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Host-Pathogen Interactions

Methods and Protocols

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Foreword

Colonization of eukaryotic hosts by bacteria causes disease in certain cases, and the distinction between commensalism, symbiosis, and opportunistic pathogenesis is blurred. Even an encounter with a primary pathogen has an unpredictable outcome and does not necessarily cause disease. This uncertainty is due to the complexity of bacterium-host interactions, which involve multiple factors, both bacterial and eukaryotic. Microbe-microbe interactions introduce further complexity into this scenario.

Genetic and molecular analysis has proven useful to dissect bacterium-host interactions in animals and plants, and in the last few decades has been enriched with novel approaches such as bioinformatics, single cell analysis, live imaging technology, and mathematical modeling. Aside from deepening our understanding of infectious diseases, multidisciplinary knowledge may inspire novel therapeutic strategies. For instance, identification of virulence functions may identify targets for future antibacterial drugs. In turn, the limitations and failures of antibacterial drugs may revive the one-century-old interest in phage therapy.

This book deals with bacterial-host interactions in pathogens and symbionts of humans and plants and describes state-of-the-art methods used in their study. Reviews of reductionist analysis in the laboratory are accompanied by descriptions of host-pathogen interactions in vivo and at the systems biology level. Altogether, the chapters of this book provide a broad, multidisciplinary view of bacterial pathogenesis from the twenty-first century perspective. The book also contains glimpses into the future of the field, with implications in human health and in agriculture.

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Preface

We live immersed in a microbial world where microorganisms compose around 30% of the estimated living biomass. It has been proposed that the first life forms appeared on our planet around 3900 million years ago, and over the course of Earth's history, they have been spreading and evolving. Recent estimations propose the existence of up to one trillion (10^{12}) microbial species, entailing a greater biodiversity than microbiologists could ever imagine. Microbes have colonized almost every niche of the planet, from the deepest ocean trenches to high layers of the atmosphere. Thus, they have adapted their metabolism to survive in habitats as extreme as boiling acid, temperatures below the pure water congelation point, or in desiccant spaces such as salt crystallizer ponds.

Subsequently to the appearance of complex forms of life, microbes developed numerous molecular adaptations to expand their ecological niches and live in close relation with them. These interactions could be favorable when the presence of the microbe contributes to the profit of its interactor, or harmful when the higher organism is damaged as a result. In any case, microbes living inside their hosts have learnt to avoid immune responses or drive them to their own benefit. According to this, the study of the interaction between superior eukaryotes (plants and animals) and microbes has become essential from environmental, economical, or health points of view. In the particular case of human pathogens, the deciphering of how such microorganisms evade immune responses or even resist conventional antibiotic treatments brings in an immediate urgency since the mortality due to lethal infections is predicted to become the leading cause of death by 2050. Therefore, the understanding of how host-pathogen interactions take place at the molecular, organism, or population level will help to discover the preventive measurements that should be taken in mind to avoid such diseases.

In a different context, the injurious interactions of microorganisms with plants for human or animal feeding deserve special attention since millions of tons per year are lost due to diverse microbial plagues. The economic impact of this wastage, aggravated by the reduction in the permissions for chemicals that can be applied to crops to fight plant pathogens, highlights the importance of host-pathogen research since it can shed light on the knowledge of these interactions to predict how an infection could occur. Besides this concern, a consideration of beneficial microorganisms must be kept in mind while its application to crops can improve their yield by means of a wide variety of plant growth-promoting activities, ranging from increasing the availability of nutrients for plants to counteracting the negative effect of specific pathogens with biocontrol activities.

In this book, we propose numerous multidisciplinary approaches employed to analyze the role of different molecules or strategies used by different guests to survive inside hosts.

The manual is mainly divided into two sections, depending on the nature of the host organism, which can be an animal or a plant host, and includes molecular biology and biochemical techniques necessary to study these host-pathogen interactions.

With respect to animal-microbial pathogen interactions, we have included several chapters focused on *Salmonella*, which is a bacterium responsible for one of the most common foodborne diseases in the world and is also used as a model of pathogenicity for interactions with animal cells. Thus, different experimental approaches to dissect the regulation of *Salmonella* virulence genes at transcriptional and translational levels are shown. Additionally,

and taking advantage of interesting *Salmonella* characteristics, such as the capacity of this bacterium to specifically colonize tumoral tissues, it has been projected that the controlled use of this bacterium could be used as an antitumoral tool. In parallel, the use of three-dimensional living models, such as organoids, to study the behavior of another gastrointestinal pathogen, *Helicobacter pylori*, is described in Chapter 1.

Other authors have highlighted the increasing importance of the use of systems biology, bioinformatics, and other -omics technologies to create models to study genetic associations in host-pathogen interactions or predict interactions between microbial proteins and effectors and their host targets.

