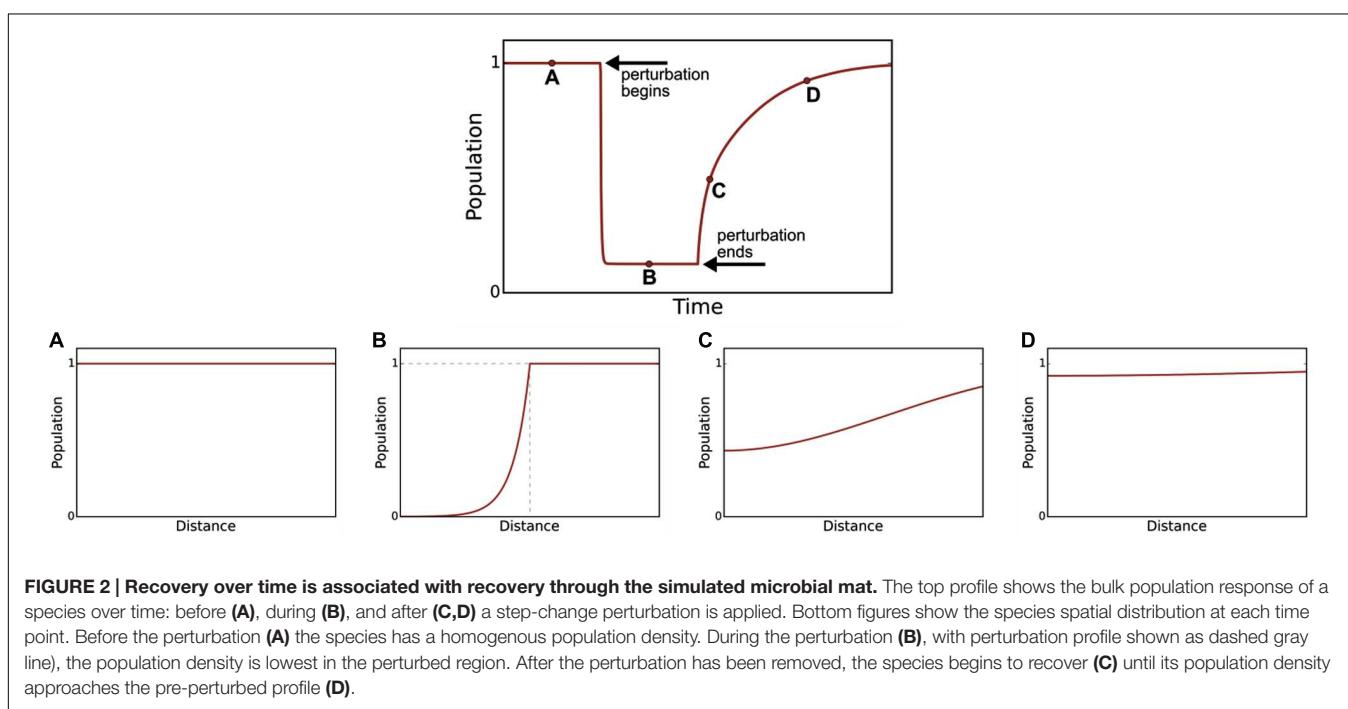
In **Figure 2**, we demonstrate how a microbial population responds to the application and removal of a perturbation. It is possible to see the relationship between temporal recovery and spatial recovery. Temporal recovery is defined as the time required for the recovery of function, in this case population density, to 99% of its original value. In cases where spatial recovery is correlated to temporal recovery, such as in our simulation (Pearson product-moment correlation coefficient, i.e., Pearson's r, of 1.0), spatial recovery metrics may be used as a proxy for quantifying resilience.
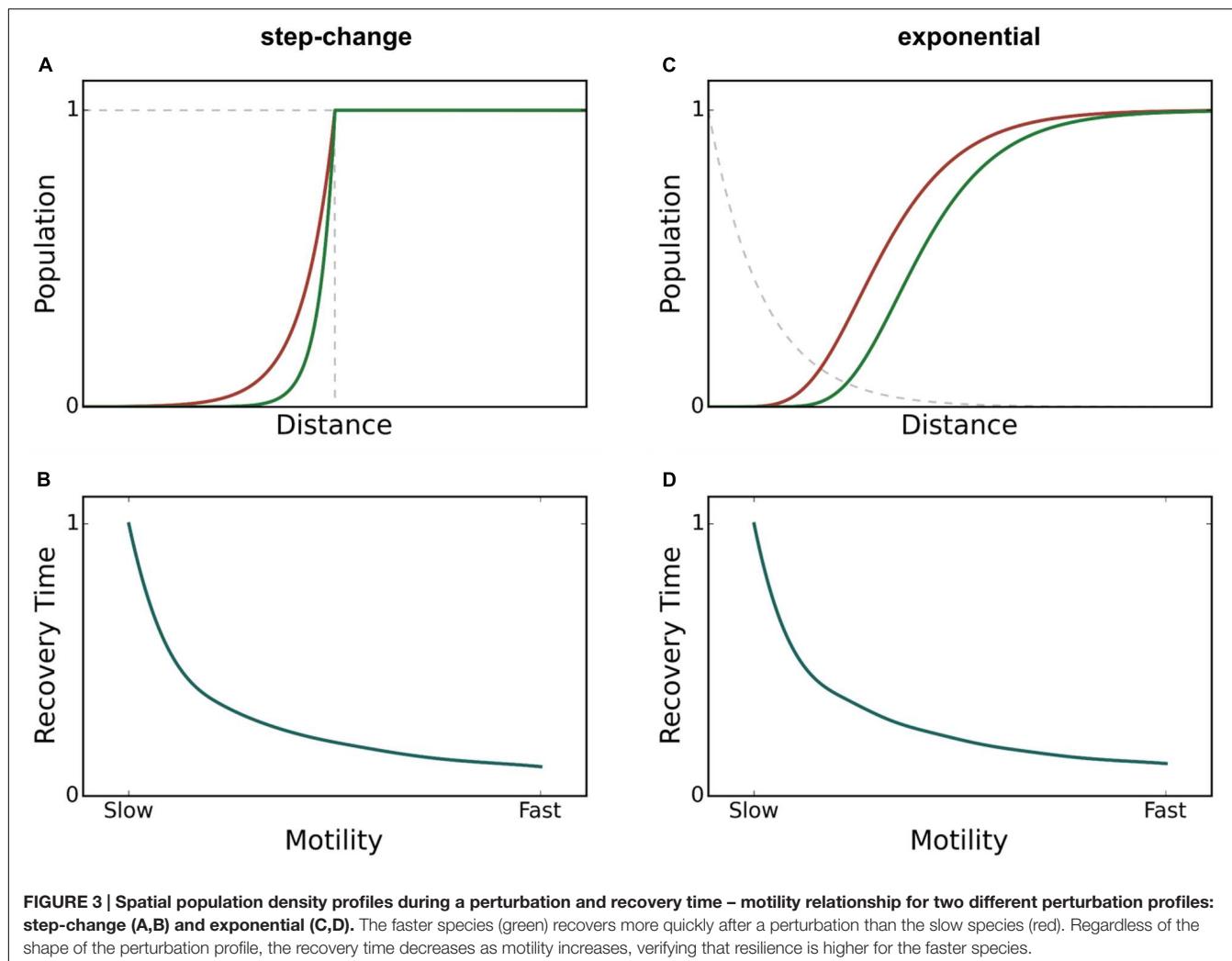
It is known that perturbations, whether occurring over time or over spatial domains, will occur frequently across gradients of varying sharpness. For example, using the example of light as the source of perturbation, attenuation follows an exponential decay profile. Even when the perturbation occurs across a gradient, rapidly motile species exhibit high resilience, meaning in this case that they recover population density more quickly than species with slow motility. However, as discussed by Dai et al. (2013), half-point recovery length may fail to accurately quantify resilience under conditions of a blurred or gradient perturbation. We tested step-change, linear, polynomial, and exponential perturbation gradients (data shown only for the exponential case) (**Figure 3**). The ability for spatial recovery to correlate to temporal recovery decreases as the perturbation profiles approaches an exponential function, where all correlation is lost (Pearson's r of 0.0). **Figure 4** demonstrates that PASMORE is able to accurately quantify spatial recovery and maintain the correlation to temporal recovery (Pearson's r of 1.0) regardless

of the perturbation profile. This was true for all perturbation gradients that we tested. Resilience arises due to the motility of the population, thus the metric should maintain its relationship to motility regardless of the perturbation to which it is exposed.

QUANTIFYING RESILIENCE WITH LIMITED PERTURBATION INFORMATION

Properly quantifying resilience using spatial metrics requires information about the perturbation's shape. This is true whether PASMORE or other metrics like half-point recovery length are used. However, in experimental systems, it may not be possible to obtain the entire profile of a perturbation gradient. Here we wanted to quantify the effect of limited information about the gradient. Therefore, we examined PASMORE for use when the exact shape of the gradient imposed by a perturbation was uncertain. As shown in **Figure 5**, we compared two cases against the full-profile PASMORE: (1) a two-point dataset to generate a linear approximation between the start and end of the observed perturbation and (2) a three-point dataset to generate two linear approximations between the start and middle and between the middle and end of the observed perturbation. Note that only the perturbation profile was approximated, not the population density, since it is assumed that the function of interest, for which resilience is to be evaluated, is quantifiable. Using sensitive measurement instruments in real systems, it is likely that more than two or three data points of the perturbation profile could be obtained, for example by using microelectrodes (Nguyen et al., 2012) or NMR imaging (Renslow et al., 2010). However, as will be demonstrated, even using a three-point

FIGURE 2 | Recovery over time is associated with recovery through the simulated microbial mat. The top profile shows the bulk population response of a species over time: before **(A)**, during **(B)**, and after **(C,D)** a step-change perturbation is applied. Bottom figures show the species spatial distribution at each time point. Before the perturbation **(A)** the species has a homogenous population density. During the perturbation **(B)**, with perturbation profile shown as dashed gray line), the population density is lowest in the perturbed region. After the perturbation has been removed, the species begins to recover **(C)** until its population density approaches the pre-perturbed profile **(D)**.

FIGURE 3 | Spatial population density profiles during a perturbation and recovery time – motility relationship for two different perturbation profiles: step-change (A,B) and exponential (C,D). The faster species (green) recovers more quickly after a perturbation than the slow species (red). Regardless of the shape of the perturbation profile, the recovery time decreases as motility increases, verifying that resilience is higher for the faster species.

approximation, the calculation of PASMORE quickly approaches the full-profile PASMORE. **Figure 5** shows that PASMORE is able to maintain the correlation to temporal recovery with a two-point approximation (Pearson's r of 0.96), and this correlation improved with a three-point dataset (Pearson's r of 0.99).

ANALYSIS OF A MICROBIAL MAT WITH INTERACTING SPECIES

We investigated how resilience as measured by PASMORE would change if two species in the mat interacted with each other. Therefore, we modified the model to simultaneously include two motile species that exhibited a difference in their rate of motility, but which was dependent on the consumption of a substrate. The substrate diffused from the top of the mat and consumption followed Monod-type kinetics, based on the diffusion and reaction parameters and setup of a previous model (Renslow et al., 2013b). Such a configuration broadly approximates the structure of a cyanobacterial mat, where the

carbon fixation upon which heterotrophs depend is maximal near the mat surface (Lindemann et al., 2013). The case where both species competed for the same substrate was compared to the case where the species consumed two independent substrates noncompetitively. **Figure 6** shows the percent change of PASMORE comparing the non-interacting to competitive interaction cases. Compared to the non-interacting case, the interacting species displayed an increase in PASMORE, and thus were found to have lower resilience. Furthermore, the species with the faster motility exhibited a larger loss of resilience compared to the slower species. This is due to the fact that, during perturbation, the species with higher resilience occupy a lower nutrient zone, while the species with lower resilience are closer to the nutrient source and are thus less affected. As such, the model illustrates a trade-off between resource availability and stress avoidance in these motile species; we anticipate that similar trade-offs will exist in many spatially organized communities where local interactions lead to gains in fitness. Finally, this simple simulation demonstrates how PASMORE can help quantify resilience in a case where previous metrics would not have been meaningful.
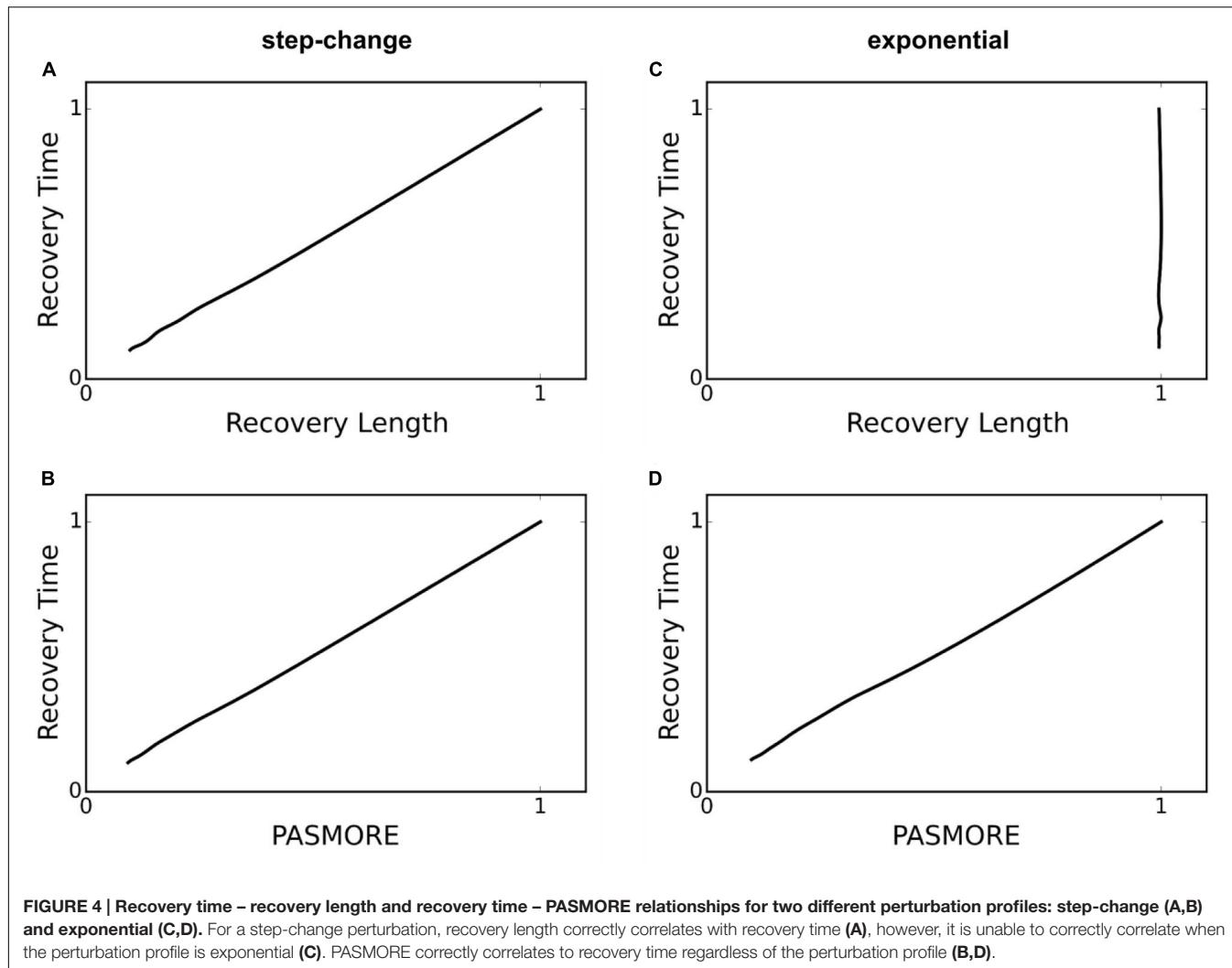
FIGURE 4 | Recovery time – recovery length and recovery time – PASMORE relationships for two different perturbation profiles: step-change (A,B) and exponential (C,D). For a step-change perturbation, recovery length correctly correlates with recovery time **(A)**, however, it is unable to correctly correlate when the perturbation profile is exponential **(C)**. PASMORE correctly correlates to recovery time regardless of the perturbation profile **(B,D)**.
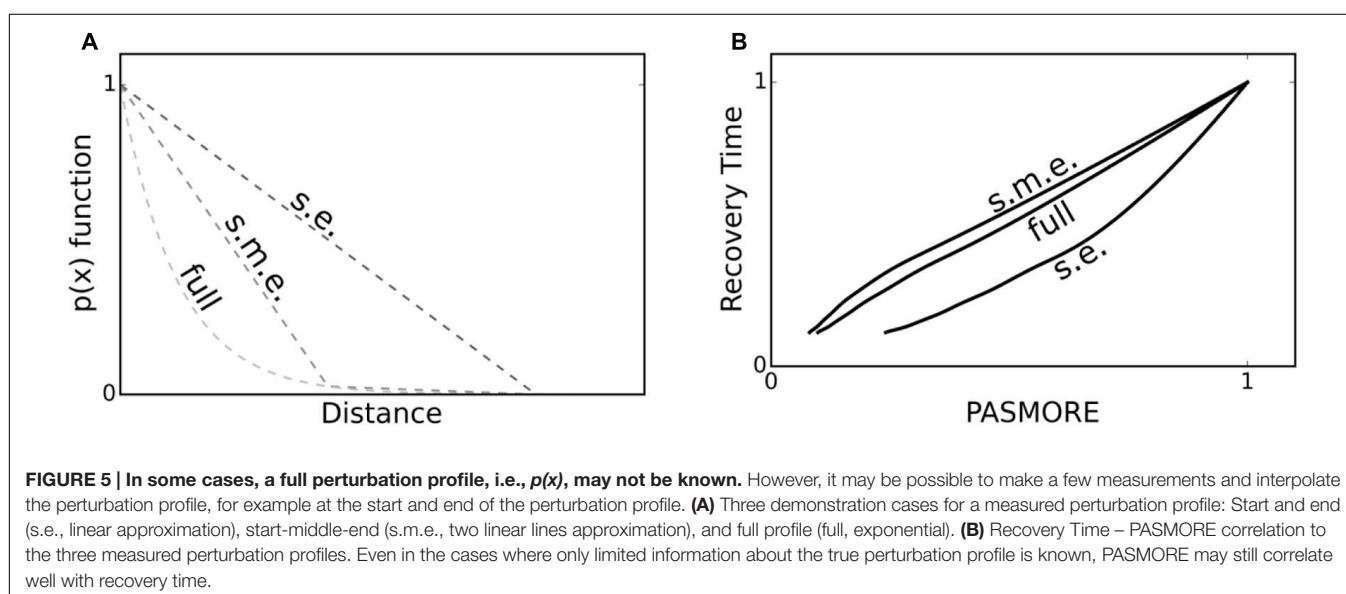
FIGURE 5 | In some cases, a full perturbation profile, i.e., _p(x)_, may not be known. However, it may be possible to make a few measurements and interpolate the perturbation profile, for example at the start and end of the perturbation profile. **(A)** Three demonstration cases for a measured perturbation profile: Start and end (s.e., linear approximation), start-middle-end (s.m.e., two linear lines approximation), and full profile (full, exponential). **(B)** Recovery Time – PASMORE correlation to the three measured perturbation profiles. Even in the cases where only limited information about the true perturbation profile is known, PASMORE may still correlate well with recovery time.
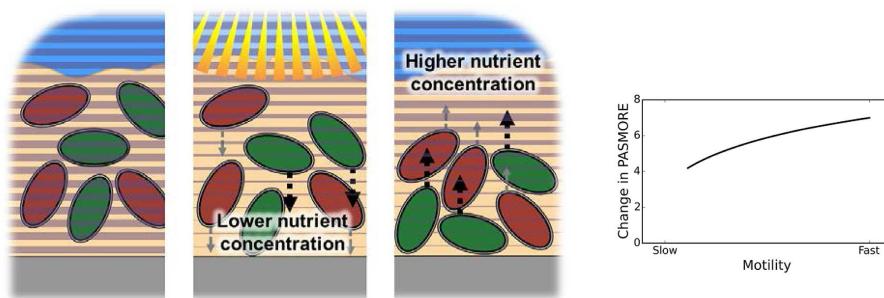
FIGURE 6 | Schematic of the model system, which has been modified to force competition between two species (one with higher resilience and one with lower resilience) by making motility dependent on consumption of a sole nutrient, which is supplied in the liquid above the microbial mat. During a perturbation, the nutrient concentration far from the perturbation becomes limited. The right plot shows the percent change of PASMORE when a slower species competes for the nutrient with a faster species, compared to a non-interacting case (i.e., when the species rely on differing nutrients). The competitive interaction led an increase in PASMORE, i.e., lower resilience, for both the slow and fast species due to the competition between species. The faster species exhibited a greater decrease in resilience compared to the slower species.

PASMORE IN THE BROADER CONTEXT OF SPATIAL RESILIENCE MEASURES

The capability to monitor spatial patterns of microbial function can help elucidate the resilience of a community in cases where temporal measurements may not have previously been practical. Spatial measures remove the need to monitor a given function over time, and thus a snapshot of the present community stability can be gauged. This type of analysis may have implications beyond the microbial world, as there are many forms of complex networks and communities that display properties of emergent phenomena with spatially-relevant functions. For example, the spatial effects of social dilemmas have been investigated for several decades (Nowak and May, 1992; Hauert, 2006) and the frequency of human cooperation and collaboration or selfishness and exploitation across spatial dimensions impacts the resilience of groups of peoples and their ability to maintain high productivity (Alvard, 2004; Jimenez et al., 2008). Furthermore, spatial patterns of human settlements and population densities may be related to community stability in the face of perturbation events such as loss of water, tillable soil, hunting ground, food, energy and other resources, as well as the related incidents of overpopulation and politico-military conflicts (Tir and Diehl, 1998; Vandam et al., 2013). In this context, densities of refugee settlements across countries and subsequent patterns of repopulation of cities after wars may reveal human community and ethno-regional group stability properties in the face of significant devastation. Other possible application areas for spatial measures of resilience could include aquifer-groundwater recharge rates (Guglielmi and Mudry, 1996; Katic and Grafton, 2011), soil and vegetation health (Seybold et al., 1999; van de Koppel and Rietkerk, 2004; Alongi, 2008; Jiang et al., 2012), permafrost vulnerability (Jorgenson et al., 2010), coral reef habitats (Nystrom and Folke, 2001; Cheal et al., 2013), and fishery management (Carpenter and Brock, 2004; Kerr et al., 2010). Indeed, understanding the resilience of biological and non-biological systems will be critical for determining the impact of climate change and the necessary policy changes to alleviate negative consequences (Bloetscher et al., 2010; Cumming, 2011). Future experiments and observations will need to be done to determine the extent to which spatial measures of resilience, such as PASMORE, may be used to provide insight into stability of complex systems beyond microbial communities. Furthermore, the ability of PASMORE to measure resilience in highly heterogeneous, discontinuous, or physically-restricted systems will need to be tested. It is unclear how barriers that constrain functional recovery, whether social, cultural, financial, political, or physical (e.g., soil and minerals at the microbial scale), will impact the capacity of PASMORE to measure resilience.

CONCLUSION

'Perturbation-adjusted spatial metric of resilience' (PASMORE) is capable of accurately quantifying spatial recovery and maintaining the correlation to temporal recovery regardless of the perturbation profile. In many environmental and ecological systems, the gradient shape of the perturbation may not be quantifiable and even the identity or type of perturbation may not be known. However, even with limited information, PASMORE can be calculated with approximation of the perturbation profile to reveal a close estimate of the system's resilience. We envision that establishing the concept of PASMORE as a practically useful metric of resilience requires further studies, including investigation of the limits of using spatial recovery in lieu of temporal recovery to quantify resilience in real natural and engineered microbial communities and relating these measurements to predictions of when systems are on the verge of collapse or nearing an irreversible transition across a tipping point. The present work provides initial foundations along this direction.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

REFERENCES

Allison, S. D., and Martiny, J. B. H. (2008). Resistance, resilience, and redundancy in microbial communities. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11512–11519. doi: 10.1073/pnas.0801925105

Alongi, D. M. (2008). Mangrove forests: resilience, protection from tsunamis, and responses to global climate change. *Estuar. Coast. Shelf Sci.* 76, 1–13. doi: 10.1016/j.ecss.2007.08.024

Alvard, M. (2004). Good hunters keep smaller shares of larger pies. *Behav. Brain Sci.* 27, 560–561. doi: 10.1017/S0140525X0422012X

Babauta, J. T., Atci, E., Ha, P. T., Lindemann, S. R., Ewing, T., Call, D. R., et al. (2014). Localized electron transfer rates and microelectrode-based enrichment of microbial communities within a phototrophic microbial mat. *Front. Microbiol.* 5:11. doi: 10.3389/fmicb.2014.00011

Behrens, S., Losekann, T., Pett-Ridge, J., Weber, P. K., Ng, W. O., Stevenson, B. S., et al. (2008). Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced element labeling-catalyzed reporter deposition fluorescence in situ hybridization (EL-FISH) and NanoSIMS. *Appl. Environ. Microbiol.* 74, 3143–3150. doi: 10.1128/AEM.00191-08

Bernstein, H. C., Beam, J. P., Kozubal, M. A., Carlson, R. P., and Inskeep, W. P. (2013). In situ analysis of oxygen consumption and diffusive transport in high-temperature acidic iron-oxide microbial mats. *Environ. Microbiol.* 15, 2360–2370. doi: 10.1111/1462-2920.12109

Bernstein, H. C., Kesaano, M., Moll, K., Smith, T., Gerlach, R., Carlson, R. P., et al. (2014). Direct measurement and characterization of active photosynthesis zones inside wastewater remediating and biofuel producing microalgal biofilms. *Bioresour. Technol.* 156, 206–215. doi: 10.1016/j.biortech.2014.01.001

Bhaya, D., Takahashi, A., and Grossman, A. R. (2001). Light regulation of type IV pilus-dependent motility by chemosensor-like elements in *Synechocystis* PCC6803. *Proc. Natl. Acad. Sci. U.S.A.* 98, 7540–7545. doi: 10.1073/pnas.131201098

Bloetscher, F., Meeroff, D. E., Heimlich, B. N., Brown, A. R., Bayler, D., and Loucraft, M. (2010). Improving resilience against the effects of climate change. *J. Am. Water Works Assoc.* 102, 36–46. doi: 10.1007/s00572-014-0595-2

Cao, B., Ahmed, B., Kennedy, D. W., Wang, Z., Shi, L., Marshall, M. J., et al. (2011). Contribution of extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms to U (VI) immobilization. *Environ. Sci. Technol.* 45, 5483–5490. doi: 10.1021/es200095j

Cao, B., Majors, P. D., Ahmed, B., Renslow, R. S., Silvia, C. P., Shi, L., et al. (2012). Biofilm shows spatially stratified metabolic responses to contaminant exposure. *Environ. Microbiol.* 14, 2901–2910. doi: 10.1111/j.1462-2920.2012.02850.x

Carey, M., and Carville, S. (2003). Scheduling and platforming trains at busy complex stations. *Transp. Res. Part A Policy Pract.* 37, 195–224. doi: 10.1016/S0965-8564(02)00012-5

Carpenter, S., Walker, B., Anderies, J. M., and Abel, N. (2001). From metaphor to measurement: resilience of what to what? *Ecosystems* 4, 765–781. doi: 10.1007/s10021-001-0045-9

Carpenter, S. R., and Brock, W. A. (2004). Spatial complexity, resilience, and policy diversity: fishing on lake-rich landscapes. *Ecol. Soc.* 9, 31.

Cheal, A. J., Emslie, M., Macneil, M. A., Miller, I., and Sweatman, H. (2013). Spatial variation in the functional characteristics of herbivorous fish communities and the resilience of coral reefs. *Ecol. Appl.* 23, 174–188. doi: 10.1890/11-2253.1

Cimellaro, G. P., Reinhorn, A. M., and Bruneau, M. (2010). Seismic resilience of a hospital system. *Struct. Infrastruct. Eng.* 6, 127–144. doi: 10.1080/15732470802663847

Cumming, G. S. (2011). Spatial resilience: integrating landscape ecology, resilience, and sustainability. *Landsc. Ecol.* 26, 899–909. doi: 10.1007/s10980-011-9623-1

Curtis, T. P., and Sloan, W. T. (2004). Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. *Curr. Opin. Microbiol.* 7, 221–226. doi: 10.1016/j.mib.2004.04.010

Czaczyk, K., and Myszka, K. (2007). Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Pol. J. Environ. Stud.* 16, 799–806.

Dai, L., Korolev, K. S., and Gore, J. (2013). Slower recovery in space before collapse of connected populations. *Nature* 496, 355–358. doi: 10.1038/nature12071

De La Fuente, I. M., Martínez, L., Pérez-Samartín, A. L., Ormaetxea, L., Amezaga, C., and Vera-López, A. (2008). Global self-organization of the cellular metabolic structure. *PLoS ONE* 3:e3100. doi: 10.1371/journal.pone.0003100

Easterling, W. E., and Kok, K. (2003). "Emergent properties of scale in global environmental modeling: are there any?," in *Scaling in Integrated Assessment*, eds J. Rotmans and D. S. Rothman (Lisse: Swets and Zeitlinger Group BV), 263–292.

Felix, M. A., and Wagner, A. (2008). Robustness and evolution: concepts, insights and challenges from a developmental model system. *Heredity* 100, 132–140. doi: 10.1038/sj.hdy.6800915

Flemming, H. C., and Wingender, J. (2001). Relevance of microbial extracellular polymeric substances (EPSs) – Part I: structural and ecological aspects. *Water Sci. Technol.* 43, 1–8.

Fuhrman, J. A. (2009). Microbial community structure and its functional implications. *Nature* 459, 193–199. doi: 10.1038/nature08058

Griffiths, B. S., and Philippot, L. (2013). Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiol. Rev.* 37, 112–129. doi: 10.1111/j.1574-6976.2012.00343.x

Guglielmi, Y., and Mudry, J. (1996). Estimation of spatial and temporal variability of recharge fluxes to an alluvial aquifer in a fore land area by water chemistry and isotopes. *Ground Water* 34, 1017–1023. doi: 10.1111/j.1745-6584.1996.tb02167.x

Häder, D.-P., and Lebert, M. (2001). *Photomovement*. Amsterdam: Elsevier Science.

Hauert, C. (2006). Spatial effects in social dilemmas. *J. Theor. Biol.* 240, 627–636. doi: 10.1016/j.jtbi.2005.10.024

Hawkes, C. V., and Keitt, T. H. (2015). Resilience vs. historical contingency in microbial responses to environmental change. *Ecol. Lett.* 18, 612–625. doi: 10.1111/ele.12451

Hibiya, K., Terada, A., Tsuneda, S., and Hirata, A. (2003). Simultaneous nitrification and denitrification by controlling vertical and horizontal microenvironment in a membrane-aerated biofilm reactor. *J. Biotechnol.* 100, 23–32. doi: 10.1016/S0168-1656(02)00227-4

Higgins, P. A. T., Mastrandrea, M. D., and Schneider, S. H. (2002). Dynamics of climate and ecosystem coupling: abrupt changes and multiple equilibria. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 357, 647–655. doi: 10.1098/rstb.2001.1043

Jiang, J., Gao, D. Z., and Deangelis, D. L. (2012). Towards a theory of ecotone resilience: coastal vegetation on a salinity gradient. *Theor. Popul. Biol.* 82, 29–37. doi: 10.1016/j.tpb.2012.02.007

Jimenez, R., Lugo, H., Cuesta, J. A., and Sanchez, A. (2008). Emergence and resilience of cooperation in the spatial prisoner's dilemma via a reward mechanism. *J. Theor. Biol.* 250, 475–483. doi: 10.1016/j.jtbi.2007.10.010

Jorgenson, M. T., Romanovsky, V., Harden, J., Shur, Y., O'donnell, J., Schuur, E. A. G., et al. (2010). Resilience and vulnerability of permafrost to climate change. *Can. J. For. Res.* 40, 1219–1236. doi: 10.1139/X10-060

Katic, P., and Grafton, R. Q. (2011). Optimal groundwater extraction under uncertainty: resilience versus economic payoffs. *J. Hydrol.* 406, 215–224. doi: 10.1016/j.jhydrol.2011.06.016

Kerr, L. A., Cadrin, S. X., and Secor, D. H. (2010). The role of spatial dynamics in the stability, resilience, and productivity of an estuarine fish population. *Ecol. Appl.* 20, 497–507. doi: 10.1890/08-1382.1

Konopka, A. (2009). What is microbial community ecology? *ISME J.* 3, 1223–1230. doi: 10.1038/ismej.2009.88

Laspidou, C. S., and Rittmann, B. E. (2002). A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. *Water Res.* 36, 2711–2720. doi: 10.1016/S0043-1354(01)00414-6

Lesne, A. (2008). Robustness: confronting lessons from physics and biology. *Biol. Rev.* 83, 509–532. doi: 10.1111/j.1469-185X.2008.00052.x

Li, T., Wu, T. D., Mazeas, L., Toffin, L., Guerquin-Kern, J. L., Leblon, G., et al. (2008). Simultaneous analysis of microbial identity and function using NanoSIMS. *Environ. Microbiol.* 10, 580–588. doi: 10.1111/j.1462-2920.2007.01478.x

Lindemann, S. R., Moran, J. J., Stegen, J. C., Renslow, R. S., Hutchison, J. R., Cole, J. K., et al. (2013). The epsomitic phototrophic microbial mat of Hot Lake, Washington: community structural responses to seasonal cycling. *Front. Microbiol.* 4:323. doi: 10.3389/fmicb.2013.00323

Lusseau, D. (2003). The emergent properties of a dolphin social network. *Proc. R. Soc. B Biol. Sci.* 270, S186–S188. doi: 10.1098/rsbl.2003.0057

Mitrovic, M., and Tadic, B. (2010). Bloggers behavior and emergent communities in Blog space. *Eur. Phys. J. B* 73, 293–301. doi: 10.1140/epjb/e2009-00431-9

Moran, J. J., Doll, C. G., Bernstein, H. C., Renslow, R. S., Cory, A. B., Hutchison, J. R., et al. (2014). Spatially tracking C-13-labelled substrate (bicarbonate) accumulation in microbial communities using laser ablation isotope ratio mass spectrometry. *Environ. Microbiol. Rep.* 6, 786–791. doi: 10.1111/1758-2229.12211

Nguyen, H. D., Renslow, R., Babauta, J., Ahmed, B., and Beyenal, H. (2012). A voltammetric flavin microelectrode for use in biofilms. *Sens. Actuators B Chem.* 161, 929–937. doi: 10.1016/j.snb.2011.11.066

Nowak, M. A., and May, R. M. (1992). Evolutionary games and spatial chaos. *Nature* 359, 826–829. doi: 10.1038/359826a0

Nystrom, M., and Folke, C. (2001). Spatial resilience of coral reefs. *Ecosystems* 4, 406–417. doi: 10.1007/s10021-001-0019-y

Pett-Ridge, J., and Weber, P. K. (2012). "NanoSIP: nanoSIMS applications for microbiology," in *Springer Protocols*, ed. A. Navid (New York, NY: Humana Press), 375–408.

Porter, M. A., Mucha, P. J., Newman, M. E. J., and Warmbrand, C. M. (2005). A network analysis of committees in the US House of Representatives. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7057–7062. doi: 10.1073/pnas.0500191102

Renslow, R., Babauta, J., Kuprat, A., Schenk, J., Ivory, C., Fredrickson, J., et al. (2013a). Modeling biofilms with dual extracellular electron transfer mechanisms. *Phys. Chem. Chem. Phys.* 15, 19262–19283. doi: 10.1039/c3cp53759e

Renslow, R., Lewandowski, Z., and Beyenal, H. (2011). Biofilm image reconstruction for assessing structural parameters. *Biotechnol. Bioeng.* 108, 1383–1394. doi: 10.1002/bit.23060

Renslow, R. S., Babauta, J. T., Dohnalkova, A. C., Boyanov, M. I., Kemner, K. M., Majors, P. D., et al. (2013b). Metabolic spatial variability in electrode-respiring Geobacter sulfurreducens biofilms. *Energy Environ. Sci.* 6, 1827–1836. doi: 10.1039/c3ee40203g

Renslow, R. S., Babauta, J. T., Majors, P. D., and Beyenal, H. (2013c). Diffusion in biofilms respiring on electrodes. *Energy Environ. Sci.* 6, 595–607. doi: 10.1039/C2EE23394K

Renslow, R. S., Majors, P. D., Mclean, J. S., Fredrickson, J. K., Ahmed, B., and Beyenal, H. (2010). In situ effective diffusion coefficient profiles in live biofilms using pulsed-field gradient nuclear magnetic resonance. *Biotechnol. Bioeng.* 106, 928–937. doi: 10.1002/bit.22755

Robinson, C. J., Bohannan, B. J. M., and Young, V. B. (2010). From structure to function: the ecology of host-associated microbial communities. *Microbiol. Mol. Biol. Rev.* 74, 453–476. doi: 10.1128/MMBR.00014-10

Scheffer, M., Bascompte, J., Brock, W. A., Brovkin, V., Carpenter, S. R., Dakos, V., et al. (2009). Early-warning signals for critical transitions. *Nature* 461, 53–59. doi: 10.1038/nature08227

Scheffer, M., Carpenter, S. R., Lenton, T. M., Bascompte, J., Brock, W., Dakos, V., et al. (2012). Anticipating critical transitions. *Science* 338, 344–348. doi: 10.1126/science.1225244

Schramm, A., Larsen, L. H., Revsbech, N. P., Ramsing, N. B., Amann, R., and Schleifer, K. H. (1996). Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.* 62, 4641–4647.

Schubert, W. (2014). Systematic, spatial imaging of large multimolecular assemblies and the emerging principles of supramolecular order in biological systems. *J. Mol. Recognit.* 27, 3–18. doi: 10.1002/jmr.2326

Seybold, C. A., Herrick, J. E., and Brejda, J. J. (1999). Soil resilience: a fundamental component of soil quality. *Soil Sci.* 164, 224–234. doi: 10.1097/00010694-199904000-00002

Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., Burgmann, H., et al. (2012). Fundamentals of microbial community resistance and resilience. *Front. Microbiol.* 3:417. doi: 10.3389/fmicb.2012.00417

Song, H.-S., Renslow, R. S., Fredrickson, J. K., and Lindemann, S. R. (2015). Integrating ecological and engineering concepts of resilience in microbial communities. *Front. Microbiol.* 6:1298. doi: 10.3389/fmicb.2015.01298

Tilman, D., Reich, P. B., Knops, J., Wedin, D., Mielke, T., and Lehman, C. (2001). Diversity and productivity in a long-term grassland experiment. *Science* 294, 843–845. doi: 10.1126/science.1060391

Tir, J., and Diehl, P. F. (1998). Demographic pressure and interstate conflict: linking population growth and density to militarized disputes and wars, 1930-89. *J. Peace Res.* 35, 319–339. doi: 10.1177/0022343398035003004

van de Koppel, J., and Rietkerk, M. (2004). Spatial interactions and resilience in arid ecosystems. *Am. Nat.* 163, 113–121. doi: 10.1086/380571

van Nes, E. H., and Scheffer, M. (2007). Slow recovery from perturbations as a generic indicator of a nearby catastrophic shift. *Am. Nat.* 169, 738–747. doi: 10.1086/516845

Vandam, R., Kaptijn, E., and Vanschoenwinkel, B. (2013). Disentangling the spatio-environmental drivers of human settlement: an eigenvector based variation decomposition. *PLoS ONE* 8:e67726. doi: 10.1371/journal.pone.0067726

Vanwonterghem, I., Jensen, P. D., Ho, D. P., Batstone, D. J., and Tyson, G. W. (2014). Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. *Curr. Opin. Biotechnol.* 27, 55–64. doi: 10.1016/j.copbio.2013.11.004

Veraart, A. J., Faassen, E. J., Dakos, V., Van Nes, E. H., Lurling, M., and Scheffer, M. (2012). Recovery rates reflect distance to a tipping point in a living system. *Nature* 481, 357–359. doi: 10.1038/nature10723

Wagner, M., Nielsen, P. H., Loy, A., Nielsen, J. L., and Daims, H. (2006). Linking microbial community structure with function: fluorescence in situ hybridization-microautoradiography and isotope arrays. *Curr. Opin. Biotechnol.* 17, 83–91. doi: 10.1016/j.copbio.2005.12.006

Wintermute, E. H., and Silver, P. A. (2010). Emergent cooperation in microbial metabolism. *Mol. Syst. Biol.* 6, 407. doi: 10.1038/msb.2010.66

Wissel, C. (1984). A universal law of the characteristic return time near thresholds. *Oecologia* 65, 101–107. doi: 10.1007/BF00384470

Xavier, J. B., and Foster, K. R. (2007). Cooperation and conflict in microbial biofilms. *Proc. Natl. Acad. Sci. U.S.A.* 104, 876–881. doi: 10.1073/pnas.0607651104

Integrating Ecological and Engineering Concepts of Resilience in Microbial Communities

Hyun-Seob Song[1], Ryan S. Renslow[2], Jim K. Fredrickson[1] and Stephen R. Lindemann[1]*

[1] Biological Sciences Division, Earth and Biological Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA, USA, [2] Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA, USA

Many definitions of resilience have been proffered for natural and engineered ecosystems, but a conceptual consensus on resilience in microbial communities is still lacking. We argue that the disconnect largely results from the wide variance in microbial community complexity, which range from compositionally simple synthetic consortia to complex natural communities, and divergence between the typical practical outcomes emphasized by ecologists and engineers. Viewing microbial communities as elasto-plastic systems that undergo both recoverable and unrecoverable transitions, we argue that this gap between the engineering and ecological definitions of resilience stems from their respective emphases on elastic and plastic deformation, respectively. We propose that the two concepts may be fundamentally united around the resilience of function rather than state in microbial communities and the regularity in the relationship between environmental variation and a community's functional response. Furthermore, we posit that functional resilience is an intrinsic property of microbial communities and suggest that state changes in response to environmental variation may be a key mechanism driving functional resilience in microbial communities.

Keywords: microbial communities, microbial ecology, resilience, resistance, robustness, stability, networks

INTRODUCTION

Microorganisms collectively exceed the biomass of all macrobiota on the planet (Whitman et al., 1998). Communities of microbes control the biogeochemical cycles upon which all macrobiota depend (Falkowski et al., 2008; Strom, 2008; Nazaries et al., 2013), and the role of microbial communities in shaping human health and physiology is also increasingly appreciated (Song et al., 2014a; Braundmeier et al., 2015; Lone et al., 2015; Sassone-Corsi and Raffatellu, 2015). Although natural microbial communities continually respond to perturbations (Konopka et al., 2014), their functioning can exhibit remarkable stability over time (Fuhrman et al., 2015), even under extreme environmental variation (Shade et al., 2012b; Lindemann et al., 2013). Comprehending the processes governing the responses of microbial communities to perturbation is critical both to ecologists concerned with predicting effects on ecosystem function (Hooper et al., 2005) and engineers designing communities for stable biotechnological processes (Lucas et al., 2015).

Though there is widespread interest in factors driving microbial community stability, the conceptual bases of stability measures, like resilience, are poorly defined. A report by the Community and Regional Resilience Institute (CARRI Report, 2013) summarized 47 definitions of resilience used in diverse scientific areas including engineering, ecology, sociology, economics,

and psychology. Ambiguity is found even within disciplines: in ecology, resilience has been discussed alongside, and sometimes interchangeably with, ∼70 other terms describing various stability measures (e.g., resistance, sustainability, and vulnerability; Grimm and Wissel, 1997). The conceptual variability in ecology surrounding stability, and resilience in particular, likely stems from system-specificity. Microbial communities span orders of magnitude in the diversity of their interacting components, from experimental or engineered systems to diverse natural communities. The metrics employed to evaluate each system's stability are largely idiosyncratic. The diversity of environments in which communities are investigated, the large array of functions of interest, and the range of research objectives concerning stability beg the question of whether a single definition of resilience can be universally applied across systems and scales for microbial communities.