Finally, three review chapters focused on animal-pathogen interactions are included. In one of them, the use of genetic association studies for the identification of alleles involved in the infection process is shown. In addition, the use of several molecular biology tools for pathogens genotyping, a valuable tool for the identification of bacterial strains as well as for epidemiological surveillance, is also discussed. Moreover, one interesting chapter provides some recommendations about the use of phage therapy as an alternative with high potential to treat diseases caused by bacterial agents.

This book also presents some techniques to study microbe-microbe relationships, since microbes have developed successful strategies, including bacteria-bacteria communication through quorum sensing systems, to regulate traits associated with the coordinated infection of higher organisms or the formation of biofilms to increase bacterial survival within the host.

Chapters included in the plant–pathogen interactions section describe tools to study virulence genes expression, techniques to identify and characterize effectors secreted through the type 3 secretion system, assays to determine phenotypes associated with plant pathogenicity, analysis of images using confocal microscopy, screening of surface polysaccharides, such as exopolysaccharides, regulated by second messengers, or the identification of small RNAs involved in the regulation of many bacterial traits associated with virulence.

Since below-ground plant-pathogen interactions are of increasing interest in recent years, a chapter is dedicated to a nondestructive split-root system designed to monitor root infection that can also be used to study plant signals, triggered by microbes and generated in the roots, which can be delivered to other roots or to above-ground tissues. Finally, considering that plant pathogens must share the space with other microbial strains, plant growth-promoting activities of rhizosphere microorganisms are also included in this book. These activities have a potential impact not only on plant growth but also in the survival of plant pathogen populations.

In summary, this book provides a huge number of molecular and bioinformatics techniques used in reputed laboratories of different countries all around the world, helpful for different kind of host-pathogen interactions studies.

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Chapter 1

Genetic Association Studies in Host–Pathogen Interaction Analysis

Jose Luis Royo and Luis Miguel Real

Abstract

Studying host–pathogen interactions at a molecular level has been always technically challenging. Identifying the different biochemical and genetic pathways involved in the different stages of infection traditionally require complex molecular biology tools and often the use of costly animal models. In this chapter we illustrate a complementary approach to address host–pathogen interactions, taking advantage of the natural interindividual genetic diversity. The application of genetic association studies allows us to identify alleles involved in infection progression or resistance. Thus, this strategy may be useful to unravel new molecular pathways underlying host–pathogen interactions. Here we present the general steps that might be followed to plan, execute, and analyze a population-based study in order to identify genetic variants affecting human exposition to pathogens.

Key words Host–pathogen genetics, Association study, Case–control study, Study design

1 Introduction

The identification of molecular pathways involved in host–pathogen interactions is a prerequisite for the development of new strategies focused on infection treatment and prevention. However, it is well established that the study of host–pathogen interactions is technically challenging and require complex molecular biology tools that are discussed along the different chapters of this volume. Studying the differential susceptibility to the infection, or the altered infection progression from a population genetics perspective may be a straightforward alternative that may help us discover novel molecular pathways underlying host–pathogen interactions. Both, low susceptibility to infections and rapid disease progression are complex traits that depend on both environmental (pathogen related) and host genetic factors. Previous works estimated the host genetic component of infectious disease susceptibility to tuberculosis, type-B hepatitis, leprosy and poliomyelitis. In these studies, authors found that genetics may explain between 32% and 53% of

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the phenotypic variance, depending on the population studied and the infectious agent [1]. These results highlight the importance of host genetic factor backgrounds, although this may have a different role depending on the pathogen [2]. One of the most relevant examples was found in the late nineties for the Human Immunodeficiency Virus (HIV). Authors found a relatively common mutation consisting on a deletion of 32 base pairs within the *CCR5* gene, a coreceptor of the HIV, that confers to the homozygotes carriers an almost absolute resistance to HIV infection [3, 4]. This finding paved the road to the development of Maravidoc™, an antagonist of CCR5, which is currently used as a treatment of HIV infection. Moreover, this discovery based the new genetic therapy strategies for the total eradication of HIV infection. Therefore, the identification of genetic variants involved in these complex traits can give us new clues about the molecular processes affecting host–pathogen interactions, and some of them might be also relevant from the clinical point of view.

Here we present the general steps that might be followed to plan, execute, and analyze a population-based study in order to identify genetic variants affecting human exposure to pathogens.

2 Case and Control Selection and Power Estimation

In the early twenties, Sir Ronald Fisher showed that a complex quantitative trait could be explained by Mendelian inheritance if several genes affect the trait [5]. A single locus with two alleles of equal frequency results in three genotypes (Fig. 1a). Assuming a simple relationship in which the allelic effects are additive, the three genotypes produce three phenotypes. For qualitative traits such as those studied by Mendel, the allelic effects showed complete dominance, and therefore only two phenotypes were observed. Assuming additive effects, two loci generate nine genotypes and five phenotypes (Fig. 1b) and three loci generate 27 genotypes and seven phenotypes [6]. Following this rationale, complex quantitative traits with a significant genetic component have a polygenic nature (Fig. 1c). The objective of genetic association studies is the identification of genetic variants involved in the appearance of these traits.

These studies are based on the fact that such variants are more frequent in those groups of individuals affected by that trait than in those groups of individuals who do not show it. This means finding loci in which cases are "identical by state" (that is, the fact of sharing a genotype because of sharing a phenotype). Therefore, it is very important to establish strict selection criteria that allow us to clearly define which individuals must be considered as cases and which ones must be considered as controls. To do so, it is important to take into account that the distribution of other variables that

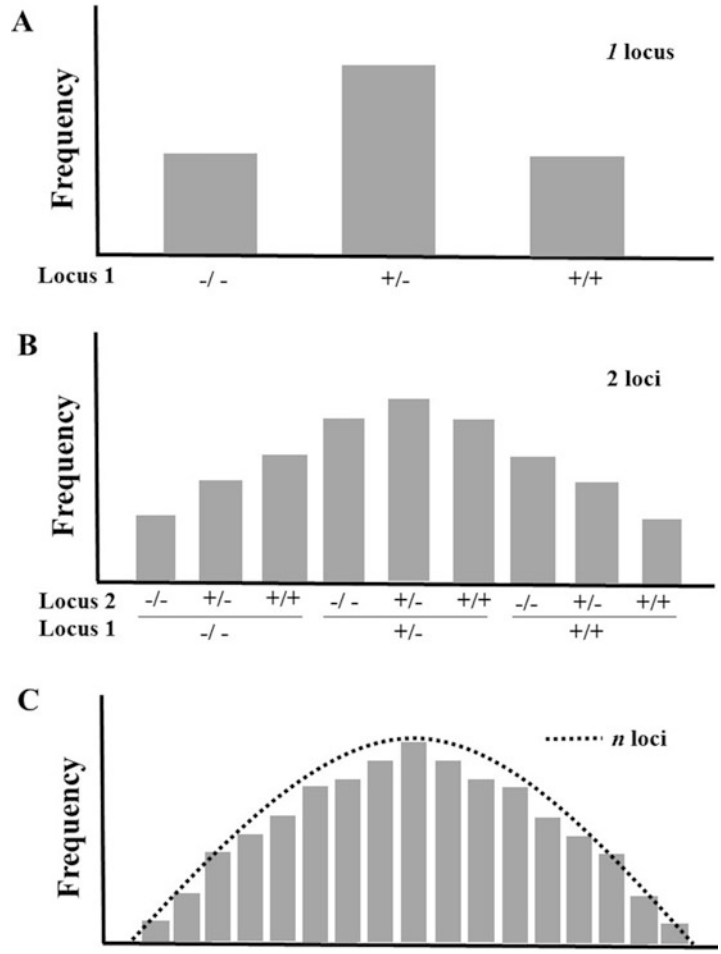

Fig. 1 Complex quantitative traits can be explained by Mendelian laws considering a polygenic nature. Increasing the number of genes (panels **a–c**) affecting a quantitative trait generates the wide spectrum of phenotypes observed in natural populations

could affect the results such as age, gender, ethnic background, should not be different between cases and controls. In order to exclude the possibility of sharing a genotype based on potential inbreeding ("identical by descent," that is, the fact of sharing a genotype because of relationship) those individuals with familiar relationship with other participant must be excluded.

The critical issue in our study will be inherent to the phenotype we may be interested in. If the cases share the same ancestral causative variant we face what is called a "founder effect" and our chances to distinguish an altered allele frequency increase. The trait under study may however, be the independent consequence of genetic changes in multiple loci (Fig. 2a). This is known as genetic

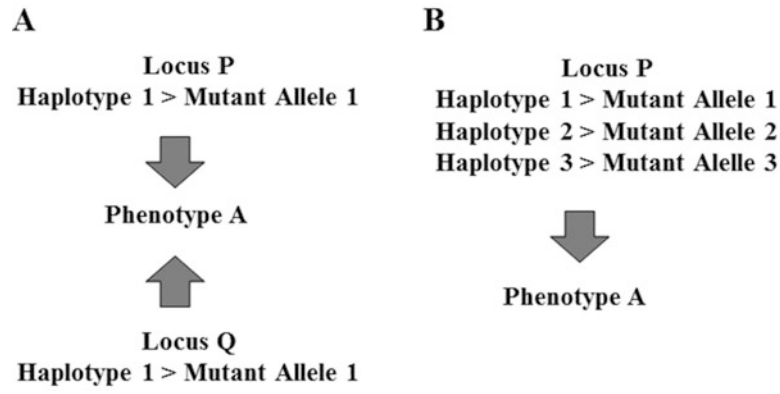

Fig. 2 Genetic versus allelic heterogeneity. Panel (**a**) reflects genetic heterogeneity, where mutant alleles influence the appearance of the studied phenotype. Panel (**b**) shows a typical case of allelic heterogeneity, where different haplotypes harbor different mutations

heterogeneity and may compromise the statistical power, since only a small proportion of our cases will be showing the mutant genotype. On top of this, each locus may have more than one mutant allele (Fig. 2b). This is called allelic heterogeneity and, since each mutant allele may be in a different haplotype, we might not be detecting the enrichment in the mutant allele in the cases subseries. In these situations, a large sample of cases and controls could increase the statistical power to detect them.