Seeking an integrated concept applicable to all microbial communities, we herein compare engineering and ecological resilience and reconcile them by arguing that resilience is an intrinsic property of complex adaptive systems which, after perturbation, recover their system-level functions and interactions with the environment, rather than their endogenous state.

ENGINEERING AND ECOLOGICAL CONCEPTS OF RESILIENCE
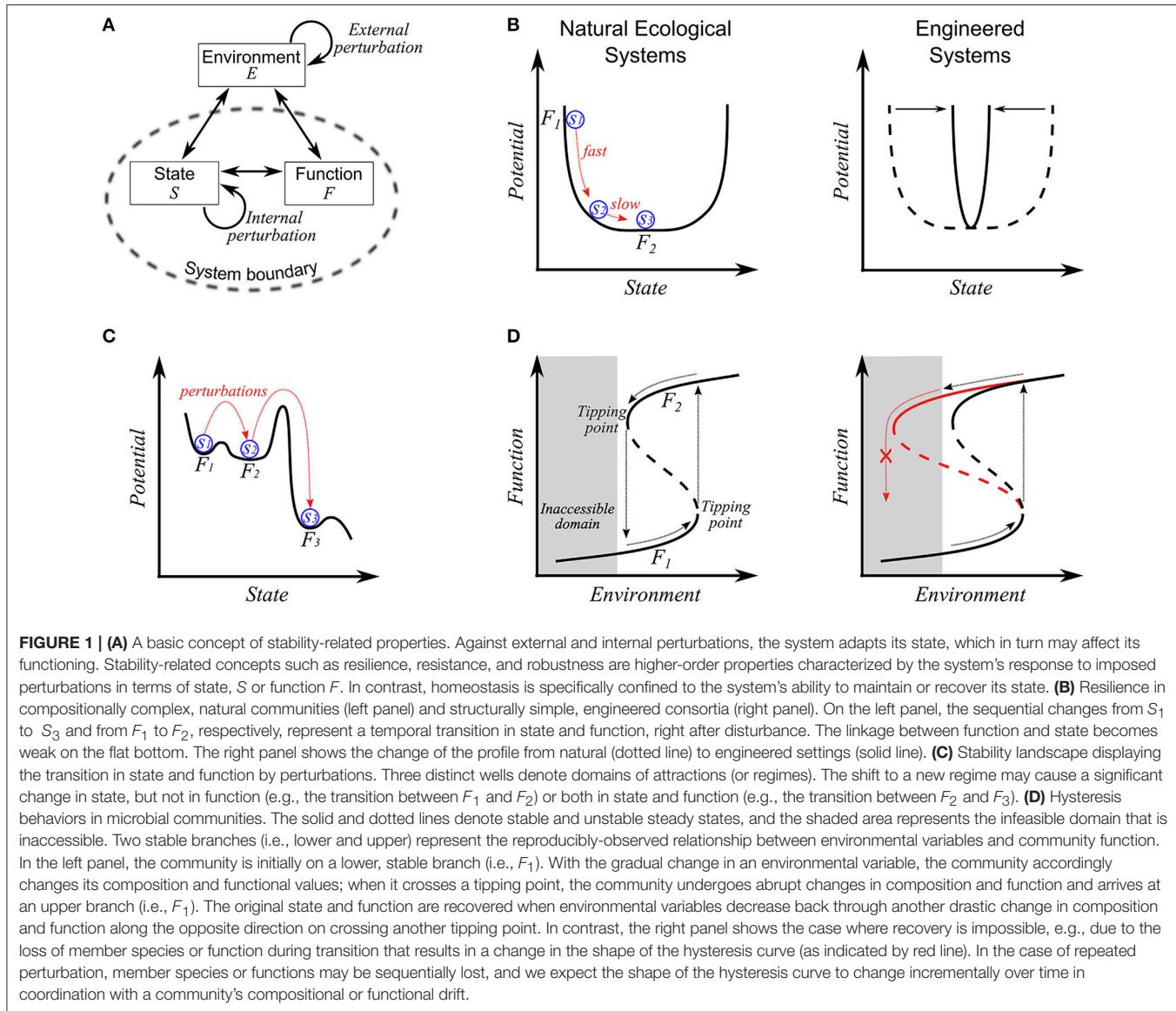
Discussion of resilience in the literature often involves the related concepts of resistance and robustness. These stability-related properties are all concerned with the relationship between an imposed perturbation and a system's response (**Figure 1A**). Resilience has been broadly articulated as a system's *ability to recover* from disturbance. Diverse interpretations emerge, however, depending on what is considered "recovery" and how that recovery is quantified. In contrast, resistance has been defined with relatively less confusion, e.g., as the degree to which a system's state or function is insensitive to disturbance (Konopka et al., 2014). As a simple distinction, resilience is concerned with the system's ability to *recover* its function post-disturbance, while resistance is concerned with the system's ability to *maintain* its function against a perturbation. In these contexts, resilience (or resistance) denotes the degree to which the *quantitative* value of any function of interest is recovered to (or maintained at) an initial or reference condition. As illustrated elsewhere (Carpenter et al., 2001), systems may display significant resilience but not appreciable resistance and vice versa. In some cases, resilience has also been used as a synonym of robustness, described as the system's ability to maintain function post-disturbance (Levin and Lubchenco, 2008). Herein we consider robustness as a more general concept of stability that is comprised of resilience, resistance, and other complementary properties (Shade et al., 2012b), i.e., resilience and resistance are key components of a system's overall robustness.

The literature provides rich discussions of resilience, which can be subdivided into two categories: engineering and ecological concepts. As discriminated by Holling in his seminal paper (Holling, 1973), engineering resilience denotes the system's ability to recover its pre-perturbed equilibrium state as measured by the *rate* of return. The ecological concept considers a system's tolerance against disturbance without shifting to a new regime governed by fundamentally different processes and mechanisms (i.e., a domain of attraction), as quantified by the overall area of the domain of attraction or the depth of the basin. A domain of attraction represents a set of states converging to a given equilibrium point (such as S3 in **Figure 1B**, left panel). Both concepts of resilience have been invoked in microbial ecology. Faithful translation of the etymon of resilience, the Latin word *resalire* (literally, "to jump back") (CARRI Report, 2013), interprets resilience as a property of elastic systems (as in physics/material sciences) that recover their original shape after disturbance. In this regard, Grimm and Calabrese (2011) equated elasticity with engineering resilience. Indeed, microbial communities share some features with elastic systems in that they sometimes undergo internal or structural deformation (e.g., changes of composition or gene expression patterns) under disturbance, yet eventually recover their original performance (e.g., recovery of microbe-driven biogeochemical processes after a forest fire; Tas et al., 2014). However, microbial communities lose elasticity (i.e., fail to recover) if the applied environmental stress exceeds a threshold, a phenomenon termed plastic deformation. The threshold across which plastic deformation occurs is variously called a tipping, yield, or bifurcation point (Veraart et al., 2012). Plastic deformation is better captured by the ecological concept of resilience. Obviously, microbial communities are neither perfectly elastic nor plastic, but are elasto-plastic systems. Thus, engineering and ecological concepts that reflect these aspects of microbial communities are complementary.

Variation in complexity between engineered and natural communities provides rationale for both the engineering and ecological concepts. Microbial communities in non-extreme natural settings typically have high compositional diversity and functional redundancy among species. As illustrated in **Figure 1B** (left panel), the community composition may vary over a certain range without affecting the function. The flat bottom of the profile implies *slow* dynamics in compositional change around a low-energy state, even in a rarely-perturbed or constant environment. Konopka et al. (2014) hypothesized that endogenous dynamics contribute to a community-level functional resilience. Relating resilience to the system's ability to persist within a domain of attraction after disturbance (i.e., ecological resilience), rather than the rate of return to the initial state, makes more sense in this circumstance. In contrast, microbial consortia with relatively lower diversity and functional redundancy should display a sharper curve (right panel of **Figure 1B**), meaning that (1) consortium-level function is maintained only at a specific compositional state and, (2) that the recovery to the original state after disturbance is relatively fast. In this second case, the rate of recovery may be a better measure of resilience (i.e., the engineering concept).

The distinction between engineering and ecological concepts of resilience fundamentally lies in their different foci: equilibrium

FIGURE 1 | (A) A basic concept of stability-related properties. Against external and internal perturbations, the system adapts its state, which in turn may affect its functioning. Stability-related concepts such as resilience, resistance, and robustness are higher-order properties characterized by the system's response to imposed perturbations in terms of state, S or function F. In contrast, homeostasis is specifically confined to the system's ability to maintain or recover its state. **(B)** Resilience in compositionally complex, natural communities (left panel) and structurally simple, engineered consortia (right panel). On the left panel, the sequential changes from S_1 to S_3 and from F_1 to F_2, respectively, represent a temporal transition in state and function, right after disturbance. The linkage between function and state becomes weak on the flat bottom. The right panel shows the change of the profile from natural (dotted line) to engineered settings (solid line). **(C)** Stability landscape displaying the transition in state and function by perturbations. Three distinct wells denote domains of attractions (or regimes). The shift to a new regime may cause a significant change in state, but not in function (e.g., the transition between F_1 and F_2) or both in state and function (e.g., the transition between F_2 and F_3). **(D)** Hysteresis behaviors in microbial communities. The solid and dotted lines denote stable and unstable steady states, and the shaded area represents the infeasible domain that is inaccessible. Two stable branches (i.e., lower and upper) represent the reproducibly-observed relationship between environmental variables and community function. In the left panel, the community is initially on a lower, stable branch (i.e., F_1). With the gradual change in an environmental variable, the community accordingly changes its composition and functional values; when it crosses a tipping point, the community undergoes abrupt changes in composition and function and arrives at an upper branch (i.e., F_1). The original state and function are recovered when environmental variables decrease back through another drastic change in composition and function along the opposite direction on crossing another tipping point. In contrast, the right panel shows the case where recovery is impossible, e.g., due to the loss of member species or function during transition that results in a change in the shape of the hysteresis curve (as indicated by red line). In the case of repeated perturbation, member species or functions may be sequentially lost, and we expect the shape of the hysteresis curve to change incrementally over time in coordination with a community's compositional or functional drift.

vs. domain of attraction; numerical values of state variables vs. relationship between structure and function; rate of recovery after perturbation vs. ability to absorb the effect of disturbance (Grimm and Calabrese, 2011). In addition, we propose that the engineering and ecological concepts represent optimization for different objectives. Microbial consortia used for bioprocessing are, in essence, a set of biocatalysts that convert substrates into products. In well-designed bioreactors, where environmental conditions are tightly controlled, collapse across a tipping point may not be the major concern. Instead, rapid recovery after minor disturbances (i.e., engineering resilience) is critical to ensure consistent product quality and profit maximization. In contrast, with respect to natural communities with complex structure and dynamics, it would be of greater importance to proactively identify threats to ecosystem functioning by predicting how much additional stress a system can absorb

without failure, a main concern in ecological resilience. In this regard, we add "maximizing system performance" vs. "preserving desirable system function" or "optimal control" vs. "monitoring and predicting" to the list of differences between engineering and ecological concepts.

The distinction in categorization of communities as "engineered" or "natural" becomes blurred in some cases. Wastewater treatment facilities (Adrados et al., 2014; Bernstein et al., 2014) and algal ponds (Park et al., 2011), for example, are engineered systems designed for a specific goal (i.e., water purification, biofuel production, or both) but are subject to environmental variations and influx of invasive species. Therefore, communities in natural and engineered environments, albeit structurally different, could be regarded as similar systems (subject to regular or episodic perturbations) to which an integrated concept of resilience could be applied.

Toward this end, the theoretical underpinnings of the engineering and ecological concepts need to be understood more fundamentally.

RESILIENCE OF COMPOSITION, FUNCTION, AND THE COMMUNITY-ENVIRONMENT RELATIONSHIP

In principle, though the resilience of any system variable could be evaluated, a more fundamental question is at what level resilience is an intrinsic property of microbial communities (i.e., endogenous state or system function) and how state changes feed back to function. This issue has been extensively addressed by Kitano in regards to the robustness of biological systems (Kitano, 2004, 2007). He made a clear distinction between homeostasis and robustness, highlighting functional robustness as a property ubiquitously observed in biological systems, which often change in internal structure and mode of operation to preserve specific system functions against perturbation. We posit that Kitano's assertion, formulated to describe the behavior of single organisms, can be extended to describe the behavior of microbial communities. Thus, the translation of Röling et al. (2007) can be extended from enzymes in the cell to microbial species in a community.

In contrast to single organisms, microbial communities rarely have obvious *physical* boundaries that circumscribe the system, although locality of interactions is a hallmark of such communities (*sensu* Konopka) (Konopka, 2009). We could, however, assume a *hypothetical* boundary encompassing all interacting species and treat the community as a supra-organism, though, in practice, community boundaries are operationally defined. The individual components that constitute such a supra-organism can then, *in toto*, be considered to be the system's state variables, which include relative abundances of individual species, energy and material exchange across species (i.e., interspecies metabolic interactions), organismal gene expression patterns, and so forth. Community-level functions are then aggregate properties, such as the total growth rate and net uptake or production rates of metabolites. In practical terms, when discussing the resilience of microbial communities, it is the observer's task to unambiguously define both the system boundary and the function of interest measured in the community within that boundary. Theoretically, any variable or component of the community may be defined as the function of interest, and resilience of that function is necessarily relative to a specific perturbation. This is the "What to What?" approach detailed by Carpenter et al. (2001), which alleviates confusion of what quantified resilience signifies by supplying details regarding the system, the observed function, the cause and scale of perturbation, and the dimension of recovery.

Following Kitano, we argue that resilience is an intrinsic property of microbial communities that recover system-level functions after perturbation, instead of recovering a given endogenous state. Two related sub-hypotheses (SH) can be distinguished. SH1: the probability of a given community function being resilient (that is, capable of regaining its pre-perturbation value) is *hierarchical* (i.e., $p_{comm} > p_{spec} > p_{subcell}$, where p_{comm}, p_{spec}, and $p_{subcell}$ denote those probabilities of community-level, species-level, and subcellular variables, respectively), and SH2: the probability gap for functional resilience at different levels of organization is unequal (i.e., $p_{comm} - p_{spec} < p_{spec} - p_{subcell}$). Together, these hypotheses imply that: (1) the intracellular state of an organism may change without affecting organism-level functions; (2) the function of organisms may change without impacting community-level functions; and (3) changes in community-level function happen at a relatively lower frequency than in organism-level function, which occur at lower frequency than intracellular state changes. While cases where this does not hold exist (e.g., Faith et al., 2011), examples for SH1 are commonly observed, e.g., changes in gene expression patterns are more susceptible to environmental variation than microbial growth rates and metabolic flux distributions (Ishii et al., 2007; Tang et al., 2009; Song et al., 2014b, 2015). Likewise, the resilience and functional stability in communities often arise as a result of significant compositional changes (Konopka et al., 2014). SH2 examples include cases where resilience of a community's composition and its function are closely linked, i.e., the gap between p_{comm} and p_{spec} is small. In a number of cases reported in the literature, the compositional resilience in microbial communities is shown to be comparable to functional resilience (Allison and Martiny, 2008; Shade et al., 2012a). Indeed, overall community function would be affected by compositional change even in cases where the role of one species can be replaced by other functionally-redundant members, unless the loss is not *quantitatively* compensated. It should be noted, however, that composition-function relationships depend on many factors including the specific function of interest, how many taxa perform the chosen function, and in what regime experimental data were collected. Thus, although we uphold the position that microbial communities are more likely to recover function, rather than composition, we regard compositional change as a potential governing mechanism of the whole system's resilience. To illustrate how community-level function can be maintained by changing internal state, we constructed a tutorial network representing a microbial community and simulated its responses to changes in environmental variables (Supplementary Material).

With a focus on the community-level performance, we restate the engineering concept of resilience as the rate of the system's recovery of its pre-perturbation *function*. This function-centered concept is linked to the ecological concept because the latter tolerates state changes that do not impact the overall function of interest. While exceptions exist, the tolerable magnitude of disturbance (the ecological concept) and the system dynamics (the engineering concept) are correlated around tipping points, i.e., systems nearing tipping points display peculiar behaviors such as slow recovery and magnified variations of state and functions (Dai et al., 2013). Thus, slow recovery of the ecosystem from disturbance may be an indicator of approaching a tipping point (Van Nes and Scheffer, 2007; Dai et al., 2012; Griffiths and Philippot, 2013; Dakos and Bascompte, 2014). The function-based view also expands the concept of a domain of attraction

in ecological resilience. The stability landscape in **Figure 1C**, for example, shows three distinct domains of attraction. Suppose that the community performance at each regime is measured as F_1, F_2, and F_3 ($F_1 \approx F_2 > F_3$), and its initial state is S_1. In this case, we can consider the community to display resilience if the system returns (after perturbation) either to Regime 1 or to Regime 2. In this regard, Regimes 1 and 2 may be taken together as essentially the same functional domain.

Assessing resilience is challenging when microbial communities are subject to slow, chronic perturbation (e.g., climate change), or periodic environmental disturbances (e.g., temperature change during diel or seasonal cycles). Under such circumstances where the system's state, functions, and stability landscape are continually changing, it becomes unclear how to define a "pre-perturbed" condition for engineering resilience and a domain of attraction for ecological resilience. With respect to environmental variations that are much slower than the system's intrinsic dynamics, microbial communities may have sufficient time to adapt their endogenous state to the environment. We argue that this is the reason why some communities develop a stable relationship with their environment. Furthermore, the regularity in the relationship between environmental variation and the community's functional response is a fundamental aspect of resilience in that it applies to both fluctuating and constant environments. That is, microbial communities can be said to exhibit resilience as long as their functional interactions with the environment are reproducibly observed (Fuhrman et al., 2015). This idea of *relational resilience* (i.e., resilience of the relationship between environmental conditions and community function) provides fresh insight into system behavior at and around tipping points. On the left panel of **Figure 1D**, two stable branches of the hysteresis curve denote two unique relationships between the community function and environment. A tipping point is then defined as the condition across which a shift in the community-environment interaction occurs. Such non-linear hysteresis behavior has been observed in laboratory (Kim et al., 2012; Song and Ramkrishna, 2013) and ecological systems, e.g., coral reef-dominated vs. macroalgae-dominated state (Hughes et al., 2010); tropical forest vs. savanna vs. treeless state (Hirota et al., 2011). Early detection of nearby tipping points in ecosystems is therefore of practical importance before transition to an undesirable state and potentially permanent loss of critical functions. It may happen that key member species are lost during steady or abrupt change in environmental conditions. The resulting reduced diversity may lead to the change of stability landscape and subsequently the shape of the hysteresis curve so that catastrophic transition is very slow to recover or even *irreversible* (the right panel of **Figure 1D**). In this case, directly restoring lost members could be required to recover the systems' original functionalities. For example, pseudomembranous colitis is a condition in which *Clostridium difficile* dominates the gut microbiome after antibiotic treatment suppresses the normal, commensal microbiota. The measurable diversity of the *C. difficile*-dominated gut community is reduced compared with healthy controls (Song et al., 2013; Schubert et al., 2014), and large populations of *C. difficile* are difficult for normal commensal organisms to displace as these organisms enter the gut. Restoring members lost from the community through fecal transplantation has been effective in rapidly restoring the dysbiotic gut community to a more normal microbiome (Weingarden et al., 2015). Although restoring lost diversity imparts resilience to the community, similar approaches may be difficult for large-scale ecosystems, highlighting the importance of detecting diversity loss through continual monitoring and predicting the effects of environmental changes on community- and ecosystem-level responses.

CONCLUSIONS AND FUTURE RESEARCH NEEDS

Moving toward an integrated framework for understanding microbial community resilience, we propose reconciling concepts of engineering and ecological resilience through (1) consideration of microbial communities as systems that undergo both elastic and plastic deformation, and (2) defining resilience as the rate of recovery of a function of interest. Refocusing on the system's fundamental characteristics (such as the community-level functions and community-environment relationships) not only minimizes conceptual variation across different resilience definitions, but also provides a deeper understanding of the intrinsic community properties. In parallel, from a practical point of view, it is also of great importance to develop rational methods for quantifying microbial community resilience and predicting approaching tipping points.

Future research will need to address several important, unresolved issues—primarily, the identification of fundamental mechanisms responsible for microbial community resilience. For example, redundancy, diversity, and modularity are frequently advanced as mechanisms for robustness in complex systems (Kitano, 2004), but in some cases, and particularly in structurally simple consortia, they may not be directly related to resilience. The question remains: what unifying mechanisms impart resilience across both structurally simple and complex microbial communities? While the concept of networked buffering offers a potential mechanism (Whitacre and Bender, 2010; Konopka et al., 2014), rigorous analysis of microbial communities has yet to be performed. Another issue is the possible occurrence of trade-offs between system robustness (or resilience) and performance (Kitano, 2007) or trade-offs between robustness with respect to distinct perturbations (e.g., the conservation principle as discussed by Doyle and colleagues; Carlson and Doyle, 1999; Csete and Doyle, 2002). One major question is to what degree do resilience mechanisms identified for microbial communities overcome such trade-offs? Finally, the principles for structural organization of microbial communities as robust networks need to be further examined, as little is known about the general topological characteristics of microbial association networks and their relationships to resilience. Critical questions include: how does the compartmentalization of genes into a network of species affect the structural and higher-order properties of microbial communities; and are microbial community properties better understood as networks of species or networks of genes? Future research focusing on

these issues will significantly advance our capability for the design, prediction, and control of microbial communities and maintenance of the critical ecosystem services they provide.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01298

REFERENCES

Adrados, B., Sánchez, O., Arias, C. A., Becares, E., Garrido, L., Mas, J., et al. (2014). Microbial communities from different types of natural wastewater treatment systems: vertical and horizontal flow constructed wetlands and biofilters. *Water Res.* 55, 304–312. doi: 10.1016/j.watres.2014.02.011

Allison, S. D., and Martiny, J. B. H. (2008). Resistance, resilience, and redundancy in microbial communities. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11512–11519. doi: 10.1073/pnas.0801925105

Bernstein, H. C., Kesaano, M., Moll, K., Smith, T., Gerlach, R., Carlson, R. P., et al. (2014). Direct measurement and characterization of active photosynthesis zones inside wastewater remediating and biofuel producing microalgal biofilms. *Bioresour. Technol.* 156, 206–215. doi: 10.1016/j.biortech.2014.01.001

Braundmeier, A. G., Lenz, K. M., Inman, K. S., Chia, N., Jeraldo, P., Walther-António, M. R., et al. (2015). Individualized medicine and the microbiome in reproductive tract. *Front. Physiol.* 6:97. doi: 10.3389/fphys.2015.00097

Carlson, J. M., and Doyle, J. (1999). Highly optimized tolerance: a mechanism for power laws in designed systems. *Phys. Rev. E* 60, 1412–1427. doi: 10.1103/PhysRevE.60.1412

Carpenter, S., Walker, B., Anderies, J. M., and Abel, N. (2001). From metaphor to measurement: resilience of what to what? *Ecosystems* 4, 765–781. doi: 10.1007/s10021-001-0045-9

CARRI Report (2013). *Definitions of Community Resilience: An Analysis.* Available online at: http://www.resilientus.org/wp-content/uploads/2013/08/definitions-of-community-resilience.pdf

Csete, M. E., and Doyle, J. C. (2002). Reverse engineering of biological complexity. *Science* 295, 1664–1669. doi: 10.1126/science.1069981

Dai, L., Korolev, K. S., and Gore, J. (2013). Slower recovery in space before collapse of connected populations. *Nature* 496, 355–358. doi: 10.1038/nature12071

Dai, L., Vorselen, D., Korolev, K. S., and Gore, J. (2012). Generic indicators for loss of resilience before a tipping point leading to population collapse. *Science* 336, 1175–1177. doi: 10.1126/science.1219805

Dakos, V., and Bascompte, J. (2014). Critical slowing down as early warning for the onset of collapse in mutualistic communities. *Proc. Natl. Acad. Sci. U.S.A.* 111, 17546–17551. doi: 10.1073/pnas.1406326111

Faith, J. J., McNulty, N. P., Rey, F. E., and Gordon, J. I. (2011). Predicting a human gut microbiota's response to diet in gnotobiotic mice. *Science* 333, 101–104. doi: 10.1126/science.1206025

Falkowski, P. G., Fenchel, T., and Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* 320, 1034–1039. doi: 10.1126/science.1153213

Fuhrman, J. A., Cram, J. A., and Needham, D. M. (2015). Marine microbial community dynamics and their ecological interpretation. *Nat. Rev. Microbiol.* 13, 133–146. doi: 10.1038/nrmicro3417

Griffiths, B. S., and Philippot, L. (2013). Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiol. Rev.* 37, 112–129. doi: 10.1111/j.1574-6976.2012.00343.x

Grimm, V., and Calabrese, J. M. (2011). "What is resilience? A short introduction," in *Viability and Resilience of Complex Systems: Concepts, Methods, and Case Studies from Ecology and Society*, eds G. Deffuant and N. Gilbert (New York, NY: Springer), 3–13.

Grimm, V., and Wissel, C. (1997). Babel, or the ecological stability discussions: an inventory and analysis of terminology and a guide for avoiding confusion. *Oecologia* 109, 323–334. doi: 10.1007/s004420050090

Hirota, M., Holmgren, M., Van Nes, E. H., and Scheffer, M. (2011). Global resilience of tropical forest and savanna to critical transitions. *Science* 334, 232–235. doi: 10.1126/science.1210657

Holling, C. S. (1973). Resilience and stability of ecological systems. *Annu. Rev. Ecol. Syst.* 4, 1–23. doi: 10.1146/annurev.es.04.110173.000245

Hooper, D. U., Chapin, F. S., Ewel, J. J., Hector, A., Inchausti, P., Lavorel, S., et al. (2005). Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecol. Monogr.* 75, 3–35. doi: 10.1890/04-0922

Hughes, T. P., Graham, N. A. J., Jackson, J. B.C., Mumby, P. J., and Steneck, R. S. (2010). Rising to the challenge of sustaining coral reef resilience. *Trends Ecol. Evol.* 25, 633–642. doi: 10.1016/j.tree.2010.07.011

Ishii, N., Nakahigashi, K., Baba, T., Robert, M., Soga, T., Kanai, A., et al. (2007). Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. *Science* 316, 593–597. doi: 10.1126/science.1132067

Kim, J. I., Song, H. S., Sunkara, S. R., Lali, A., and Ramkrishna, D. (2012). Exacting predictions by cybernetic model confirmed experimentally: steady state multiplicity in the chemostat. *Biotechnol. Prog.* 28, 1160–1166. doi: 10.1002/btpr.1583

Kitano, H. (2004). Biological robustness. *Nat. Rev. Genet.* 5, 826–837. doi: 10.1038/nrg1471

Kitano, H. (2007). Towards a theory of biological robustness. *Mol. Syst. Biol.* 3, 137. doi: 10.1007/978-3-540-31339-7_4

Konopka, A. (2009). What is microbial community ecology? *ISME J.* 3, 1223–1230. doi: 10.1038/ismej.2009.88

Konopka, A., Lindemann, S., and Fredrickson, J. (2014). Dynamics in microbial communities: unraveling mechanisms to identify principles. *ISME J.* 9, 1488–1495. doi: 10.1038/ismej.2014.251

Levin, S. A., and Lubchenco, J. (2008). Resilience, robustness, and marine ecosystem-based management. *Bioscience* 58, 27–32. doi: 10.1641/B580107

Lindemann, S. R., Moran, J. J., Stegen, J. C., Renslow, R. S., Hutchison, J. R., Cole, J. K., et al. (2013). The epsomitic phototrophic microbial mat of Hot Lake, Washington: community structural responses to seasonal cycling. *Front. Microbiol.* 4:323. doi: 10.3389/fmicb.2013.00323

Lone, A. G., Atci, E., Renslow, R., Beyenal, H., Noh, S., Fransson, B., et al. (2015). Staphylococcus aureus induces hypoxia and cellular damage in porcine dermal explants. *Infect. Immun.* 83, 2531–2541. doi: 10.1128/IAI.03075-14

Lucas, R., Kuchenbuch, A., Fetzer, I., Harms, H., and Kleinsteuber, S. (2015). Long-term monitoring reveals stable and remarkably similar microbial communities in parallel full-scale biogas reactors digesting energy crops. *FEMS Microbiol. Ecol.* 91:fiv004. doi: 10.1093/femsec/fiv004

Nazaries, L., Pan, Y., Bodrossy, L., Baggs, E. M., Millard, P., Murrell, J. C., et al. (2013). Evidence of microbial regulation of biogeochemical cycles from a study on methane flux and land use change. *Appl. Environ. Microbiol.* 79, 4031–4040. doi: 10.1128/AEM.00095-13

Park, J. B. K., Craggs, R. J., and Shilton, A. N. (2011). Wastewater treatment high rate algal ponds for biofuel production. *Bioresour. Technol.* 102, 35–42. doi: 10.1016/j.biortech.2010.06.158

Röling, W. F. M., Van Breukelen, B. M., Bruggeman, F. J., and Westerhoff, H. V. (2007). Ecological control analysis: being(s) in control of mass flux and metabolite concentrations in anaerobic degradation processes. *Environ. Microbiol.* 9, 500–511. doi: 10.1111/j.1462-2920.2006.01167.x

Sassone-Corsi, M., and Raffatellu, M. (2015). No vacancy: how beneficial microbes cooperate with immunity to provide colonization resistance to pathogens. *J. Immunol.* 194, 4081–4087. doi: 10.4049/jimmunol.1403169

Schubert, A. M., Rogers, M. A. M., Ring, C., Mogle, J., Petrosino, J. P., and Schloss, P.D. (2014). Microbiome data distinguish patients with *Clostridium difficile* infection and non-*C. difficile*-associated diarrhea from healthy controls. *MBio* 5:e01021. doi: 10.1128/mBio.01021-14

Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., Bürgmann, H., et al. (2012a). Fundamentals of microbial community resistance and resilience. *Front. Microbiol.* 3:417. doi: 10.3389/fmicb.2012.00417

Shade, A., Read, J. S., Youngblut, N. D., Fierer, N., Knight, R., Kratz, T. K., et al. (2012b). Lake microbial communities are resilient after a whole-ecosystem disturbance. *ISME J.* 6, 2153–2167. doi: 10.1038/ismej.2012.56

Song, H.-S., Cannon, W. R., Beliaev, A. S., and Konopka, A. (2014a). Mathematical modeling of microbial community dynamics: a methodological review. *Processes* 2, 711–752. doi: 10.3390/pr2040711

Song, H.-S., McClure, R. S., Bernstein, H. C., Overall, C. C., Hill, E. A., and Beliaev, A. S. (2015). Integrated *in silico* analyses of regulatory and metabolic networks of *Synechococcus* sp. PCC 7002 reveal relationships between gene centrality and essentiality. *Life* 5, 1127–1140. doi: 10.3390/life5021127

Song, H.-S., and Ramkrishna, D. (2013). Complex nonlinear behavior in metabolic processes: global bifurcation analysis of *Escherichia coli* growth on multiple substrates. *Processes* 1, 263–278. doi: 10.3390/pr1030263

Song, H.-S., Reifman, J., and Wallqvist, A. (2014b). Prediction of metabolic flux distribution from gene expression data based on the flux minimization principle. *PLoS ONE* 9:e112524. doi: 10.1371/journal.pone.0112524

Song, Y., Garg, S., Girotra, M., Maddox, C., Von Rosenvinge, E. C., Dutta, A., et al. (2013). Microbiota dynamics in patients treated with fecal microbiota transplantation for recurrent *Clostridium difficile* infection. *PLoS ONE* 8:e81330. doi: 10.1371/journal.pone.0081330

Strom, S. L. (2008). Microbial ecology of ocean biogeochemistry: a community perspective. *Science* 320, 1043–1045. doi: 10.1126/science.1153527

Tang, Y. J., Martin, H. G., Deutschbauer, A., Feng, X. Y., Huang, R., Llora, X., et al. (2009). Invariability of central metabolic flux distribution in *Shewanella*

oneidensis MR-1 under environmental or genetic perturbations. *Biotechnol. Prog.* 25, 1254–1259. doi: 10.1002/btpr.227

Tas, N., Prestat, E., McFarland, J. W., Wickland, K. P., Knight, R., Berhe, A. A., et al. (2014). Impact of fire on active layer and permafrost microbial communities and metagenomes in an upland Alaskan boreal forest. *ISME J.* 8, 1904–1919. doi: 10.1038/ismej.2014.36

Van Nes, E. H., and Scheffer, M. (2007). Slow recovery from perturbations as a generic indicator of a nearby catastrophic shift. *Am. Nat.* 169, 738–747. doi: 10.1086/516845

Veraart, A. J., Faassen, E. J., Dakos, V., van Nes, E. H., Lürling, M., and Scheffer, M. (2012). Recovery rates reflect distance to a tipping point in a living system. *Nature* 481, 357–359. doi: 10.1038/nature11029

Weingarden, A., González, A., Vázquez-Baeza, Y., Weiss, S., Humphry, G., Berg-Lyons, D., et al. (2015). Dynamic changes in short-and long-term bacterial composition following fecal microbiota transplantation for recurrent *Clostridium difficile* infection. *Microbiome* 3, 10. doi: 10.1186/s40168-015-0070-0

Whitacre, J. M., and Bender, A. (2010). Networked buffering: a basic mechanism for distributed robustness in complex adaptive systems. *Theor. Biol. Med. Model.* 7:20. doi: 10.1186/1742-4682-7-20

Whitman, W. B., Coleman, D. C., and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6578–6583. doi: 10.1073/pnas.95.12.6578

Incorporating the soil environment and microbial community into plant competition theory

Po-Ju Ke [1, 2]* and Takeshi Miki [2, 3]

[1] Department of Biology, Stanford University, Stanford, CA, USA, [2] Institute of Oceanography, National Taiwan University, Taipei, Taiwan, [3] Research Center for Environmental Changes, Academia Sinica, Taipei, Taiwan

Plants affect microbial communities and abiotic properties of nearby soils, which in turn influence plant growth and interspecific interaction, forming a plant-soil feedback (PSF). PSF is a key determinant influencing plant population dynamics, community structure, and ecosystem functions. Despite accumulating evidence for the importance of PSF and development of specific PSF models, different models are not yet fully integrated. Here, we review the theoretical progress in understanding PSF. When first proposed, PSF was integrated with various mathematical frameworks to discuss its influence on plant competition. Recent theoretical models have advanced PSF research at different levels of ecological organizations by considering multiple species, applying spatially explicit simulations to examine how local-scale predictions apply to larger scales, and assessing the effect of PSF on plant temporal dynamics over the course of succession. We then review two foundational models for microbial- and litter-mediated PSF. We present a theoretical framework to illustrate that although the two models are typically presented separately, their behavior can be understood together by invasibility analysis. We conclude with suggestions for future directions in PSF theoretical studies, which include specifically addressing microbial diversity to integrate litter- and microbial-mediated PSF, and apply PSF to general coexistence theory through a trait-based approach.

Keywords: coexistence, litter-mediated feedback, Lotka-Volterra model, microbial-mediated feedback, invasibility analysis, spatial-explicit model

Introduction

One of the fundamental goals of plant ecology is to understand the maintenance mechanisms of plant species coexistence (Chesson, 2000). Many hypotheses have been proposed, including the resource competition theory (Hsu et al., 1977; Tilman et al., 1981), two-way trade-off models (Tilman, 1994), and the three-way trade-off strategy model (Grime, 1977). Other theories, such as the neutral theory (Hubbell, 1997) and the metacommunity theory (Leibold et al., 2004), address stochastic and spatial processes as well as deterministic processes. These theoretical frameworks and subsequent accepted viewpoints in plant ecology implicitly rely on environmental determinism (i.e., a unidirectional effect of the abiotic environment on the plant community and unidirectional adaptation of organisms to the given environment). This concept is widely accepted despite the fact Tansley (1935), a plant ecologist, first proposed the term "ecosystem" precisely to address feedback among organisms and the abiotic environment. The resource competition theory (Tilman, 1982) examines the effect of individuals on environmental resource availability, however its competitive

outcome depends on rates of external resource supply. An alternative investigative approach in plant community ecology, plant-soil-feedback (PSF), emphasizes the bidirectional interaction between growth limiting factors in soil and plant population and community dynamics (Berendse, 1994; Bever et al., 1997). Plants can affect soil properties, which in turn alter plant growth, survival, reproduction, and competition, subsequently acting as the driving forces for community composition and function. In this sense, Jones et al. (1994) appropriately characterized plants as fundamental ecosystem engineers.

Theoretical advances in PSF research have primarily focused on two major drivers: soil microbes and soil nutrients (see Ehrenfeld et al., 2005 for other factors, and van der Putten et al., 2013 for a comprehensive empirical review). Soil microbes interact with plants, providing diverse functional roles and are responsible for "microbial-mediated" PSF. Plants 'cultivate' local microbial communities surrounding plant roots (i.e., species-specific root-associated microbes, including beneficial and detrimental groups), which in turn affects plant performance (Bever et al., 1997; Bever, 2003). On the other hand, the feedback mechanisms mediating soil nutrient availability and the subsequent nutrient cycling between above- and below-ground ecosystem components are often referred to as "litter-mediated" PSF. Soil nutrient availability is determined by organic carbon and plant litter mineralization, which is controlled by plant species specific traits (e.g., litter production rate and its chemistry, Binley and Giardina, 1998) and can influence plant competition outcome (Berendse et al., 1987, 1989; Berendse, 1994). Although, the two mechanisms are commonly discussed individually, under some scenarios, the two main mechanisms are shown to interact and are in fact tightly linked. For example, litter-mediated PSF is also mediated by soil microbial decomposers such as saprophytic bacteria and fungi (Miki et al., 2010; Miki, 2012).

Although, empirical studies have increased our general understanding of PSF, many ecological models are constructed to examine PSF processes for specific species or ecosystems. General PSF mathematical theory remains under-explored, thus hindering comprehensive understanding of the importance of PSF and the roles of belowground microbial-mediated processes in structuring terrestrial ecosystems. Clearly, PSF theory requires development to better understand various plant community properties, including the number of coexisting species (e.g., Molofsky and Bever, 2002; Mazzoleni et al., 2010), species abundance and distribution (e.g., Mack and Bever, 2014), transient successional patterns (e.g., Fukami and Nakajima, 2013), and species spatial and temporal distribution (e.g., Molofsky et al., 2002).

In this article, we summarize how PSF concepts have been integrated into ecological modeling studies, with particular focus on topics related to plant species coexistence and spatio-temporal dynamics (see **Table 1** for a list of theoretical studies along with their corresponding modeling framework category). We subsequently reanalyzed two fundamental mechanistic PSF models (i.e., Berendse, 1994; Bever et al., 1997), which are the foundation of current theoretical models, and compared

them with the classic Lotka-Volterra competition model. Our results demonstrated how the application of invasibility analysis elucidated behaviors of different PSF models by generating coexistence criteria with similar ecological interpretation. Moreover, invasibility analysis provided superior ideas for soil-centered (or microbe-centered) views in PSF studies, resulting in a revised PSF metric which will aid in future theoretical development in microbial ecology. Finally, we proposed new research directions to integrate litter- and microbial-mediated PSF, and apply trait-based modeling approaches to predict species' PSF and coexistence.

Theoretical Development and Modeling Achievements for PSF

PSF Effects on Plant Competition Outcome and Species Coexistence

Theoretical PSF studies were first proposed to examine how PSF contributes to the coexistence of competing plant species. Bever et al. (1997) (hereafter Bever's model) conducted pioneering research leading to the microbial-mediated PSF model, which incorporated reciprocal interactions and frequency dependency among plants and soil microbial communities. Bever's model proposed that the final fate of a plant community can be predicted by the overall effect of soil microbial communities on both plant species (i.e., an interaction coefficient, I_S). Bever's model predicted that two plant species can coexist cyclically due to microbial-mediated PSF when both species generate PSF which decreases its relative growth rate (i.e., negative microbial-mediated PSF in terms of negative I_S). However, the model predicted that single species dominance is reached when both plants species generate PSF which increases its relative growth rate (i.e., positive microbial-mediated PSF in terms of positive I_S). It is important to note that the community-level outcome of PSF (i.e., predicted by the sign of I_S) cannot be directly inferred by the interaction type between soil microbes and its host (Bever et al., 1997). For example, if the mycorrhizal fungi delivers more benefit to the competitor compared to that to its host, competing plant species can coexist as PSF would decrease the relative growth rate of its host (i.e., negative I_S despite positive plant-microbe interaction; Bever, 1999, 2002; Umbanhowar and McCann, 2005). Likewise, pathogens can increase the relative growth rate of its host and result in single species dominance if it has stronger suppression on the growth of the competitor (i.e., positive I_S despite negative interaction with its host). A later version of Bever's model incorporated PSF into a two species Lotka-Volterra model and demonstrated coexistence can be promoted by a negative PSF, even under strong competitive interactions and fitness differences between the two plant species (Bever, 2003). Individual-based simulation models incorporating the PSF concept made similar predictions (Bonanomi et al., 2005; Petermann et al., 2008), and further suggested that the magnitude of population oscillations depends on negative PSF strength. However, Revilla et al. (2013) performed a complete analysis of Bever's model and suggested population cycling under negative PSF (i.e., in terms of negative J_S, a modified

TABLE 1 | Theoretical plant-soil feedback models reviewed in this article.

Author (year)	Main PSF mechanisms (model type)[†]	Study detail (plant community processes)[§]
Adler and Muller-Landau, 2005	Microbial-PSF[a]	Plant and natural enemy dispersal distance (SS, SD)
Aguilera, 2011	Microbial-PSF[b]	Density-dependency of plant microbe interactions (PI)
Berendse et al., 1987	Litter-PSF[b]	Species-specific litter chemistry (SC)
Berendse et al., 1989	Litter-PSF[b]	Species-specific litter chemistry (SC)
Berendse, 1994	Litter-PSF[b]	Species-specific litter chemistry (SC)
Bever et al., 1997	Microbial-PSF[b]	LV-type plant-microbe interactions (SC)
Bever, 1999	Microbial-PSF[b]	LV-type plant-microbe interactions (SC)
Bever, 2003	Microbial-PSF[b]	LV-type plant-microbe interactions (SC)
Bever et al., 2010	Microbial-PSF[b]	Microbial-mediated plant niche partitioning and PSF (SC)
Bonanomi et al., 2005	No specific mechanism[a]	Negative PSF and population dynamics (SC, SD)
Clark et al., 2005	Litter-PSF[b]	Species-specific litter chemistry (SC)
Daufresne and Hedin, 2005	Litter-PSF[b]	Resource ratio hypothesis and nutrient cycling (SC)
Dickie et al., 2005	Microbial-PSF[a]	Distance-dependent interaction strength (SS)
Eppinga et al., 2006	Microbial-PSF[b]	Nonlinear LV-type plant-microbe interactions (PI)
Eppinga et al., 2011	Litter-PSF[b]	Species-specific litter chemistry and trait evolution (PI)
Eppstein et al., 2006	No specific mechanism[a]	Community dynamics and frequency dependency (SS, SC)
Eppstein and Molofsky, 2007	No specific mechanism[b]	Invasion dynamics and frequency dependency (PI, SC)
Fukami and Nakajima, 2013	No specific mechanism[a]	Transient dynamics and delayed convergence (SD)
Fukano et al., 2013	Microbial-PSF[c]	Disturbance regime (PI)
Kulmatiski et al., 2011	Microbial-PSF[b]	Multi-species LV-type plant microbe interaction (SC)
Kulmatiski et al., 2012	Microbial-PSF[c]	Multi-species biomass-explicit plant-microbe interaction (BEF)
Loeuille and Leibold, 2014	No specific mechanism[a]	Species diversification and macro-ecological patterns (SD)
Levine et al., 2006	Microbial-PSF[d]	Spatial scale and invasion velocity (PI, SS)
Mack and Bever, 2014	Microbial-PSF[a]	Plant dispersal and PSF interactions scale (SRA, SS)
Mangan et al., 2010	Microbial-PSF[a]	Negative PSF and Janzen-Connell hypothesis (SD, SRA)
Mazzoleni et al., 2010	Litter-PSF[b]	Autotoxicity and latitudinal diversity gradient (SD)
Miki and Kondoh, 2002	Litter-PSF[b]	Species-specific litter chemistry (PI, SC)
Miki et al., 2010	Litter- and microbial- PSF[b]	Decomposer diversity (SC)
Miki, 2012	Litter- and microbial- PSF[b]	Decomposer diversity (PI)
Molofsky et al., 2001	No specific mechanism[a]	Coexistence under positive PSF (SS, SC)
Molofsky and Bever, 2002	No specific mechanism[a]	Positive PSF and unsuitable habitats (SS, SD)
Molofsky et al., 2002	No specific mechanism[a]	Plant dispersal and PSF interactions scale (SS, SC)
Mordecai, 2013a	Microbial-PSF[c]	Generalist pathogen and pathogen spillover (SC)
Mordecai, 2013b	Microbial-PSF[c]	Generalist pathogen and pathogen spillover (SC)
Mordecai, 2015	Microbial-PSF[c]	Generalist pathogen and storage effect (SC)
Petermann et al., 2008	Microbial-PSF[a]	Janzen-Connell hypothesis (SD)
Revilla et al., 2013	Microbial-PSF[b]	LV-type plant-microbe interactions (SC)
Schnitzer et al., 2011	No specific mechanism[b]	LV-type plant-microbe interactions (BEF)
Sedio and Ostling, 2013	Microbial-PSF[a]	Natural enemy host specificity and Janzen-Connell hypothesis (SD)
Suding et al., 2013	No specific mechanism[a]	Invasion dynamics and enemy release (PI)
Turnbull et al., 2010	Microbial-PSF[c]	Invasive species spread (PI)
Umbanhowar and McCann, 2005	Microbial-PSF[b]	Plant-mycorrhizal fungi interactions (SC)
Zee and Fukami, 2015	No specific mechanism[a]	Species loss following habitat fragmentation (SD)

Studies are selected if it considers the effect of microbial-mediated PSF and/or litter-mediated PSF on plant competition outcome or community structure. As a result, models investigating the effect of plant-microbial interaction and nutrient cycling on the growth of a single plant species, as well as those focusing on disease dynamics, are not included.