To study the low susceptibility or resistance to infection, the cases are normally considered those individuals who have been in contact with a pathogen but that were not infected (exposed but not infected or ENI), whereas controls are represented by infected individuals. This scenario is the ideal one, since genetic differences between both groups are maximized (Fig. 3). As controls we can also use nonselected individuals from general population when ENI individuals are scarce. Because ENI individuals exist as part of the general population, this kind of controls will reduce the probability of finding statistical significant differences (discussed below). However, the results obtained will be more robust since they were observed in spite of the existence of a bias in the control group. Another advantage of using nonselected population controls is that there is a lower probability of bias due to the selection processes in this group.

As aforementioned, finding ENI individuals is not an easy work. Moreover, a bad selection of these individuals could render no results. Those individuals who have a genetic resistance to the infection must be selected according with the probability of having contact with the pathogen to certificate its condition of resistant. For instance, to select hepatitis C virus (HCV) ENI individuals it

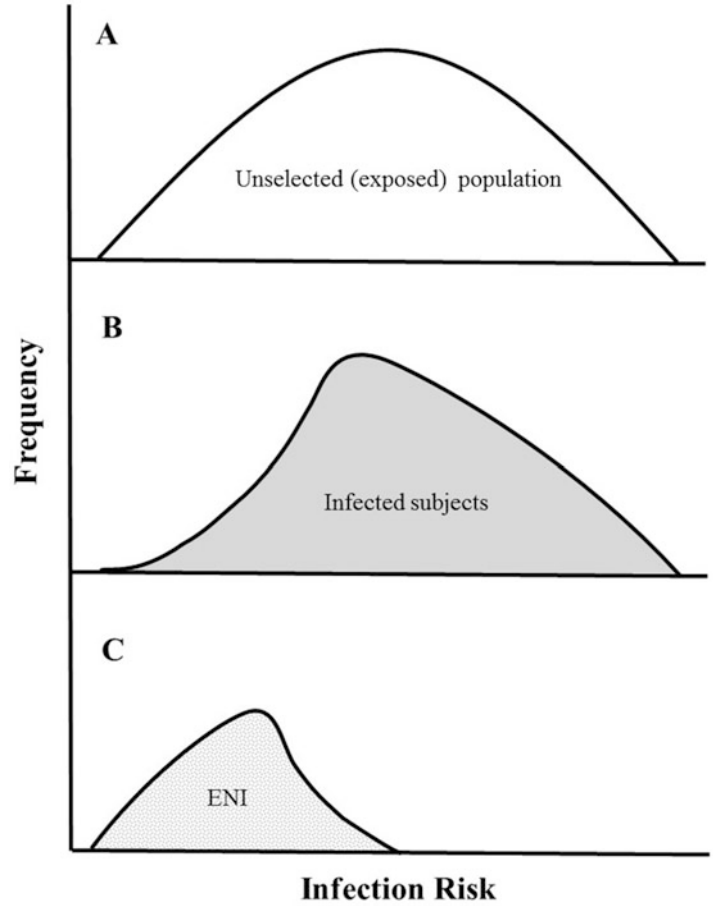

Fig. 3 Graphical representation of the infection risk of a population. Panel (**a**) represents a theoretical normal distribution of a hypothetical random population. Panels (**b**) and (**c**) represent the bias on the infection risk, therefore changing the genetic pool when compared to the random population

has been proposed a score mainly based on the frequency and duration of risk habits [7]. Similarly, In the case of the resistance to HIV infection, different criteria has been established to select those HIV ENI individuals mainly based in the period of time practicing risk habits [8, 9].

Alternatively, it could be assumed that genetic variants that confer resistance must be absent in infected individuals but present in the general population. In that case, it is possible to select as cases and controls those infected individuals and nonselected individuals from the general population respectively. These studies can only be used to detect genetic variants with a strong effect because the statistical power of these studies is low. Therefore, this strategy has been used when resistant individuals are very scarce [4, 10].

Another important question is the number of individuals necessary to carry out genetic association studies. Whenever a genetic comparison between two populations is performed, two potential results can arise. We may find a statistically significant difference (*p*-value obtained lower than our *alpha* threshold) or not (*p*-value greater than our *alpha*). Then, we may consider that we might have observed a false negative result given a low statistical power. Statistical power can be defined as the likelihood of assuming that cases and controls have the same genetic distribution when they are truly different. This is a type II error or a false negative result. Calculating the statistical power of your study is always recommended a priori. Some easy tools can be used (Episheet20015, Kenneth Rothman, Spread sheets for the analysis of Epidemiological data; www.krothman.org/episheet.xls). Intuitively, we can assume that the larger the sample size and the less conservative we are in setting an *alpha*, the higher sensitive our study will be. This means that we will be able to detect as statistically significant even small changes in allele frequencies. Statistical power also depends on parameters such as the allele frequency in the study population and the case–-control ratio. Since economical issues must also be taken into account it is generally assumed as correct to have 80% power.