[†] *Model type: [a] stochastic cellular automata; [b] ordinary differential equations; [c] difference equations; [d] integrodifference equations. [§] Plant community process: SC, species coexistence; PI, plant invasion; BEF, biodiversity-ecosystem functioning relationship; SRA, species relative abundance; SD, species diversity; SS, spatial structure.*

version of I_S in Bever's model) might occur in the form of heteroclinic cycles, which can enable stochastic extinction in real empirical systems. Recent theoretical studies with an emphasis on microbial-mediated PSF have also extended Bever's

model to multiple species (Bonanomi et al., 2005; Petermann et al., 2008; Kulmatiski et al., 2011, 2012). For example, a three-species version of Bever's model showed PSF played a critical role in predicting rank order abundance of experimental

plant communities, and the PSF model made better predictions compared with a pure competition model (Kulmatiski et al., 2011).

Theoretical studies on litter-mediated PSF investigated the influence of plant litter quality on soil nutrient availability, and how changes in soil nutrient availability alter plant competition (pioneered by Berendse et al., 1987, 1989; Berendse, 1994). When plants compete for a single growth limiting factor in the soil (e.g., inorganic nitrogen), a difference in plant growth response to different nutrient levels (i.e., a tradeoff) is often necessary for litter-mediated PSF to alter competitive outcomes (Miki and Kondoh, 2002; Clark et al., 2005). Berendse (1994) used ordinary differential equations to build simple ecosystem models to demonstrate community-level outcomes depended on a combination of the plant species' litter quality and nutrient uptake strategies. Plant species with growth advantages in nutrient-rich soils reinforced their dominance by producing rapidly decomposing litter. Similarly, plant species more competitive in nutrient-poor sites increased their dominance by producing slowly decomposing litter. Both trait combinations resulted in nutrient availability that favors the resident plant (i.e., positive litter-mediated PSF), leading to competitive exclusion of its competitor (Berendse, 1994), or alternative stable states differing in species composition (Clark et al., 2005) or species richness (Miki and Kondoh, 2002; Miki et al., 2010). In contrast, coexistence was facilitated if plant species influenced the nutrient cycle to reinforce the persistence of its competitor (i.e., negative litter-mediated PSF). Some studies integrated litter-mediated PSF with Tilman's (1982) resource ratio theory to consider multiple limiting factors in the soil and plant stoichiometry (Daufresne and Hedin, 2005; Eppinga et al., 2011). This theoretical framework also demonstrated that whether litter-mediated PSF enhances or suppresses coexistence was dependent on the trait combination of competing plants species (Daufresne and Hedin, 2005). Simple litter-mediated PSF models were also extended to examine more detailed nutrient cycling, emphasizing the importance of environmental factors (Miki and Kondoh, 2002), litter quality attributes other than decomposition rates (e.g., the recycled proportion, Clark et al., 2005), different plant-available nutrient types (Clark et al., 2005; Daufresne and Hedin, 2005), and litter effects other than soil nutrient availability (Eppinga et al., 2011) on community outcomes driven by litter-mediated PSF.

PSF Models that Go Beyond Species Coexistence

Recent theoretical studies go beyond discussing coexistence of few plant species, and have applied PSF as a mechanism to explain other macro-scale community patterns (see Bever et al., 2010; van der Putten et al., 2013 and references therein). The relationship between PSF and plant diversity is one topic that has received a great deal of interest. Many empirical studies revealed negative microbial-mediated PSF acted as a mechanism for the Janzen-Connell hypothesis (Janzen, 1970; Connell, 1971), contributing to negative density-dependent (Bell et al., 2006; Yamazaki et al., 2008; Bagchi et al., 2010, 2014) and distance-dependent (Augspurger, 1983; Packer and Clay, 2000; Swamy and Terborgh, 2010) seedling mortality. These mortality patterns resulted from

negative PSF, which enhanced plant diversity; and simulation models suggested the greatest diversity was attained when natural enemies were host-specific (Sedio and Ostling, 2013). Furthermore, a species' relative abundance in a community was predicted by the PSF strength it experienced; plant species with lower abundance suffered stronger negative PSF (Klironomos, 2002; Mangan et al., 2010; but see Reinhart, 2012). Mangan et al. (2010) applied a spatially explicit cellular automata model to confirm the positive relationship between species' PSF strength and species relative abundance by parameterizing the model with field-measured PSF strength. Additional work on the model confirmed the positive relationship was robust for different forms of life-history tradeoffs (e.g., tradeoffs between mortality and establishment rates) (Mangan et al., 2010; Mack and Bever, 2014).

Large-scale studies suggested that the stronger negative microbial-mediated PSF at lower latitude regions contributed to its higher plant species richness (Johnson et al., 2012). Mazzoleni et al. (2010) constructed an ecosystem model considering "resource-waste" (i.e., "autotoxicity" via intra-specific toxic compounds, Mazzoleni et al., 2007) during litter decomposition as a source for negative litter-mediated PSF. Autotoxicity has the potential to suppress plant growth directly by harming plant tissue or indirectly by exacerbating detrimental pathogen effects (Mazzoleni et al., 2007, 2010; Bonanomi et al., 2010). The ecosystem model indicated that as litter decomposition rate increased from higher to lower latitude, so did the level of autotoxicity, generating stronger negative density-dependency and supporting higher species richness at lower latitudes. The model also showed that washing of autotoxicity removed negative PSF effects and decreased species richness, a potential mechanism to explain differences in plant species richness between flooded and non-flooded communities at the same latitude (Mazzoleni et al., 2010).

At the interface between community and ecosystem ecology, PSF was also proposed to contribute to the biodiversity-ecosystem functioning relationship, with particular focus on productivity (i.e., higher plant diversity leads to increased productivity, also known as overyielding). Loreau and Hector (2001) attributed an asymptotic productivity increase to either sampling effect or niche complementary due to a rise in species diversity (Loreau and Hector, 2001; but see Hector et al., 1999; Miki, 2009; Cardinale et al., 2012 for diversity-productivity patterns other than asymptotically increase). Soil microbes, particularly host-specific pathogens, were recently proposed as another potential mechanism involved in the diversity-productivity relationship. Studies showed that negative density-dependent effect of pathogens were diluted as plant species diversity increased since the proportion of self-cultivated soil decreased, resulting in higher plant productivity (Maron et al., 2011; Schnitzer et al., 2011). Empirical studies supported this mechanism; compared with monocultures, plants overyield when grown in a soil mixture cultivated with different plant species (Kulmatiski et al., 2012; Hendriks et al., 2013), while sterilizing the soil eliminated the positive relationship (Maron et al., 2011; Schnitzer et al., 2011). In addition, Hendriks et al. (2015) found spatial heterogeneity in soil biota created by highly diverse

communities also contributed to plant avoidance of host-specific pathogens. Schnitzer et al. (2011) used a simple Lotka-Volterra type model to illustrate that the classic asymptotic diversity-productivity relationship appeared only in the presence of host-specific pathogens. The pathogen effects were stronger, and the plant productivity saturation point occurred at higher plant diversity, when operating together with niche complementary (i.e., comparing neutral and non-neutral models). Based on Bever's PSF model, Kulmatiski et al. (2012) developed a biomass-explicit multi-species PSF model to directly examine the influence of PSF on biomass production. Their model suggested that dilution of species-specific soil biota effects, resulting from increased plant diversity, can result in over- and under-yielding, depending on the sign of species' PSF (i.e., negative or positive, respectively). In addition, the negative relationship between PSF strength and over-yielding became stronger with increasing species richness. Empirical results supported model predictions, however results also indicated further information regarding plant community structure (e.g., presence or absence of nitrogen-fixing plants) would provide useful information for future studies (Kulmatiski et al., 2012). Although, theoretical studies linking microbial-mediated PSF and diversity-productivity relationships are growing, to our knowledge models related to this topic have not explicitly considered litter-mediated PSF and nutrient cycling.

Finally, the impact of PSF on invasion success has been another rapidly growing research area (empirical studies reviewed in Mitchell et al., 2006; Reinhart and Callaway, 2006; Inderjit and van der Putten, 2010; Suding et al., 2013). In general, PSF facilitates invasion when exotic plant species experience weaker negative PSF (or benefit from stronger positive PSF) compared to native plant species (Reinhart and Callaway, 2006). Keane and Crawley (2002) proposed the "enemy-release hypothesis," which stated that by migrating from their native range, exotic species escaped species-specific specialized natural pathogens. Consequently, exotic species experienced reduced negative microbial-mediated PSF in the new region and became successful invaders (Keane and Crawley, 2002; Klironomos, 2002; Mitchell and Power, 2003; Callaway et al., 2004). Further, theoretical studies suggested the effectiveness of enemy-release depended on the diversity of the native community (Turnbull et al., 2010), the functional response between plant growth and soil microbial density (Aguilera, 2011), the disturbance regime (Fukano et al., 2013), and the invader competitive ability on the native species' cultivated soil environment (Eppstein and Molofsky, 2007; Turnbull et al., 2010; Suding et al., 2013). In some cases, soil-borne pathogens might still facilitate invasion, even if the invader is not enemy-released. This happens when the invader attracts generalist pathogens, which have stronger negative effects on native plant species, a scenario termed the "enemy-accumulation hypothesis." This scenario was first proposed by Eppinga et al. (2006) using a non-linear extension of Bever's model to explain the success of *Ammophila arenaria* invasion in California, which was a successful invader despite suffering similar pathogen suppression as that in its native European range (Bever et al., 1997; Beckstead and Parker, 2003). The effects of generalist pathogens on plant

invasion have also been studied under the "pathogen spillover" hypothesis, where invasive plant species with greater pathogen tolerance gain advantage by transmitting the shared pathogens to less tolerant native plant species (Beckstead et al., 2010). Pathogen spillover can lead to competitive exclusion of natives, coexistence, or priority effects (Mordecai, 2013a,b). In addition to microbial-mediated PSF, litter-mediated PSF can also influence the outcome of exotic plant invasion. Specific combinations of litter decomposability and nutrient uptake strategies will generate positive litter-mediated PSF for the invader (Miki and Kondoh, 2002). When linking litter-mediated PSF with invasion, studies have also discussed its effect on resources other than soil nutrient. The effect of invader's litter on the local light environment has been of particular interest. Results of studies indicated that invaders' litter accumulation will decrease light availability by shading, while simultaneously increasing soil nutrient availability via litter decomposition. Studies suggested that the combined impact of invader litter facilitated invasion non-additively when the invader is a weaker competitor for soil nutrients but a better competitor for light (Farrer and Goldberg, 2009; Eppinga et al., 2011).

The Importance of Spatial Scale on PSF Effects

One simplification made in many PSF models is the assumption of a well-mixed soil environment, which neglects the fact that plant dispersal and PSF usually operate locally. However, empirical evidence often revealed that the interaction between plants and soil microbes are highly distance-dependent and can influence plant spatial patterning (Augspurger, 1983; Packer and Clay, 2000; Reinhart et al., 2003; Dickie et al., 2005; Swamy and Terborgh, 2010). Eppstein et al. (2006) applied spatially explicit models to investigate the importance of space on PSF outcomes; results differed from the well-mixed models and provided new insights into the value of PSF and plant spatial patterning. In addition, the prediction from Bever's model regarding PSF sign and plant competition outcome had been extensively studied (Bever et al., 1997; Molofsky et al., 2001, 2002; Molofsky and Bever, 2002; also reviewed in Bever, 2003 and Bever et al., 2012). Molofsky et al. (2002) showed that under a spatially explicit modeling framework the prediction that negative PSF facilitated plant coexistence was generally not altered (but see Bever et al., 1997 for the case when two plants exhibited different dispersal ability). Plant coexistence was maintained by negative PSF, while a large array of different spatial patterns was observed, ranging from near random, clumped, to band formation distributions. The spatial patterning of competing plant species depends on the relative spatial scale of dispersal and PSF, as well as the negative PSF strength (Molofsky et al., 2002). However, the prediction that positive PSF leads to single species dominance was altered when considering the spatial aspects of PSF. Studies demonstrated that under certain initial conditions, positive PSF can lead to the formation of self-maintained monomorphic patches, promoting long-term coexistence at the regional scale via the maintenance of spatial heterogeneity (Molofsky et al., 2001; Molofsky and Bever, 2002). Moreover, the probability of species coexistence was greater when positive PSF existed compared with the absence of PSF (i.e., a neutral case), and coexistence was

more effectively maintained when positive PSF operated at a local scale (Molofsky et al., 2001; Molofsky and Bever, 2002). This unique prediction was maintained even when the model was generalized to consider asymmetric frequency-dependent strength between competing plant species (Eppstein et al., 2006). Dickie et al. (2005) demonstrated that mycorrhizal-generated positive PSF and plant-plant competition showed different distance-dependent patterns. By integrating empirical evidence into a spatially explicit model, results indicated a net mycorrhizal effect facilitating seedling growth occurred only at low plant densities, thus potentially promoting species coexistence and diversity (Dickie et al., 2005). These theoretical achievements suggested that when spatial structure of interactions was considered, the predicted species monoculture associated with positive PSF was mitigated and coexistence was maintained at larger spatial scales.

In addition to the coexistence patterns between two plant species, other macro-scale community patterns generated by PSF have also been examined under a spatial framework. By considering spatial structure and heterogeneous landscape (i.e., with unsuitable habitats conditions), Molofsky and Bever (2002) suggested that positive PSF can maintain community species diversity for a much longer time period if PSF operated at a local scale. For negative microbial-mediated PSF to effectively promote species richness, local microbial and plant dispersion was required to generate clumped adult distribution and strong negative density-dependency for seedling survival (Adler and Muller-Landau, 2005; Petermann et al., 2008). The positive relationship between species' PSF strength and its relative abundance (Mack and Bever, 2014), as well as evolutionary diversification (Loeuille and Leibold, 2014), were also only observed when the scale of negative PSF and plant dispersal were local. For exotic plant invasion, positive PSF created by the invader can promote its invasiveness when operating at a local scale, despite the counteracting positive PSF created by natives (Eppstein et al., 2006). However, Levine et al. (2006) showed for positive PSF to accelerate invasion velocity, the spatial scale of soil modification must be larger than that of the plant-plant competition.

Former studies typically considered positive or negative PSF separately, however recent models have begun to consider the spatial consequences of a complex PSF scenario (Fukami and Nakajima, 2013), where PSF affects plant growth positively for some species-pair, and negatively for others. Fukami and Nakajima (2013) evaluated compositional variation between patches with different initial compositions, and suggested a complex PSF scenario could maintain the initial variability in species composition for a longer period of time, contributing to regional plant diversity compared to a simpler PSF scenario. A complex PSF scenario also alleviated diversity loss caused by habitat fragmentation, and such buffering effects were greatest when plants dispersed locally. The mechanisms for such a buffering effect is because complex PSF decreased both spatial clustering of species distribution prior to habitat fragmentation and extinction probability after fragmentation, which would both be pronounced in the absence of PSF if plants dispersed locally (Zee and Fukami, 2015). In conclusion,

spatially explicit PSF models generated many useful predictions that cannot be revealed if a well-mixed soil environment was assumed, and suggested that the structuring ability of PSF strongly depends on the spatial scale of PSF and plant dispersal.

Temporal Dynamics of PSF and its Effect on Plant Community Succession

The influence of PSF has primarily been examined in studies that focused on community properties in a single temporal snapshot, or implicitly assumed the community is at equilibrium (but see Mordecai, 2015, for the effects of generalist pathogens on plant species coexistence through a temporal storage effect). However, PSF also contributes to temporal dynamics and the development of plant communities. Most of our current knowledge in this area derives from successional studies (reviewed in Kardol et al., 2013; van der Putten et al., 2013). The following two concepts were proposed for the roles of PSF during succession: (i) the predictable and directional changes in PSF sign and strength (Kardol et al., 2006); and (ii) random-emergence of PSF (Fukami and Nakajima, 2013). The first dominant concept suggested that during succession, negative PSF was experienced by early-successional plant species and facilitated replacement by late-successional plant species (van der Putten et al., 1993; Berendse, 1994; Bonanomi et al., 2005; Clark et al., 2005). In a well-known empirical example, Kardol et al. (2006) demonstrated that during secondary succession of abandoned grasslands, directional changes in PSF strength occurred and early-successional plant species mainly experienced negative PSF, while late-successional species primarily experienced positive PSF, which stabilized the plant community composition. This directional sequence of PSF occurrence resulted from nonrandom linkage between plants' above- and below-ground PSF-related traits; early-arriving plant species generally exhibited fast growth rates, but poor defense against pathogens, while late-arriving species were typically slow growing and mycorrhizal-associated species (Kardol et al., 2006, 2007, 2013).

While directional change in PSF strength across plant succession (i.e., from negative to positive PSF) has attracted much attention, empirical evidence supporting other hypotheses has also been reported (e.g., Reynolds et al., 2003; Sikes et al., 2012). A new idea proposed by Fukami and Nakajima (2013) focused on the priority effects generated by PSF, and how different PSF scenarios would influence community trajectories among patches with different immigration histories. Their main conclusion was when PSF operated in a complex manner such that the direction and strength of species' PSF was not determined by its successional stage (e.g., PSF for early-successional species were not necessarily negative), initial species variation among patches would be maintained, and more plant species could colonize a patch and persist longer before local extinction. This results in a long-lasting transient phase characterized by high species turnover (i.e., a large number of local colonization and extinction events) and high regional diversity before reaching the final community structure (Fukami and Nakajima, 2013). Therefore, the model indicated that when PSF occurred idiosyncratically, with a weak correlation between its strength and the species'

successful stage, PSF delayed succession and the speed for local communities to converge to a late successional stage community. The different successional trajectories resulting from different PSF scenarios represented alternative transient states in plant community development, which is defined as communities varying in structure or function before reaching a stable state (Fukami and Nakajima, 2011). A promising direction for future research is to integrate the directional and random-emergence paradigm for changes in PSF during succession, and test predictions for successional trajectories using chronosequences from different ecosystems.

Linkage Between Mechanistic-PSF Models and the Lotka-Volterra Competition Model

The influence of PSF on plant community dynamics has been incorporated into theoretical ecological modeling, however many of these models are phenomenological and not restricted to specific PSF mechanisms (**Table 1**). In terms of specific PSF mechanisms, it can be traced back to two fundamental models: microbial- (Bever et al., 1997) and litter-mediated PSF (Berendse, 1994). Here, we reintroduce these two mechanistic-PSF models with more unified formulations. We reanalyzed both models using invasibility analysis (Bever, 2003; Revilla et al., 2013) to show how the final community state can be classified into four possible outcomes, a scenario comparable to the classic Lotka-Volterra competition model. We believe this method provides a systematic way to evaluate PSF models, regardless of the specific topics which they originally focused on.

Lotka-Volterra Competition Model and Invasibility Analysis for PSF Models

Our analytical framework can first be illustrated by considering the phenomenological Lotka-Volterra competition model for two plant species (with species density represented by P_A and P_B, respectively), which is written as follows:

$$\frac{dP_A}{dt} = r_A P_A \left(1 - \frac{P_A + c_B P_B}{T_P} \right) \tag{1}$$

$$\frac{dP_B}{dt} = r_B P_B \left(1 - \frac{c_A P_A + P_B}{T_P} \right). \tag{2}$$
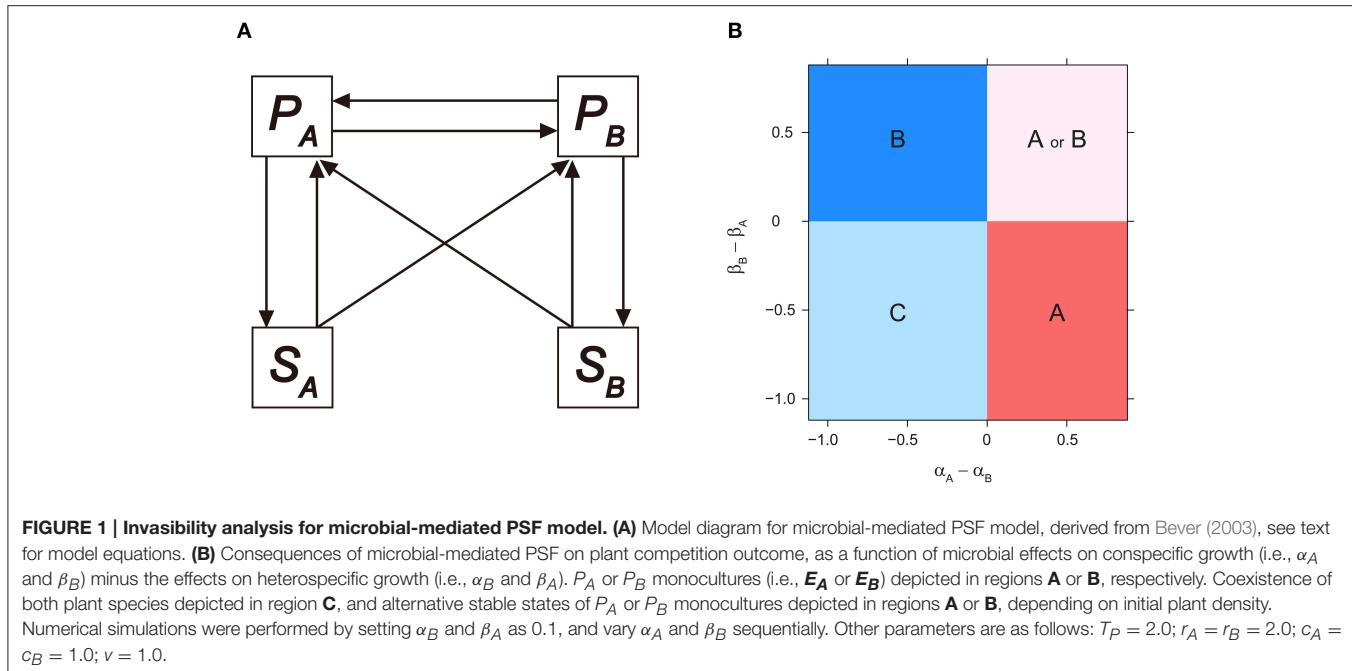
Here, r_i represents intrinsic population growth rate for plant species i ($i = A$ or B), T_P denotes the carrying capacity for plants (for simplicity, we assumed identical parameter values for both species). Coefficient c_j measures the negative effects of space competition from heterospecies j relative to the negative density dependent effect of conspecies i (i.e., $j = B$ or A when $i = A$ or B, respectively). If $c_j = 1$, species are identical in terms of competition for space (i.e., a neutral case when intra- and inter-specific competition have similar magnitudes). The final outcome in Lotka-Volterra type competition can be predicted by applying invasibility analysis. That is, evaluating species' per capita growth rate (i.e., invader fitness) when its density is low while the competitor is at its monoculture equilibrium (Chesson, 2000). When invader fitness is positive, the invasibility criteria is fulfilled

and population size increases (or recovers) from low density. A plant species monoculture occurs when only one species can invade the other. Coexistence is achieved when two plant species are mutually invasive (i.e., each species can invade the habitat dominated by the other species and no species can competitively exclude the other), while founder control (i.e., competition outcome depends on the initial plant density conditions) occurs when both invader species are unable to invade the other. From Equations (1) and (2), the invasibility criterion for P_A to invade a monoculture of P_B (i.e., $E_B(P_A^* = 0, P_B^* = T_P)$) is $c_B < 1$, while that for P_B to invade monoculture of P_A (i.e., $E_A(P_A^* = T_P, P_B^* = 0)$) is $c_A < 1$. Here, E_i is the mathematical notation for the plant monoculture equilibrium of P_i, and P_i^* within the parenthesis represents the density of P_i at the specific equilibrium. Therefore, the parameter space of c_A and c_B can be partitioned into the following four regions: (i) P_A monocultures ($c_B < 1$ but $c_A > 1$); (ii) P_B monocultures ($c_B > 1$ but $c_A < 1$); (iii) coexistence (both c_B and $c_A < 1$); (iv) founder control (i.e., alternative stable state of either P_A or P_B monoculture; both c_B and $c_A > 1$). These results are consistent with those obtained from local stability analysis; coexistence was possible when impacts on heterospecific growth are weaker than those on conspecific growth (i.e. c_B and $c_A < 1$; Chesson, 2000).

The framework of invasibility analysis can be applied to mechanistic-PSF models. Consider a two plant species (i.e., P_A and P_B) and two corresponding soil components (e.g., either soil nutrients or microbial communities) that influence plant population dynamics. When the community is at equilibrium for the P_A monoculture (i.e. $E_A(P_A^* > 0, P_B^* = 0)$), the corresponding soil component is determined by the parameters related to the dominant resident species (in this case P_A), but is independent of the invader (P_B) parameters. The invasion criterion for P_B to invade the P_A monoculture can be derived from its invader fitness. Importantly, this invasibility criterion includes parameters determining how P_B is affected directly by P_A and indirectly by P_A-cultivated soil components, but does not include parameters related to how P_B affects the soil. The criterion implies that the important invasion determinants are interactions between resident species and resident-cultivated soil. Therefore, by combining mutual invasibility conditions for PSF models, we can categorize the trait parameter space related to P_A- and P_B-soil interactions into different consequences of monoculture and/or coexistence.

Microbial-mediated PSF Models and Invasibility Analyses

Microbial-mediated PSF was first incorporated into the Lotka-Volterra model by Bever et al. (1997) and Bever (2003), and further extended by other studies (Eppinga et al., 2006; Bever et al., 2010; Aguilera, 2011; Kulmatiski et al., 2011; Revilla et al., 2013). Bever's groundbreaking models (Bever et al., 1997; Bever, 2003) captured the bidirectional interaction of PSF; different plant species interacted with the soil microbial community differently and developed a species-specific microbial community association, and changes in microbial communities had differential influences on different plant species (Bever et al., 1997; see **Figure 1A** for model diagram adapted from

FIGURE 1 | Invasibility analysis for microbial-mediated PSF model. (A) Model diagram for microbial-mediated PSF model, derived from Bever (2003), see text for model equations. **(B)** Consequences of microbial-mediated PSF on plant competition outcome, as a function of microbial effects on conspecific growth (i.e., α_A and β_B) minus the effects on heterospecific growth (i.e., α_B and β_A). P_A or P_B monocultures (i.e., E_A or E_B) depicted in regions **A** or **B**, respectively. Coexistence of both plant species depicted in region **C**, and alternative stable states of P_A or P_B monocultures depicted in regions **A** or **B**, depending on initial plant density. Numerical simulations were performed by setting α_B and β_A as 0.1, and vary α_A and β_B sequentially. Other parameters are as follows: $T_P = 2.0$; $r_A = r_B = 2.0$; $c_A = c_B = 1.0$; $v = 1.0$.

Bever, 2003). The microbial community density specifically associated with P_A and P_B was represented by N_α and N_β, respectively. The dimensionality of the soil microbial community was reduced by focusing on the relative abundance of different soil microbial communities, which were represented by $S_A = N_\alpha/(N_\alpha + N_\beta)$ and $S_B = N_\beta/(N_\alpha + N_\beta)$ for P_A- and P_B-associated soil microbial communities, respectively (Bever et al., 1997). The model also assumed linear frequency dependency between plants and soil microbial communities (but see Eppinga et al., 2006 and Aguilera, 2011 for a non-linear case). When integrating microbial-mediated PSF in the Lotka-Volterra model, the population dynamics for plants can be written as follows (Bever, 2003):

$$\frac{dP_A}{dt} = r_A P_A \left(1 + \alpha_A S_A + \beta_A S_B - \frac{P_A + c_B P_B}{T_P} \right) \quad (3)$$

$$\frac{dP_B}{dt} = r_B P_B \left(1 + \alpha_B S_A + \beta_B S_B - \frac{c_A P_A + P_B}{T_P} \right). \quad (4)$$

The parameters α_A and α_B measure how S_A influences the per-capita growth rate of P_A and P_B, respectively, while β_A and β_B measure how S_B influences the per-capita growth rates of P_A and P_B, respectively. The feedback parameters α_A and β_B denote the effect of microbial community on its associated host and are termed direct (or intraspecific) PSF. Alternatively, the microbial community can affect its associated host by influencing the growth of competing plant species. Such interactions are termed indirect (or interspecific) PSF, and are indicated by α_B and β_A. The sign and magnitude of these feedback parameters are determined by both plant and microbial traits that influence microbial composition (e.g., detrimental pathogens or beneficial mycorrhiza), and

interaction strength between plants and microbes (e.g., host specificity of microbes) (Bever et al., 1997, 2010; Bever, 2002).

Under the assumption of linear frequency dependency, the sizes of soil microbial communities (i.e., N_α and N_β) were assumed to increase linearly with the relative abundance of its associated host plant as:

$$\frac{dN_\alpha}{dt} = \frac{P_A}{P_A + P_B} \cdot N_\alpha \quad (5)$$

$$\frac{dN_\beta}{dt} = v \frac{P_B}{P_A + P_B} \cdot N_\beta, \quad (6)$$

where v represents the relative influence of P_B on the microbial community against that of P_A. One way to interpret v is the rate that P_B, relative to P_A, converts the microbial community to its specific associated composition (Kulmatiski et al., 2011), which can be determined by plant exudate and microbial response (reviewed in Bais et al., 2006; Bever et al., 2012). Since, $S_A + S_B = 1$, the dynamics of relative abundance in different microbial communities can be derived by applying the quotient rule of calculus to Equation (5) as follows:

$$\frac{dS_A}{dt} = S_A (1 - S_A) \left(\frac{P_A}{P_A + P_B} - v \frac{P_B}{P_A + P_B} \right). \quad (7)$$

In regard to plant species composition, monoculture equilibrium of either P_A or P_B are derived from Equations (3), (4), and (7) as $E_A(P_A^* = T_P (1 + \alpha_A), P_B^* = 0)$ and $E_B(P_A^* = 0, P_B^* = T_P (1 + \beta_B))$, respectively. The corresponding soil equilibrium states are $S_A^* = 1$ and $S_A^* = 0$, respectively. Based on local stability analysis, Bever et al. (1997) proposed that the community outcome was predicted by the sign of an interaction coefficient, I_S, which was

defined as $I_S = \alpha_A - \beta_A - \alpha_B + \beta_B$. The necessary condition for coexistence to be maintained by PSF was $I_S < 0$. In contrast, PSF resulted in a monoculture of either plant species when $I_S > 0$. Here, applying a similar invasibility analysis used in Bever (2003) and Revilla et al. (2013), we calculated plant P_A would invade the P_B monoculture (i.e., E_B) when:

$$\lim_{P_A \to +0} \frac{1}{P_A} \frac{dP_A}{dt}\bigg|_{E_B} = r_A \left[1 + \beta_A - \frac{c_B T_P (1 + \beta_B)}{T_P} \right] > 0. \quad (8)$$

As we were interested in the effects of PSF strength on the invasion criteria, we assume $c_A = c_B = 1$ (i.e., a neutral case such that the two plants are identical in terms of competition for space). The invasibility criterion for P_A can be written as $\beta_B - \beta_A < 0$. This criterion implies that P_A can invade E_B if the dominating P_B-cultivated soil has less facilitative (or more negative) effects on P_B compared to that on the invading P_A (i.e., P_B-cultivated soil has a relatively negative effect on P_B compared to that on P_A). On the other hand, plant P_B can invade the monoculture of P_A (i.e., E_A) when:

$$\lim_{P_B \to +0} \frac{1}{P_B} \frac{dP_B}{dt}\bigg|_{E_A} = r_B \left[1 + \alpha_B - \frac{c_A T_P (1 + \alpha_A)}{T_P} \right] > 0, \quad (9)$$

which gives the invasion criterion as $\alpha_A - \alpha_B < 0$ when $c_A = c_B = 1$. Consequently, P_B can invade E_A if the dominant P_A-cultivated soil has a relatively negative effect on P_A. Note that these criteria are determined by the sign of direct PSF (i.e., α_A and β_B) minus indirect PSF (i.e., α_B and β_A), and can be interpreted as the differential effect of the cultivated soil on its host compared to the effects on the other plant species. The parameter space of $\alpha_A - \alpha_B$ and $\beta_B - \beta_A$ can therefore be partitioned into four regions based on these invasibility criteria (also confirmed by numerical simulations, **Figure 1B**). Coexistence (region C in **Figure 1B**) is possible when both plant species support a microbial community which has stronger facilitative effects on the competitor compared to that on itself, or stronger suppressive effects on itself than on the competitor (i.e., both $\alpha_A - \alpha_B$ and $\beta_B - \beta_A < 0$). Alternatively, microbial-mediated PSF leads to a plant species monoculture (region A and B in **Figure 1B**) when only one plant cultivates the microbial community to have a positive effect on itself (i.e., only $\beta_B - \beta_A$ or $\alpha_A - \alpha_B < 0$ for P_A- and P_B-monoculture, respectively). Finally, when both plant species experience positive feedback from its associated microbial community (i.e., both $\alpha_A - \alpha_B$ and $\beta_B - \beta_A > 0$), the system generates alternative stable state of monoculture of either plant species (region A or B in **Figure 1B**). Note that by relaxing the $c_A = c_B = 1$ assumption, similar simple boundaries are produced, although the invasion criteria becomes $\beta_B - \beta_A < 1 - c_B$ for P_A and $\alpha_A - \alpha_B < 1 - c_A$ for P_B (see also Bever, 2003 and Revilla et al., 2013 for model analysis when relaxing this assumption).

We argue that applying the invasibility analysis to Bever's fundamental microbial-mediated PSF model (Bever, 2003) provides a more transparent interpretation of PSF. For example, consider conditions where $\alpha_A > \alpha_B$ and $\beta_A > \beta_B$ (i.e., P_A has

greater growth advantages compared to P_B, despite competitive equivalence for space). Dominance of P_A can be successfully predicted by criteria derived from invasibility analysis, but not by the sign of I_S (e.g., positive coexistence equilibrium does not exist under some parameter region despite $I_S < 0$). In addition, unlike local stability analysis, which can only pinpoint the local asymptotically stable criteria of the coexistence equilibrium, invasibility analysis relies on species' per capita growth rate when rare (i.e., at low population density) and identifies the criteria for either stable or fluctuating coexistence (Chesson, 2000).

Litter-mediated PSF Models and Invasibility Analysis

In addition to microbial-mediated PSF, another branch of mechanistic-PSF models focus on plants ability to alter soil biochemical properties through litter-mediated PSF (Binley and Giardina, 1998). Berendse (1994, see also Berendse et al., 1987, 1989) proposed the litter-mediated PSF model, which was further extended by other studies (Miki and Kondoh, 2002; Clark et al., 2005; Miki et al., 2010; Eppinga et al., 2011). A simplified version of the model (**Figure 2A**, modified from Berendse, 1994) incorporates plant growth limitations by soil nutrients and nutrient cycling processes into the Lotka-Volterra model as follows:

$$\frac{dP_A}{dt} = \frac{v_A N}{K_A + N}\left(1 - \frac{P_A + c_B P_B}{T_P}\right)P_A - m_A P_A \quad (10)$$
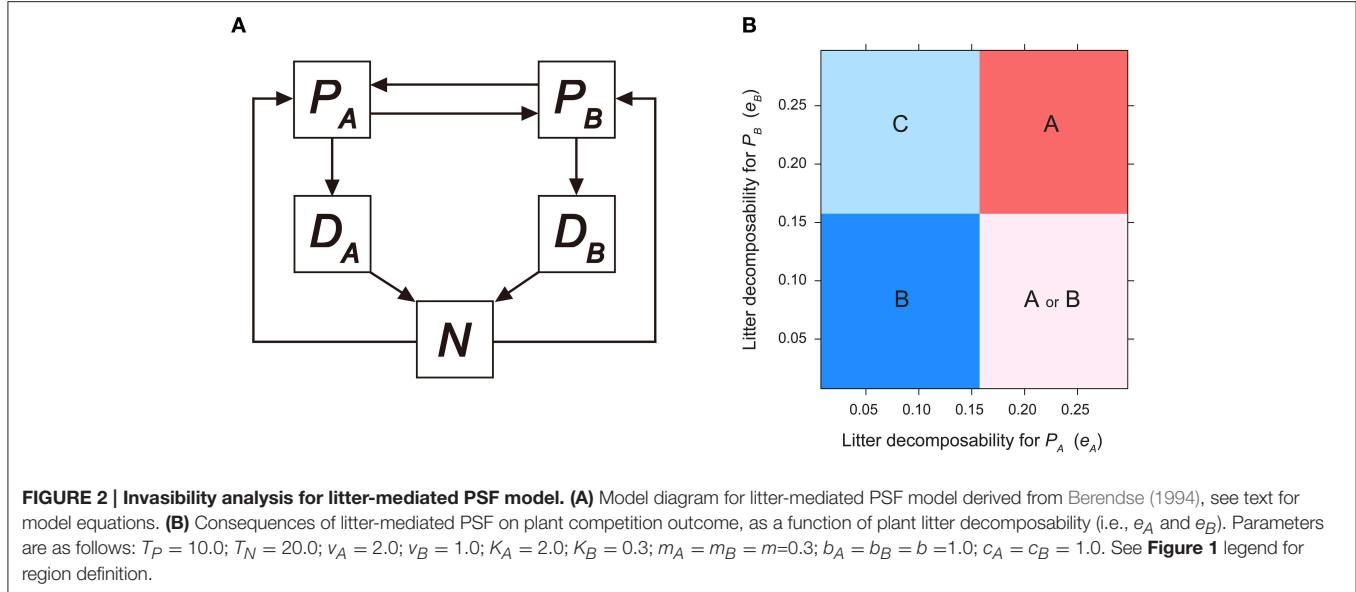
$$\frac{dP_B}{dt} = \frac{v_B N}{K_B + N}\left(1 - \frac{c_A P_A + P_B}{T_P}\right)P_B - m_B P_B \quad (11)$$

Here, v_i and $K_i (i = A$ or $B)$ represent the maximum rate and half-saturation constant for plant species i $(i = A$ or $B)$ to uptake plant-available nutrients in the soil, N. The term $v_i N/(K_i + N)$ in Equations (10) and (11) represent how the per capita growth rate of P_i is influenced by nutrient availability; the term in the parenthesis denotes growth limitation due to space competition, and the last term represents plant mortality (with species-specific mortality rate, m_i). To ensure potential coexistence of plant species, in the following analysis we considered trade-offs between nutrient uptake strategies: P_A (or P_B) tends to be competitively superior with high nutrient (low nutrient) levels (Miki and Kondoh, 2002; Clark et al., 2005). When parameterizing the model, we set $v_A > v_B$ while $v_A/K_A < v_B/K_B$.

Dead plant materials from P_A and P_B enter the plant-unavailable litter pool, with nutrient content denoted by D_A and D_B, respectively. The amount of nutrient transferred was calculated by the number of dead individuals multiplied by nutrient content per individual, b_i (i.e., the first term in Equations (12) and (13)).

$$\frac{dD_A}{dt} = b_A m_A P_A - e_A D_A \quad (12)$$

$$\frac{dD_B}{dt} = b_B m_B P_B - e_B D_B \quad (13)$$

FIGURE 2 | Invasibility analysis for litter-mediated PSF model. (A) Model diagram for litter-mediated PSF model derived from Berendse (1994), see text for model equations. **(B)** Consequences of litter-mediated PSF on plant competition outcome, as a function of plant litter decomposability (i.e., e_A and e_B). Parameters are as follows: $T_P = 10.0$; $T_N = 20.0$; $v_A = 2.0$; $v_B = 1.0$; $K_A = 2.0$; $K_B = 0.3$; $m_A = m_B = m = 0.3$; $b_A = b_B = b = 1.0$; $c_A = c_B = 1.0$. See **Figure 1** legend for region definition.

$$\frac{dN}{dt} = e_A D_A + e_B D_B - \frac{b_A v_A N}{K_A + N}\left(1 - \frac{P_A + c_B P_B}{T_P}\right)P_A$$
$$- \frac{b_B v_B N}{K_B + N}\left(1 - \frac{c_A P_A + P_B}{T_P}\right)P_B. \quad (14)$$

The second term in Equations (12) and (13) represents nutrient release from biological components through plant litter decomposition, with species-specific decomposition rate, e_i. For simplicity, it is assumed that litter chemical quality determines its decomposition rate (Cornwell et al., 2008), excluding the role of saprophytic microbes (but see Knops et al., 2002; Miki et al., 2010; Miki, 2012). Mineralized nutrients are returned to the plant-available nutrient pool and taken up by plants for population growth. Under this simple framework, the dynamic system was assumed to be closed, such that $b_A P_A(t) + b_B P_B(t) + D_A(t) + D_B(t) + N(t) = const. \equiv T_N$, where T_N represents the system's total nutrient content (but see Menge et al., 2009 for an opened ecosystem model).