3 Gene Variant Selection

Every variant that has been described in the genome may not affect protein expression or enzyme activity. Several bioinformatics approaches have been proposed in order to evaluate only those genetic markers with a higher likelihood of being associated to a certain phenotype. However, the reality is that functional variants must be captured using statistical approaches. The main principle that should be clear is that whenever during evolution a functional variant appeared, it laid in a particular haplotype and no other. As centuries passed, meiotic events subjected that particular chromosome to a number of recombination events that split the information in linkage blocks (Fig. 2). Nowadays, finding a common genetic variant that is overrepresented on a particular subset of subjects suggests that together with this variant, on the same haplotype from its linkage disequilibrium block, there is a functional variant that explains our phenomenon. Depending on the project strategy, the genetic component of the phenotype under study and, obviously, the available budget, researchers may choose to conduct a candidate-gene approach or a genome-wide association study. Candidate gene studies may be also designed using two strategies. One involves the analysis of potentially functional variants, based on previously reported information or bioinformatics analyses. The second approach involves the selection of tag makers. This is a subset of genetic markers with the capacity of

"summarizing" the haplotypic diversity of the population. Using the first approach we may have significant results with relatively small sample sizes, however if an unreported or unknown functional variant is present in a nonanalyzed haplotype, we face a negative result even when the gene is highly involved in the studied phenotype. On the contrary, basing our study on single-tag nucleotide polymorphism (SNP) normally require larger series of patients and controls. The advantage in this way comes from the certainty of analyzing the entire haplotypic diversity of a selected gene. The higher exponent of this strategy is the genome-wide association study (GWAS). The most remarkable characteristic of these studies is that they are considered "hypothesis-free." This means that no prior assumption about the relation of a gene with a specific phenotype shall be done. GWAS involve hundreds of thousands and sometimes millions of SNPs distributed all over the genome. We can therefore assure that a high proportion of the haplotypic diversity is captured. The major drawback is, as can be anticipated, data analysis. There are some software such as Plink (http://pngu.mgh.harvard.edu/purcell/plink/) [11] specially designed for performing these genetic studies. However, given the large amount of assays, the number of false positives is huge. A common strategy is to use more stringent p-values, following for instance multiple testing corrections. The consequence is that this increases the number of false negative results. The natural design therefore involves an initial GWAS, the selection of a list of the best candidates followed by a replication study on an independent patient series. This is one of the reasons why these studies may be extremely costly.

4 Data Analysis Strategies

Normally, polymorphisms that do not reach >90% of genotyping call rate must be discarded. Similarly, when a high number of polymorphisms are being analyzed, samples that are not genotyped for the 80% of the polymorphisms must be also discarded. Moreover, and depending on the cohort size, we recommend to include a 3–5% of sample duplicates, normally at the end of the well plates, in order to detect potential genotyping errors. Once >95% concordance is assured, Hardy–Weinberg equilibrium (HWE) should be assayed for cases and controls. If the genotypic distribution is in accordance with HWE, especially in controls, we could assume that we have not introduced bias in their selection processes. Moreover, the HWE is an additional control of the genotyping work since a deviation of this equilibrium could be indicating a cross-contamination of DNA samples. These assumptions are applicable to the control group, since, a deviation of the HWE exclusively

found among cases could indicate a high effect of the genetic variant in the complex trait analyzed.

Genetic association studies are based in the comparison of genotypic distribution between cases and controls. This allows us to know if a genetic variant is more represented in a group, and therefore, if it is associated with the presence of the trait analyzed. To do that, when a small number of SNPs (few dozens) are used, we can employ simple web assisted tools such as the one hosted at the Institute of Human Genetics from the Helmholtz Research Center (University of Munich; https://ihg.gsf.de/ihg/snps.html), or SNPstats, developed at the University of Barcelona (http://bioinfo.iconcologia.net/SNPstats, [12]). These platforms perform a systematic comparison of the genotype distribution between cases and controls, assuming recessive, dominant, and codominant models of inheritance in order to determine the best-fitting genetic model. For larger number of SNPs (hundreds to thousands), specialized software shall be used. The most extended one is, as aforementioned, Plink (http://pngu.mgh.harvard.edu/purcell/plink/) [11], a command-based application that has been proven to be extremely powerful. All these tools also calculate the HWE for cases and controls. For a further characterization and subsequent analyses, we recommend the use of more conventional statistical software such as SPSS (IBM Corporation, Somers, NY, USA) or STATA (STATA Corporation, College Station, TX, USA).

5 Limitations of Genetic Association Studies

The frequency of false positive results in genetic association studies is an inherent problem in this kind of work. Two strategies are applied to avoid this problem: Multiple testing correction and validation of the obtained results in a different sample.