The community outcome of the litter-mediated PSF model can be characterized by combining local stability analysis and invasibility analysis. We set $c_A = c_B = 1$, $m_A = m_B = m$, and $b_A = b_B = b$ to examine the effects of litter decomposition rates, e_A and e_B. The monoculture equilibrium of plant species i exhibits the following form: $E_i(P_i^* = T_P - T_P m(K_i + N_i^*)/(v_i N_i^*), P_j^* = 0)$, where N_i^* is the corresponding equilibrium for soil nutrient pool when the system is at monoculture of P_i ($i = A$ or B). By applying invasibility analysis to the litter-mediated PSF model, we derive that plant P_B can invade the P_A-monoculture (i.e., E_A) when:

$$N_A^* < \frac{K_A v_A - K_B v_B}{v_A - v_B} \equiv \hat{N}, \quad (15)$$

while the condition for P_A to invade E_B is calculated as follows:

$$N_B^* > \hat{N}. \quad (16)$$

To solve N_i^* (and corresponding plant litter decomposition rate), which fulfill Equations (15) and (16), we substitute Equations (10–13) into Equation (14) to derive the quadratic equation of N_i^* for each monoculture equilibrium as follows:

$$f_i\left(N_i^*\right) \equiv (e_i v_i) N_i^{*2} + \left[(e_i + m)(v_i - m)bT_P - e_i v_i T_N\right]N_i^*$$
$$+ \left[-K_i bm T_P (e_i + m)\right] = 0. \quad (17)$$

The above equation has one unique positive root due to the positive quadratic coefficient but negative constant term. The inequality $f_A(\hat{N}) > 0$ must be satisfied to obtain the N_A^* fulfilling the invasibility criterion for P_B (i.e., Equation (15)), resulting in the following:

$$e_A < \frac{mbT_P v_A \left[(K_A v_B - K_B v_A) - m(K_A - K_B)\right]}{v_A(K_A v_B - K_B v_A)\left\{T_N - \left[(v_A - m) - \frac{(v_A - v_B)K_A m}{(K_A v_B - K_B v_A)}\right]\frac{bT_P}{v_A} - \left[\frac{K_A v_B - K_B v_A}{v_A - v_B}\right]\right\}} \equiv e_A^*, \quad (18)$$

assuming that T_N is sufficiently large and $m < (K_A v_B - K_B v_A)/(K_A - K_B)$. This indicates when litter decomposition rate of P_A is sufficiently low, the soil nutrient pool size of P_A-cultivated soils is not maintained at higher levels, and consequently cannot prevent P_B invasion (which is assumed to be competitively superior under low soil nutrients). Similarly, $f_B(N_B^*)$ has a root satisfying Equation (16), and thus P_A can invade, when $f_B(\hat{N}) < 0$. This leads to the following inequality:

$$e_B > \frac{mbT_Pv_B\left[(K_Av_B - K_Bv_A) - m(K_A - K_B)\right]}{v_B(K_Av_B - K_Bv_A)\left\{T_N - \left[(v_B - m) - \frac{(v_A - v_B)K_Bm}{(K_Av_B - K_Bv_A)}\right]\frac{bT_P}{v_B} - \left[\frac{K_Av_B - K_Bv_A}{v_A - v_B}\right]\right\}} \equiv e_B^*, \tag{19}$$
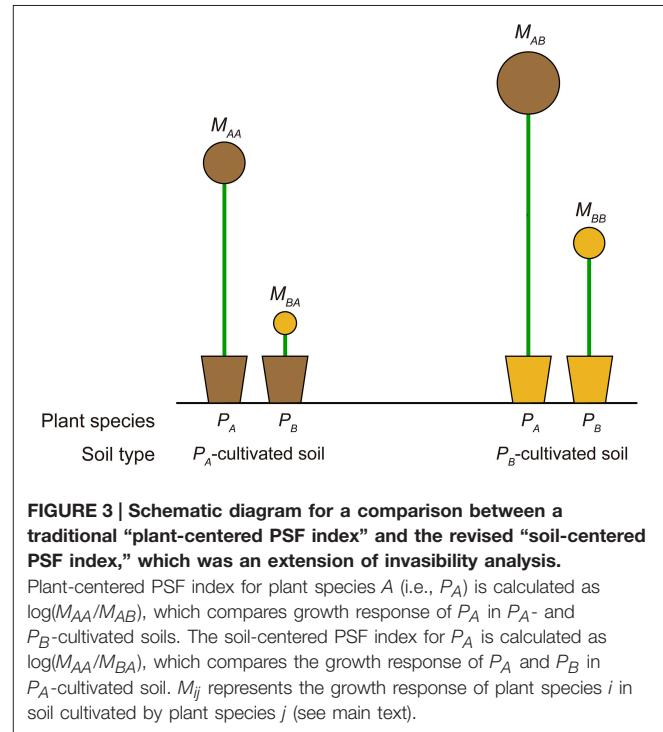
suggesting that P_A can invade $\boldsymbol{E_B}$ when litter decomposition rate of P_B is sufficiently fast (i.e., $e_B > e_B^*$). When $e_B > e_B^*$, the soil nutrient pool size of P_B-cultivated soil is maintained at higher levels and provides P_A a growth advantage during invasion. When the two invasibility criteria are combined, the parameter space of e_A and e_B is categorized into four regions (**Figure 2B**). Coexistence (region C in **Figure 2B**) is possible when litter produced by P_A decomposes at a sufficiently slower rate, but litter of P_B decomposes sufficiently faster (i.e., $e_A < e_A^*$, and $e_B > e_B^*$). Dominance of P_A (region A in **Figure 2B**) occurs when $e_A < e_A^*$, and dominance of P_B (region B in **Figure 2B**) occurs when $e_B > e_B^*$. When litter produced by P_A decomposes at a sufficiently faster rate and P_B litter decomposes sufficiently slow (i.e., $e_A > e_A^*$, and $e_B < e_B^*$), the system has alternative stable states of either $\boldsymbol{E_A}$ or $\boldsymbol{E_B}$, depending on the initial plant species density (region A or B in **Figure 2B**).

The Effectiveness of Invasion Analysis and a Revised PSF Index

Based on invasibility analysis, we derived coexistence criteria for both microbial- and litter-mediated PSF models. Invasiveness of one plant species depends on how the resident-cultivated soil affects the growth of the invader compared to the resident, and the competition outcome is then assessed by the mutual invasion between plant species. Invasion is possible when the resident-cultivated soil microbial community has less positive effect on the resident species (e.g., $\alpha_A - \alpha_B < 0$ for microbial-mediated PSF), or when nutrient cycling has reduced benefit for the resident species (e.g., $e_A < e_A^*$ for litter-mediated PSF). The rationale for these criteria is consistent with theoretical models, which indicate invasiveness is determined by the growth response of the invader in resident soil (Eppstein and Molofsky, 2007; Turnbull et al., 2010; Suding et al., 2013).

PSF strength for a target plant species is commonly quantified in empirical studies by comparing the growth response of the target plant species in soils cultivated by different species (i.e., a "plant-centered PSF index"). For example, let M_{ij} represent the growth response of plant species i in plant species j soil; the plant-centered PSF index is commonly calculated as $\log(M_{ii}/M_{ij})$. The invasibility analysis result provided here, which suggested that the competition outcome depended on how the resident-cultivated soil influenced the invader's growth compared with that of the resident, indicated the need for a revision of this metric. Based on the analytical results provided in the previous section, we argue the PSF quantified by comparing the competing plant species' growth responses in the target plant-cultivated soil were a better predictor for competition outcomes (i.e., a "soil-centered PSF index" for plant species i, calculated as $\log(M_{ii}/M_{ji})$). These soil-centered indices have been used in studies regarding a home-field advantage in litter decomposition (e.g., Ayres et al., 2009;

Milcu and Manning, 2011), but to the best of our knowledge, not yet in empirical PSF studies. **Figure 3** provides an illustrative example why the revised metric should be considered. Application of the commonly used PSF metric indicated that PSF for P_A was negative (i.e., $\log(M_{AA}/M_{AB}) < 0$), while that for P_B was positive (i.e., $\log(M_{BB}/M_{BA}) > 0$), predicting a P_B monoculture. However, in this example P_A was outcompeting P_B in soil environment cultivated by both P_A and P_B. The actual competition outcome should be the competitive exclusion of P_B by P_A, which can be accurately predicted by the soil-centered PSF index derived from the invasibility analysis (i.e., soil-centered PSF index for P_A was $\log(M_{AA}/M_{BA}) > 0$, while that for P_B was $\log(M_{BB}/M_{AB}) < 0$). In fact, out of all 24 possible plant growth response outcomes of a two-plant transplant experiment, the traditional plant-centered PSF index only correctly predicted half of the competitive outcomes, while the soil-centered PSF index correctly predicted all outcomes (see Figures S1–S4). However, we noted that when comparing growth response of different plants, biomass should first be normalized against their growth in uncultivated soils lacking plant growth (e.g., field-collected bare soil or autoclaved soils) to examine intrinsic biomass differences between different plant species. This reference soil choice can be critical, and sometimes challenging, however we believe studies can reveal a higher controlling power of PSF on community structure by quantifying PSF strength using a revised PSF index.

FIGURE 3 | Schematic diagram for a comparison between a traditional "plant-centered PSF index" and the revised "soil-centered PSF index," which was an extension of invasibility analysis. Plant-centered PSF index for plant species A (i.e., P_A) is calculated as $\log(M_{AA}/M_{AB})$, which compares growth response of P_A in P_A- and P_B-cultivated soils. The soil-centered PSF index for P_A is calculated as $\log(M_{AA}/M_{BA})$, which compares the growth response of P_A and P_B in P_A-cultivated soil. M_{ij} represents the growth response of plant species i in soil cultivated by plant species j (see main text).

Future Directions

Toward an Integration of Microbial- and Litter-mediated PSF

The majority of PSF modeling papers to date has specifically addressed a single PSF mechanism or were phenomenological studies and did not assume a specific mechanism. The influence of PSF on plant and microbial communities can be better predicted by integrating microbial- and litter-mediated PSF in future research (de Deyn et al., 2004; Manning et al., 2008). Bever et al. (2010) reviewed the role of root-associated microbes in structuring plant resource partitioning, however plant nutrient uptake was not coupled with nutrient cycling in their framework. The role of saprophytic microbes in litter-mediated PSF is an interesting and important starting point to combine the two mechanisms, as microbial decomposers ultimately drive litter decomposition (Knops et al., 2002; Miki et al., 2010; Miki, 2012). The microbial decomposer community has been emphasized during discussions of differential litter decomposition rates at different sites (Ayres et al., 2009; Austin et al., 2014), and predictions of carbon storage in response to climate change (Allison et al., 2010; Wieder and Allison, 2013). However, the effects of decomposer community structure on plant competition outcomes are rarely explicitly discussed within the context of PSF. Recent theoretical studies revealed that community structure of microbial decomposers had critical consequences when predicting plant community structure and their responses to anthropogenic disturbance via litter-mediated PSF (Miki et al., 2010; Miki, 2012). In particular, Miki et al. (2010) demonstrated that functional diversity of microbial decomposers can weaken the positive litter-mediated PSF controlled by plant litter quality, thus facilitating plant coexistence.

Microbial decomposers are engaged in a broad range of interactions, which also provides linkages between litter- and microbial-mediated PSF. For example, in addition to providing nutrients via their active control on litter decomposition, microbial decomposers are directly involved in nutrient competition with plants (i.e., through immobilization). The competition strength depends on the plant's nutrient uptake strategies and litter quality (Daufresne and Loreau, 2001; Schimel and Bennett, 2004; Ushio et al., 2009). Miki et al. (2010) demonstrated these traits therefore influence the outcome of litter-mediated PSF, other than simply altering decomposition rates. Root-associated microbes, which were more commonly discussed in microbial-mediated PSF, might also indirectly influence litter-mediated PSF. For example, the competition between plants and microbial decomposers could be modified when mycorrhizal fungi have the ability to take up organic nutrients. Studies have reported nutrient uptake by ectomycorrhizal and ericoid mycorrhizal fungi, which resulted in decomposer carbon starvation and hence slower litter decomposition rates accompanied by increased soil carbon storage (Read and Perez-Moreno, 2003; Orwin et al., 2011; Averill et al., 2014). In addition to indirect modification of litter decomposition rates, root-associated microbes also altered plant litter input through their effects on plant productivity (Mitchell, 2003; van der Heijden et al., 2008; Orwin et al., 2011; Ke et al.,

2015). In conclusion, the functional composition and community dynamics of microbes can interact with litter-mediated PSF, even when the microbes themselves are not directly engaged in litter decomposition. Our studies support an explicit consideration of the interdependency between the two PSF mechanisms, which can provide new predictions related to both plant and microbial community dynamics.

Predicting Species' PSF Strength through a Trait-based Ecological Approach

PSF acts as a structuring force in plant and microbial communities, therefore one important task for ecologists is to improve our prediction of species' PSF strength. The trait-based approach is a promising new approach, which has been widely applied to study plant community assembly via aboveground interactions (McGill et al., 2006; de Bello et al., 2010; Adler et al., 2013). Recent studies also applied trait-based approaches to elucidate microbial community assembly and the evolution of microbial life forms (Aguilar-Trigueros et al., 2014; Crowther et al., 2014). However, attempts to apply such approach remain scarce for PSF studies (van der Putten et al., 2013) (but see Baxendale et al., 2014). Ke et al. (2015) employed a trait-based approach to a theoretical PSF study, which assessed the sensitivity of PSF strength to various plant and microbial traits. Results revealed that the relative importance of litter decomposability compared to other plant demographic traits in determining PSF strength changes with the community composition of root-associated microbes. Baxendale et al. (2014) provided the first empirical data that associated PSF with plant foliar traits mentioned by the leaf economic spectrum (Wright et al., 2004). Results suggested that trait-based plant classification was most informative when plants are grown in mixtures (i.e., under interspecific competition), and plants responded positively to nutrient environments cultivated by plants with similar traits. One ultimate goal for trait-based studies is to predict shifts in ecological processes, such as PSF along abiotic gradients (McGill et al., 2006). The above studies suggested some traits might lose their impact along the abiotic gradient, as trait influence was also determined by biotic interactions with other plant and microbial species. We propose that additional studies linking plant and microbial traits to PSF strength or predicting species' PSF under different conditions are warranted.

Combining PSF with General Coexistence Theory

Empirical and theoretical ecologists have long puzzled over the maintenance mechanisms of plant species coexistence. Chesson (2000) proposed that species coexistence was maintained by a combination of equalizing and stabilizing forces. This can be defined as equalizing forces that minimize differences in species' intrinsic growth rates, while stabilizing forces that limit species' per capita growth rates as species become common (Chesson, 2000; Adler et al., 2007). The establishment of a theoretical link between PSF-driven coexistence and classic coexistence theories is our final suggestion for future PSF research. Under Chesson's (2000) coexistence framework, PSF has been most commonly thought to act as a stabilizing mechanism by generating negative density-dependence (Bell et al., 2006;

Yamazaki et al., 2008; Bagchi et al., 2010, 2014) or nutrient usage differentiation (Clark et al., 2005; Daufresne and Hedin, 2005; Bever et al., 2010). However, PSF can also facilitate plant coexistence through equalizing mechanisms. For example, if the competitive inferior species can form mutualistic associations with beneficial microbes (e.g., mycorrhizal fungi), which act in a density-independent manner, the presence of PSF can decrease fitness differences between competing species. Similar, equalizing examples in pathogens were also thoroughly reviewed (Mordecai, 2011, 2013a). If such equalizing forces of PSF are strong, plant coexistence can be maintained despite weak stabilizing forces by host specific pathogens or resource partitioning (Adler et al., 2007; Bever et al., 2010). The invasibility analysis provided in the present review is a viable starting point, as the invader's long-term low-density growth rate was one most important metric in Chesson's framework (Chesson, 2000). Future theoretical studies can continue separating the equalizing and stabilizing forces in current PSF models, and mechanistically link plant and microbial traits to the two coexistence mechanisms.

In conclusion, PSF research has attracted attention from many empirical ecologists and demonstrated notable success in using PSF to predict plant community patterns. Theoretical models, based on fundamental mechanistic-PSF models, have also substantially contributed to our knowledge. We believe the integration of multiple PSF mechanisms into the well-developed theoretical framework in ecology, such as stage-structure (e.g., Ke et al., 2015) and metacommunity (e.g., Loeuille and Leibold, 2014) models will produce new insights to understand plant and microbial community structure and dynamics.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01066

References

Adler, F. R., and Muller-Landau, H. C. (2005). When do localized natural enemies increase species richness? *Ecol. Lett.* 8, 438–447. doi: 10.1111/j.1461-0248.2005.00741.x

Adler, P. B., Fajardo, A., Kleinhesselink, A. R., and Kraft, N. J. B. (2013). Trait-based tests of coexistence mechanisms. *Ecol. Lett.* 16, 1294–1306. doi: 10.1111/ele.12157

Adler, P. B., HilleRisLambers, J., and Levine, J. M. (2007). A niche for neutrality. *Ecol. Lett.* 10, 95–104. doi: 10.1111/j.1461-0248.2006.00996

Aguilar-Trigueros, C. A., Powell, J. R., Anderson, I. C., Antonovics, J., and Rillig, M. C. (2014). Ecological understanding of root-infecting fungi using trait-based approaches. *Trends Plant Sci.* 19, 432–438. doi: 10.1016/j.tplants.2014.02.006

Aguilera, A. G. (2011). The influence of soil community density on plant-soil feedbacks: an important unknown in plant invasion. *Ecol. Model.* 222, 3413–3420. doi: 10.1016/j.ecolmodel.2011.06.018

Allison, S. D., Wallenstein, M. D., and Bradford, M. A. (2010). Soil-carbon response to warming dependent on microbial physiology. *Nat. Geosci.* 3, 336–340. doi: 10.1038/ngeo846

Augspurger, C. K. (1983). Seed dispersal of the tropical tree, *Platypodium Elegans*, and the escape of its seedlings from fungal pathogens. *J. Ecol.* 71, 759–771. doi: 10.2307/2259591

Austin, A. T., Vivanco, L., González-Arzac, A., and Pérez, L. I. (2014). There's no place like home? An exploration of the mechanisms behind plant litter-decomposer affinity in terrestrial ecosystems. *New Phytol.* 204, 307–314. doi: 10.1111/nph.12959

Averill, C., Turner, B. L., and Finzi, A. C. (2014). Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature* 505, 543–545. doi: 10.1038/nature12901

Ayres, E., Steltzer, H., Simmons, B. L., Simpson, R. T., Steinweg, J. M., Wallenstein, M. D., et al. (2009). Home-field advantage accelerates leaf litter decomposition in forests. *Soil Biol. Biochem.* 41, 606–610. doi: 10.1016/j.soilbio.2008.12.022

Bagchi, R., Gallery, R. E., Gripenberg, S., Gurr, S. J., Narayan, L., Addis, C. E., et al. (2014). Pathogens and insect herbivores drive rainforest plant diversity and composition. *Nature* 506, 85–88. doi: 10.1038/nature12911

Bagchi, R., Swinfield, T., Gallery, R. E., Lewis, O. T., Gripenberg, S., Narayan, L., et al. (2010). Testing the Janzen-Connell mechanism: pathogens cause overcompensating density dependence in a tropical tree. *Ecol. Lett.* 13, 1262–1269. doi: 10.1111/j.1461-0248.2010.01520.x

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Baxendale, C., Orwin, K. H., Poly, F., Pommier, T., and Bardgett, R. D. (2014). Are plant-soil feedback responses explained by plant traits? *New Phytol.* 204, 408–423. doi: 10.1111/nph.12915

Beckstead, J., Meyer, S. E., Connolly, B. M., Huck, M. B., and Street, L. E. (2010). Cheatgrass facilitates spillover of a seed bank pathogen onto native grass species. *J. Ecol.* 98, 168–177. doi: 10.1111/j.1365-2745.2009.01599.x

Beckstead, J., and Parker, I. M. (2003). Invasiveness of *Ammophila arenaria*: release from soil-borne pathogens? *Ecology* 84, 2824–2831. doi: 10.1890/02-0517

Bell, T., Freckleton, R. P., and Lewis, O. T. (2006). Plant pathogens drive density-dependent seedling mortality in a tropical tree. *Ecol. Lett.* 9, 569–574. doi: 10.1111/j.1461-0248.2006.00905.x

Berendse, F. (1994). Litter decomposability – a neglected component of plant fitness. *J. Ecol.* 82, 187–190. doi: 10.2307/2261398

Berendse, F., Bobbink, R., and Rouwenhorst, G. (1989). A comparative study on nutrient cycling in wet heathland ecosystems II. Litter decomposition and nutrient mineralization. *Oecologia* 78, 338–348. doi: 10.1007/BF00379107

Berendse, F., Oudhof, H., and Bol, J. (1987). A comparative study on nutrient cycling in wet heathland ecosystems I. Litter production and nutrient losses from the plant. *Oecologia* 74, 174–184. doi: 10.1007/BF00379357

Bever, J. D. (1999). Dynamics within mutualism and the maintenance of diversity?: inference from a model of interguild frequency dependence. *Ecol. Lett.* 2, 52–62. doi: 10.1046/j.1461-0248.1999.21050.x

Bever, J. D. (2002). Negative feedback within a mutualism: host-specific growth of mycorrhizal fungi reduces plant benefit. *Proc. Biol. Sci.* 269, 2595–2601. doi: 10.1098/rspb.2002.2162

Bever, J. D. (2003). Soil community feedback and the coexistence of competitors: conceptual frameworks and empirical tests. *New Phytol.* 157, 465–473. doi: 10.1046/j.1469-8137.2003.00714.x

Bever, J. D., Dickie, I. A., Facelli, E., Facelli, J. M., Klironomos, J., Moora, M., et al. (2010). Rooting theories of plant community ecology in microbial interactions. *Trends Ecol. Evol.* 25, 468–478. doi: 10.1016/j.tree.2010.05.004

Bever, J. D., Platt, T. G., and Morton, E. R. (2012). Microbial population and community dynamics on plant roots and their feedbacks on plant community. *Annu. Rev. Microbiol.* 66, 265–283. doi: 10.1146/annurev-micro-092611-150107

Bever, J. D., Westover, K. M., and Antonovics, J. (1997). Incorporating the soil community into plant population dynamics: the utility of the feedback approach. *J. Ecol.* 85, 561–573. doi: 10.2307/2960528

Binley, D., and Giardina, C. (1998). Why do tree species affect soil? the warp and woof of tree-soil interaction. *Biogeochemistry* 42, 89–106. doi: 10.1023/A:1005948126251

Bonanomi, G., Antignani, V., Capodilupo, M., and Scala, F. (2010). Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. *Soil Biol. Biochem.* 42, 136–144. doi: 10.1016/j.soilbio.2009.10.012

Bonanomi, G., Giannino, F., and Mazzoleni, S. (2005). Negative plant-soil feedback and species coexistence. *Oikos* 111, 311–321. doi: 10.1111/j.0030-1299.2005.13975.x

Callaway, R. M., Thelen, G. C., Rodriguez, A., and Holben, W. E. (2004). Soil biota and exotic plant invasion. *Nature* 427, 731–733. doi: 10.1038/nature02322

Cardinale, B. J., Duffy, J. E., Gonzalez, A., Hooper, D. U., Perrings, C., Venail, P., et al. (2012). Biodiversity loss and its impact on humanity. *Nature* 489, 326–326. doi: 10.1038/nature11373

Chesson, P. (2000). Mechanisms of maintenance of species diversity. *Annu. Rev. Ecol. Syst.* 31, 343–366. doi: 10.1146/annurev.ecolsys.31.1.343

Clark, B. R., Hartley, S. E., Suding, K. N., and de Mazancourt, C. (2005). The effect of recycling on plant competitive hierarchies. *Am. Nat.* 165, 609–622. doi: 10.1086/430074

Connell, J. H. (1971). "On the role of natural enemies in preventing competitive exclusion in some marine animals and in rain forest trees," in *Dynamics of Populations*, eds P. J. den Boer and G. R. Gradwell (Wageningen: Centre for Agricultural Publishing and Documentation), 298–312.