As mentioned, multiple testing corrections are applied in large-scale genetic association studies such as GWAS. As a consequence, GWAS approach requires large samples to detect low penetrant mutations [13]. This is another reason why these studies are costly. In spite of this, the results obtained by large or low scale genetic association studies must be validated in an independent series or, alternatively, make a random selection in two groups and determine if the difference observed is consistently detected in both subseries with identical genetic effect.

In spite of these problems, we have to take into account that an interaction between different genetic variants could be underlying the effect of the host genetics on the onset of complex traits (epistasis). In that case, it is necessary to employ tools that allow us to identify such genetic interactions. Some efforts has been performed to address this issue [14–16] but this is still a challenge.

Finally, the advantage of the association studies is to determine which are the most important genes involved in a particular trait. Therefore, although these studies do not give clues about the mechanism that underlie the association, they point out where we must focus the researches to understand these mechanisms.

6 Illustrative Examples

The hepatitis C virus (HCV) is spontaneously cleared by the 30% of the infected individuals, whereas the rest of them become chronically infected. Using a GWAS approach, the rs8099917 genetic variant, close to *IFNL3* and *INFL4* genes, was associated to this outcome [17]. Previously, other GWAS associated the rs12979860 variant, in strong linkage disequilibrium with rs8099917 and mapped within *IFNL4* and close to *INFL3*, with the response to treatment against HCV [18]. In fact, rs12979860 genotyping provided useful information for individualizing the management of patients with chronic hepatitis C [19, 20]. These approaches point out that these interferons are important to understand the interaction of human immune system and HCV. Interestingly, none of these variants have been proven to have functional role, but these studies focused the attention in the molecular mechanism that have to be explored. Consequently, it has been reported that rs368234815, a genetic variant in high linkage disequilibrium with rs12979860, has a functional effect that could explain the association found [21]. In addition, it has been hypothesized that these interferons could also have an important role in the susceptibility of infection by HIV. Recently, a candidate gene approach based on this hypothesis has reported an association between a lower susceptibility to HIV infection and the rs368234815 genetic variant [8]. This result reinforces the importance of these molecules in the fight against these retroviruses.

Following similar strategies, more than 100 genetic variants have been associated with severe malaria risk in a recent GWAS [22–24]. Among them, polymorphisms linked to *USP38*, *FREM3*, *SDC1*, *DDC*, and *LOC727982* genes were putatively associated with differential susceptibility to severe malaria in a validation study [25]. This is an example of how unexpected genes identified by GWAS approaches that have been validated in a different sample are related to the progression of infectious diseases.

7 Conclusions

Our goal with this chapter is to illustrate the different tools available for population genetics to discover genes or pathways involved in host–pathogen interactions. A schematic diagram is represented in