Cornwell, W. K., Cornelissen, J. H. C., Amatangelo, K., Dorrepaal, E., Eviner, V. T., Godoy, O., et al. (2008). Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. *Ecol. Lett.* 11, 1065–1071. doi: 10.1111/j.1461-0248.2008.01219.x

Crowther, T. W., Maynard, D. S., Crowther, T. R., Peccia, J., Smith, J. R., and Bradford, M. A. (2014). Untangling the fungal niche: the trait-based approach. *Front. Microbiol.* 5:579. doi: 10.3389/fmicb.2014.00579

Daufresne, T., and Hedin, L. O. (2005). Plant coexistence depends on ecosystem nutrient cycles: extension of the resource-ratio theory. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9212–9217. doi: 10.1073/pnas.0406427102

Daufresne, T., and Loreau, M. (2001). Ecological stoichiometry, primary producer-decomposer interactions, and ecosystem persistence. *Ecology* 82, 3069–3082. doi: 10.1890/0012-9658(2001)082[3069:ESPPDI]2.0.CO;2

de Bello, F., Lavorel, S., Díaz, S., Harrington, R., Cornelissen, J. H. C., Bardgett, R. D., et al. (2010). Towards an assessment of multiple ecosystem processes and services via functional traits. *Biodivers. Conserv.* 19, 2873–2893. doi: 10.1007/s10531-010-9850-9

de Deyn, G. B., Raaijmakers, C. E., and van der Putten, W. H. (2004). Plant community development is affected by nutrients and soil biota. *J. Ecol.* 824–834. doi: 10.1111/j.0022-0477.2004.00924.x

Dickie, I. A., Schnitzer, S. A., Reich, P. B., and Hobbie, S. E. (2005). Spatially disjunct effects of co-occurring competition and facilitation. *Ecol. Lett.* 8, 1191–1200. doi: 10.1111/j.1461-0248.2005.00822.x

Ehrenfeld, J. G., Ravit, B., and Elgersma, K. (2005). Feedback in the plant-soil system. *Annu. Rev. Environ. Resour.* 30, 75–115. doi: 10.1146/annurev.energy.30.050504.144212

Eppinga, M. B., Kaproth, M. A., Collins, A. R., and Molofsky, J. (2011). Litter feedbacks, evolutionary change and exotic plant invasion. *J. Ecol.* 99, 503–514. doi: 10.1111/j.1365-2745.2010.01781.x

Eppinga, M. B., Rietkerk, M., Dekker, S. C., and de Ruiter, P. C. (2006). Accumulation of local pathogens: a new hypothesis to explain exotic plant invasion. *Oikos* 114, 168–176. doi: 10.1111/j.2006.0030-1299.14625.x

Eppstein, M. J., Bever, J. D., and Molofsky, J. (2006). Spatio-temporal community dynamics induced by frequency dependent interactions. *Ecol. Modell.* 197, 133–147. doi: 10.1016/j.ecolmodel.2006.02.039

Eppstein, M. J., and Molofsky, J. (2007). Invasiveness in plant communities with feedbacks. *Ecol. Lett.* 10, 253–263. doi: 10.1111/j.1461-0248.2007.01017.x

Farrer, E. C., and Goldberg, D. E. (2009). Litter drives ecosystem and plant community changes in cattail invasion. *Ecol. Appl.* 19, 398–412. doi: 10.1890/08-0485.1

Fukami, T., and Nakajima, M. (2011). Community assembly: alternative stable states or alternative transient states? *Ecol. Lett.* 14, 973–984. doi: 10.1111/j.1461-0248.2011.01663.x

Fukami, T., and Nakajima, M. (2013). Complex plant-soil interactions enhance plant species diversity by delaying community convergence. *J. Ecol.* 101, 316–324. doi: 10.1111/1365-2745.12048

Fukano, Y., Tachiki, Y., Yahara, T., and Iwasa, Y. (2013). Soil disturbances can suppress the invasion of alien plants under plant-soil feedback. *Ecol. Model.* 260, 42–49. doi: 10.1016/j.ecolmodel.2013.03.022

Grime, J. P. (1977). Evidence for the existence of three primary strategies in plants and its relevance of ecological and evolutionary theory. *Am. Nat.* 111, 1169–1194. doi: 10.1086/283244

Hector, A., Schmid, B., Beierkuhnlein, C., Caldeira, M. C., Diemer, M., Dimitrakopoulos, P. G., et al. (1999). Plant diversity and productivity experiments in European grassland. *Science* 286, 1123–1127. doi: 10.1126/science.286.5442.1123

Hendriks, M., Mommer, L., de Caluwe, H., Smit-tiekstra, A. E., van der Putten, W. H., and de Kroon, H. (2013). Independent variations of plant and soil mixtures reveal soil feedback effects on plant community overyielding. *J. Ecol.* 101, 287–297. doi: 10.1111/1365-2745.12032

Hendriks, M., Visser, E. J. W., Visschers, I. G. S., Aarts, B. H. J., de Caluwe, H., Smit-Tiekstra, A. E., et al. (2015). Root responses of grassland species to spatial heterogeneity of plant-soil feedback. *Funct. Ecol.* 29, 177–186. doi: 10.1111/1365-2435.12367

Hsu, S. B., Hubbell, S., and Waltman, P. (1977). A mathematical theory for single-nutrient competition in continuous cultures of micro-organisms. *SIAM J. Appl. Math.* 32, 366–383. doi: 10.1137/0132030

Hubbell, S. (1997). A unified theory of biogeography and relative species abundance and its application to tropical rain forests and coral reefs. *Coral Reefs* 16, S9–S21. doi: 10.1007/s003380050237

Inderjit, and van der Putten, W. H. (2010). Impacts of soil microbial communities on exotic plant invasions. *Trends Ecol. Evol.* 25, 512–519. doi: 10.1016/j.tree.2010.06.006

Janzen, D. H. (1970). Herbivores and the number of tree species in tropical forest. *Am. Nat.* 104, 501–528

Johnson, D. J., Beaulieu, W. T., Bever, J. D., and Clay, K. (2012). Conspecific negative density dependence and forest diversity. *Science* 336, 904–907. doi: 10.1126/science.1220269

Jones, C. G., Lawton, J. H., and Schachak, M. (1994). Organisms as ecosystems engineers. *Oikos* 69, 373–386. doi: 10.2307/3545850

Kardol, P., Bezemer, T. M., and van der Putten, W. H. (2006). Temporal variation in plant-soil feedback controls succession. *Ecol. Lett.* 9, 1080–1088. doi: 10.1111/j.1461-0248.2006.00953.x

Kardol, P., Cornips, N. J., van Kempen, M. M. L., Bakx-Schotman, J. M. T., and van der Putten, W. H. (2007). Microbe-mediated plant–soil feedback causes historical contingency effects in plant community assembly. *Ecol. Monogr.* 77, 147–162. doi: 10.1890/06-0502

Kardol, P., De Dyne, G. B., Laliberte, E., Mariotte, P., and Hawkes, C. V. (2013). Biotic plant-soil feedbacks across temporal scales. *J. Ecol.* 101, 309–315. doi: 10.1111/1365-2745.12046

Ke, P.-J., Miki, T., and Ding, T.-S. (2015). The soil microbial community predicts the importance of plant traits in plant-soil feedback. *New Phytol.* 206, 349–341. doi: 10.1111/nph.13215

Keane, R. M., and Crawley, M. J. (2002). Exotic plant invasion and the enemy release hypothesis. *Trends Ecol. Evol.* 17, 164–170. doi: 10.1016/S0169-5347(02)02499-0

Klironomos, J. N. (2002). Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature* 417, 67–70. doi: 10.1038/417067a

Knops, J. M. H., Bradley, K. L., and Wedin, D. A. (2002). Mechanisms of plant species impact on ecosystem nitrogen cycling. *Ecol. Lett.* 5, 454–466. doi: 10.1046/j.1461-0248.2002.00332.x

Kulmatiski, A., Beard, K. H., and Heavilin, J. (2012). Plant-soil feedbacks provide an additional explanation for diversity-productivity relationships. *Proc. Biol. Sci.* 279, 3020–3026. doi: 10.1098/rspb.2012.0285

Kulmatiski, A., Heavilin, J., and Beard, K. H. (2011). Testing predictions of a three-species plant-soil feedback model. *J. Ecol.* 99, 542–550. doi: 10.1111/j.1365-2745.2010.01784.x

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., et al. (2004). The metacommunity concept: a framework for multi-scale community ecology. *Ecol. Lett.* 7, 601–613. doi: 10.1111/j.1461-0248.2004.00608.x

Levine, J. M., Pachepsky, E., Kendall, B. E., Yelenik, S. G., and Lambers, J. H. R. (2006). Plant-soil feedbacks and invasive spread. *Ecol. Lett.* 9, 1005–1014. doi: 10.1111/j.1461-0248.2006.00949.x

Loeuille, N., and Leibold, M. A. (2014). Effects of local negative feedbacks on the evolution of species within metacommunities. *Ecol. Lett.* 17, 563–573. doi: 10.1111/ele.12258

Loreau, M., and Hector, A. (2001). Partitioning selection and complementarity in biodiversity experiments. *Nature* 412, 72–76. doi: 10.1038/35083573

Mack, K. M. L., and Bever, J. D. (2014). Coexistence and relative abundance in plant communities are determined by feedbacks when the scale of feedback and dispersal is local. *J. Ecol.* 102, 1195–1201. doi: 10.1111/1365-2745.12269

Mangan, S. A., Schnitzer, S. A., Herre, E. A., Mack, K. M. L., Valencia, M. C., Sanchez, E. I., et al. (2010). Negative plant-soil feedback predicts tree-species relative abundance in a tropical forest. *Nature* 466, 752–755. doi: 10.1038/nature09273

Manning, P., Morrison, S. A., Bonkowski, M., and Bardgett, R. D. (2008). Nitrogen enrichment modifies plant community structure via changes to plant-soil feedback. *Oecologia* 157, 661–673. doi: 10.1007/s00442-008-1104-0

Maron, J. L., Marler, M., Klironomos, J. N., and Cleveland, C. C. (2011). Soil fungal pathogens and the relationship between plant diversity and productivity. *Ecol. Lett.* 14, 36–41. doi: 10.1111/j.1461-0248.2010.01547.x

Mazzoleni, S., Bonanomi, G., Giannino, F., Incerti, G., Dekker, S. C., and Rietkerk, M. (2010). Modelling the effects of litter decomposition on tree diversity patterns. *Ecol. Modell.* 221, 2784–2792. doi: 10.1016/j.ecolmodel.2010.08.007

Mazzoleni, S., Bonanomi, G., Giannino, F., Rietkerk, M., Dekker, S. C., and Zucconi, F. (2007). Is plant biodiversity driven by decomposition processes? An emerging new theory on plant diversity. *Community Ecol.* 8, 103–109. doi: 10.1556/ComEc.8.2007.1.12

McGill, B. J., Enquist, B. J., Weiher, E., and Westoby, M. (2006). Rebuilding community ecology from functional traits. *Trends Ecol. Evol.* 21, 178–185. doi: 10.1016/j.tree.2006.02.002

Menge, D. N. L., Pacala, S. W., and Hedin, L. O. (2009). Emergence and maintenance of nutrient limitation over multiple timescales in terrestrial ecosystems. *Am. Nat.* 173, 164–175. doi: 10.1086/595749

Miki, T. (2009). A new graphical model for untangling complex relationships among environment, biodiversity, and ecosystem functioning. *Ecol. Res.* 24, 937–941. doi: 10.1007/s11284-008-0552-7

Miki, T. (2012). Microbe-mediated plant–soil feedback and its roles in a changing world. *Ecol. Res.* 27, 509–520. doi: 10.1007/s11284-012-0937-5

Miki, T., and Kondoh, M. (2002). Feedbacks between nutrient cycling and vegetation predict plant species coexistence and invasion. *Ecol. Lett.* 5, 624–633. doi: 10.1046/j.1461-0248.2002.00347.x

Miki, T., Ushio, M., Fukui, S., and Kondoh, M. (2010). Functional diversity of microbial decomposers facilitates plant coexistence in a plant-microbe-soil feedback model. *Proc. Nat. Acad. Sci. U.S.A.* 107, 14251–14256. doi: 10.1073/pnas.0914281107

Milcu, A., and Manning, P. (2011). All size classes of soil fauna and litter quality control the acceleration of litter decay in its home environment. *Oikos* 120, 1366–1370. doi: 10.1111/j.1600-0706.2010.19418.x

Mitchell, C. E. (2003). Trophic control of grassland production and biomass by pathogens. *Ecol. Lett.* 6, 147–155. doi: 10.1046/j.1461-0248.2003.00408.x

Mitchell, C. E., Agrawal, A. A., Bever, J. D., Gilbert, G. S., Hufbauer, R. A., Klironomos, J. N., et al. (2006). Biotic interactions and plant invasions. *Ecol. Lett.* 9, 726–740. doi: 10.1111/j.1461-0248.2006.00908.x

Mitchell, C. E., and Power, A. G. (2003). Release of invasive plants from fungal and viral pathogens. *Nature* 421, 625–627. doi: 10.1038/nature01317

Molofsky, J., and Bever, J. D. (2002). A novel theory to explain species diversity in landscapes: positive frequency dependence and habitat suitability. *Proc. Biol. Sci.* 269, 2389–2393. doi: 10.1098/rspb.2002.2164

Molofsky, J., Bever, J. D., and Antonovics, J. (2001). Coexistence under positive frequency dependence. *Proc. Biol. Sci.* 268, 273–277. doi: 10.1098/rspb.2000.1355

Molofsky, J., Bever, J. D., Antonovics, J., and Newman, T. J. (2002). Negative frequency dependence and the importance of spatial scale. *Ecology* 83, 21–27. doi: 10.2307/2680117

Mordecai, E. A. (2011). Pathogen impacts on plant communities: unifying theory, concepts, and empirical work. *Ecology* 81, 429–441. doi: 10.1890/10-2241.1

Mordecai, E. A. (2013a). Consequences of pathogen spillover for cheatgrass-invaded grassland coexistence, competitive exclusion, or priority effects. *Am. Nat.* 181, 737–747. doi: 10.1086/670190

Mordecai, E. A. (2013b). Despite spillover, a shared pathogen promotes native plant persistence in a cheatgrass-invaded grassland. *Ecology* 94, 2744–2753. doi: 10.1890/13-0086.1

Mordecai, E. A. (2015). Pathogens impact on plant diversity in variable environments. *Oikos* 124, 414–420. doi: 10.1111/oik.01328

Orwin, K. H., Kirschbaum, M. U. F., St John, M. G., and Dickie, I. A. (2011). Organic nutrient uptake by mycorrhizal fungi enhances ecosystem carbon storage: a model-based assessment. *Ecol. Lett.* 14, 493–502. doi: 10.1111/j.1461-0248.2011.01611.x

Packer, A., and Clay, K. (2000). Soil pathogens and spatial patterns of seedling mortality in a temperate tree. *Nature* 404, 278–281. doi: 10.1038/35005072

Petermann, J. S., Fergus, A. J. F., Turnbull, L. A., and Schmid, B. (2008). Janzen-Connell effects are widespread and strong enough to maintain diversity. *Ecology* 89, 2399–2406. doi: 10.1890/07-2056.1

Read, D. J., and Perez-Moreno, J. (2003). Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? *New Phytol.* 157, 475–492. doi: 10.1046/j.1469-8137.2003.00704

Reinhart, K. O. (2012). The organization of plant communities: negative plant-soil feedbacks and semiarid grasslands. *Ecology* 93, 2377–2385. doi: 10.1890/12-0486.1

Reinhart, K. O., and Callaway, R. M. (2006). Soil biota and invasive plants. *New Phytol.* 170, 445–457. doi: 10.1111/j.1469-8137.2006.01715.x

Reinhart, K. O., Packer, A., van der Putten, W. H., and Clay, K. (2003). Plant-soil biota interactions and spatial distribution of black cherry in its native and invasive ranges. *Ecol. Lett.* 6, 1046–1050. doi: 10.1046/j.1461-0248.2003.00539.x

Revilla, T. A., Veen, G. F., Eppinga, M. B., and Weissing, F. J. (2013). Plant-soil feedback and the coexistence of competing plants. *Theor. Ecol.* 6, 99–113. doi: 10.1007/s12080-012-0163-3

Reynolds, H. L., Packer, A., Bever, J. D., and Clay, K. (2003). Grassroot ecology: plant-microbe-soil interactions as drivers of plant community structure and dynamics. *Ecology* 84, 2281–2291. doi: 10.1890/02-0298

Schimel, J. P., and Bennett, J. (2004). Nitrogen mineralization: challenges of a changing paradigm. *Ecology* 85, 591–602. doi: 10.1890/03-8002

Schnitzer, S. A., Klironomos, J. N., HilleRisLambers, J., Kinkel, L. L., Reich, P. B., Xiao, K., et al. (2011). Soil microbes drive the classic plant diversity-productivity pattern. *Ecology* 92, 296–303. doi: 10.1890/10-0773.1

Sedio, B. E., and Ostling, A. M. (2013). How specialised must natural enemies be to facilitate coexistence among plants? *Ecol. Lett.* 16, 995–1003. doi: 10.1111/ele.12130

Sikes, B. A., Maherali, H., and Klironomos, J. N. (2012). Arbuscular mycorrhizal fungal communities change among three stages of primary sand dune succession but do not alter plant growth. *Oikos* 121, 1791–1800. doi: 10.1111/j.1600-0706.2012.20160.x

Suding, K. N., Harpole, W. S., Fukami, T., Kulmatiski, A., Macdougall, A. S., Stein, C., et al. (2013). Consequences of plant-soil feedbacks in invasion. *J. Ecol.* 101, 298–308. doi: 10.1111/1365-2745.12057

Swamy, V., and Terborgh, J. W. (2010). Distance-responsive natural enemies strongly influence seedling establishment patterns of multiple species in an Amazonian rain forest. *J. Ecol.* 98, 1096–1107. doi: 10.1111/j.1365-2745.2010.01686.x

Tansley, A. G. (1935). The use and abuse of vegetational concepts and terms. *Ecology* 16, 284–307.

Tilman, D. (1982). *Resource Competition and Community Structure*. Princeton, NJ: Princeton University Press.

Tilman, D. (1994). Competition and biodiversity in spatially structured habitats. *Ecology* 75, 2–16. doi: 10.2307/1939377

Tilman, D., Mattson, M., and Langer, S. (1981). Competition and nutrient kinetics along a temperature gradient: an experimental test of a mechanistic approach to niche theory. *Limnol. Oceanogr.* 26, 1020–1033. doi: 10.4319/lo.1981.26.6.1020

Turnbull, L. A., Levine, J. M., Fergus, A. J. F., and Petermann, J. S. (2010). Species diversity reduces invasion success in pathogen-regulated communities. *Oikos* 119, 1040–1046. doi: 10.1111/j.1600-0706.2009.17914.x

Umbanhowar, J., and McCann, K. (2005). Simple rules for the coexistence and competitive dominance of plants mediated by mycorrhizal fungi. *Ecol. Lett.* 8, 247–252. doi: 10.1111/j.1461-0248.2004.00714.x

Ushio, M., Miki, T., and Kitayama, K. (2009). Phenolic control of plant nitrogen acquisition through the inhibition of soil microbial decomposition processes: a plant-microbe competition model. *Microbes Environ.* 24, 180–187. doi: 10.1264/jsme2.ME09107

van der Heijden, M. G. A., Bardgett, R. D., and van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol. Lett.* 11, 296–310. doi: 10.1111/j.1461-0248.2007.01139.x

van der Putten, W. H., Bardgett, R. D., Bever, J. D., Bezemer, T. M., Casper, B. B., Fukami, T., et al. (2013). Plant-soil feedbacks: the past, the present and future challenges. *J. Ecol.* 101, 265–276. doi: 10.1111/1365-2745.12054

van der Putten, W. H., van Dijk, C., and Peters, B. A. M. (1993). Plant-specific soil-borne diseases contribute to succession in foredune vegetation. *Nature* 362, 53–56. doi: 10.1038/362053a0

Wieder, W. R., and Allison, S. D. (2013). Global soil carbon projections are improved by modelling microbial processes. *Nature Clim. Change* 3, 909–912. doi: 10.1038/NCLIMATE1951

Wright, I. J., Reich, P. B., Westoby, M., Ackerly, D. D., Baruch, Z., Bongers, F., et al. (2004). The worldwide leaf economics spectrum. *Nature* 12, 821–827. doi: 10.1038/nature02403

Yamazaki, M., Iwamoto, S., and Seiwa, K. (2008). Distance- and density-dependent seedling mortality caused by several diseases in eight tree species co-occurring in a temperate forest. *Plant Ecol.* 201, 181–196. doi: 10.1007/s11258-008-9531-x

Zee, P. C., and Fukami, T. (2015). Complex organism-environment feedback buffer species diversity against habitat fragmentation. *Ecography* 38, 1–10. doi: 10.1111/ecog.01027

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