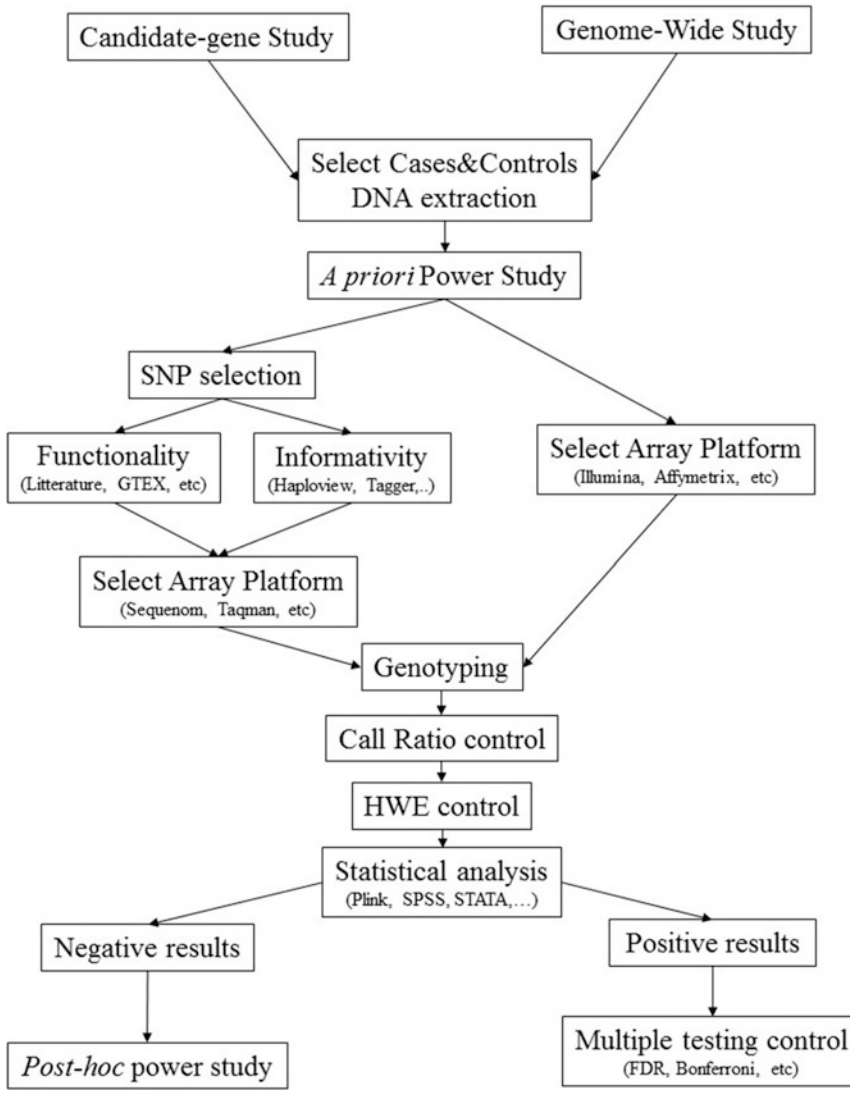

Fig. 4 Genetic association studies analysis flowchart

Fig. 4. The strategy successfully followed led to the identification of a handful of human genetic variants that confer some individuals a certain degree of protection against pathogens. Importantly, the hypothesis free association studies or GWAS applied to this field can identify unexpected important relations that would be hard to discover using other approaches. This fact accelerates the knowledge of host genetic interactions and open ways for the development of new therapeutic strategies.

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Chapter 2

Bacterial Genotyping Methods: From the Basics to Modern

Margarita M. Ochoa-Díaz, Silvana Daza-Giovannetty, and Doris Gómez-Camargo

Abstract

Bacterial genotyping methods have been used in several areas of microbiology and have facilitated the identification of bacterial strains, as well as the study of virulence and resistance factors, epidemiological surveillance, among others. Constituting, in this way, as complementary or alternative to phenotypic methods. This chapter reviews some of the methods of bacterial genotyping used nowadays, explains briefly how they are performed, and highlights some limitations and advantages they have.

Key words Bacteria, Genotyping techniques, Microbiological techniques

1 Introduction

In modern microbiology, the identification of bacterial strains is essential and permits to achieve effective clinical interventions on public health. On the other hand, the increase in virulence and transmissibility, antibiotics resistance, and the possibility of genetic manipulation for bioterrorism lead to the importance of having these tools [1, 2].

For bacterial strain typing, there are phenotypic and genotypic methods. Within the phenotypic methods, the identification of the strains is based on phenotypic characteristics including the morphology of the colonies in different isolation media, biochemical tests, serology, pathogenicity, and antibiotic susceptibility. However, these variables are often not enough to differentiate between strains that are closely related or are very similar [2].

Molecular methods have been instituted as complementary, alternative or even phenotypic reference procedures, because the previous ones have some inherent problems: not all strains of the same species show homogeneous characteristics, the same strain can generate different patterns in repeated tests, and also they

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have limitations related to the construction of a reference database, among others [3].

Limitations that have been observed related to phenotypic methods include the requirement of a long time to obtain the results and also the interpretation is usually very subjective, for this reason they sometimes may not be conclusive [1].

As the need to enter beyond the phenotypic characteristics gained importance, in the 80s there began the search for stable genes that allowed to establish phylogenetic relationships among bacteria, such as those coding for ribosomal subunits 5S, 16S, 23S, and their intergenic spaces. Following this, three basic categories were established in which the current typing methods can be classified, including: DNA band patterns, DNA sequencing, and methods based on DNA hybridization [2, 3].

Methods of genotyping determination have been used to identify the characteristics of the bacteria including antibiotics susceptibility and their resistance mechanisms for an early-effective treatment and also for patient prophylaxis. Related to phenotypic tests, which are still used and are in many cases very useful, it is important to recognize their limitations, especially in cases where there is a wide variety on the same species, which occurs mainly due to three genetics events: horizontal gene transfer, gene loss or acquisition and recombination [2, 4, 5].

2 *R*estriction *F*ragment *L*ength *P*olymorphism (RFLP)

RFLPs markers were the first molecular markers used for the development of genetic maps. This was reported in 1980 by Botstein et al. [6].

In this technique, the genes or their fragments are analyzed (this is generated by hybridization with a labeled probe). The procedure begins with the digestion of DNA through restriction enzymes (endonucleases); then the fragments are amplified by PCR and finally they are separated by electrophoresis to observe their differences in size (Fig. 1) [7, 8].

Some advantages and limitations of the RFLP are listed in Table 1 [7].

3 *P*ulsed-*F*ield *G*el *E*lectrophoresis (PFGE)

In 1984 Schwartz and Cantor [9] devised a way to separate large molecules of DNA. This was the beginning for the development of the pulsed-field gel electrophoresis (PFGE), in which, with the influence of two electric fields alternating their polarity, the DNA of the bacterial cells, which is concentrated in an agarose gel, migrates. The field strength is not uniform and changes constantly.

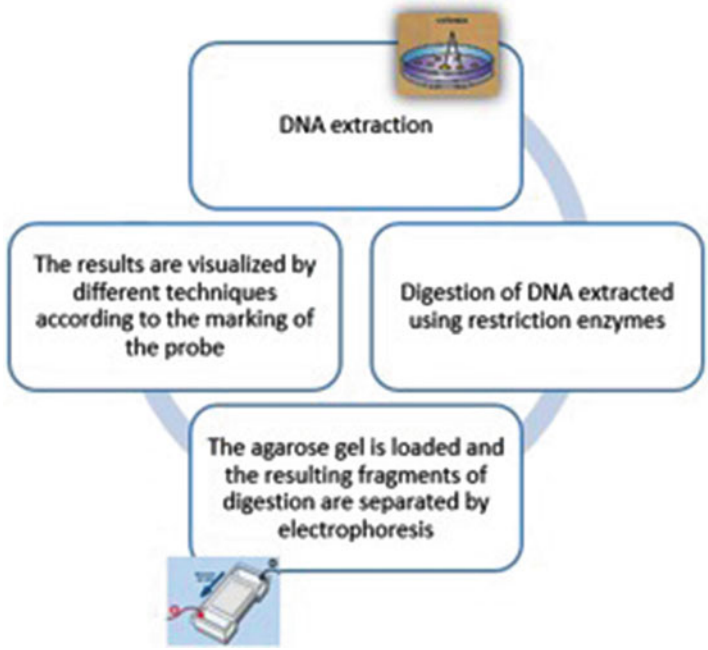

Fig. 1 Scheme of restriction fragment length polymorphism procedure (RFLP)

Table 1
Advantages and limitations of the use of restriction fragment length polymorphism (RFLP)

Advantages	Limitations
It is considered a simple technique	It is necessary to have a large amount of DNA
It is reproducible	Does not allow automation
It is not radioactive	It takes time and is expensive
	Discriminatory power limited by locus–specific

For this reason it refers to "pulses" [10]. The DNA is digested by enzymes and the fragments obtained are separated according to their size, then the gel is stained and the DNA can be observed under ultraviolet light (Fig. 2). The resulting pattern can be compared with others that are included in a database to finally achieve the identification of the bacterium (https://www.cdc.gov/pulsenet/pathogens).

PFGE has several advantages and limitations. Some of them are listed in Table 2.

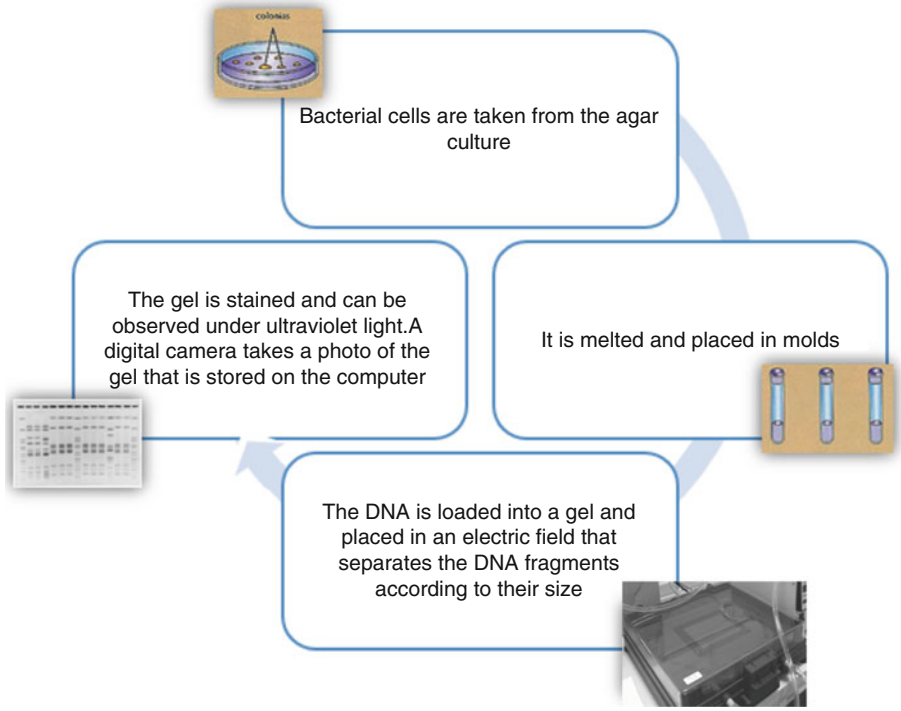

Fig. 2 Pulsed-field gel electrophoresis (PFGE)

Table 2
Advantages and limitations of the use of pulsed-field gel electrophoresis (PFGE)

Advantages	Limitations
High concordance in relation to epidemiology	Needs a lot of time
Can be applied as a subtyping method	Not discrimination between unrelated isolates
Stable and reproducible DNA patterns	DNA patterns may vary among technicians
	The separation in each part of the gel cannot be optimized
	Same size bands do not always come from the same part of the chromosome
	Some strains cannot be typified

4 *M*ultiple *L*ocus *Va*riable-Number Tandem Repeat Analysis (MLVA)

In this technique, an imprint of the DNA of the isolated bacteria is generated. It is generally used after having performed the PFGE to have a more detailed result. The method consists of searching for tandem repeats in variable numbers (VNTR) in the genome and

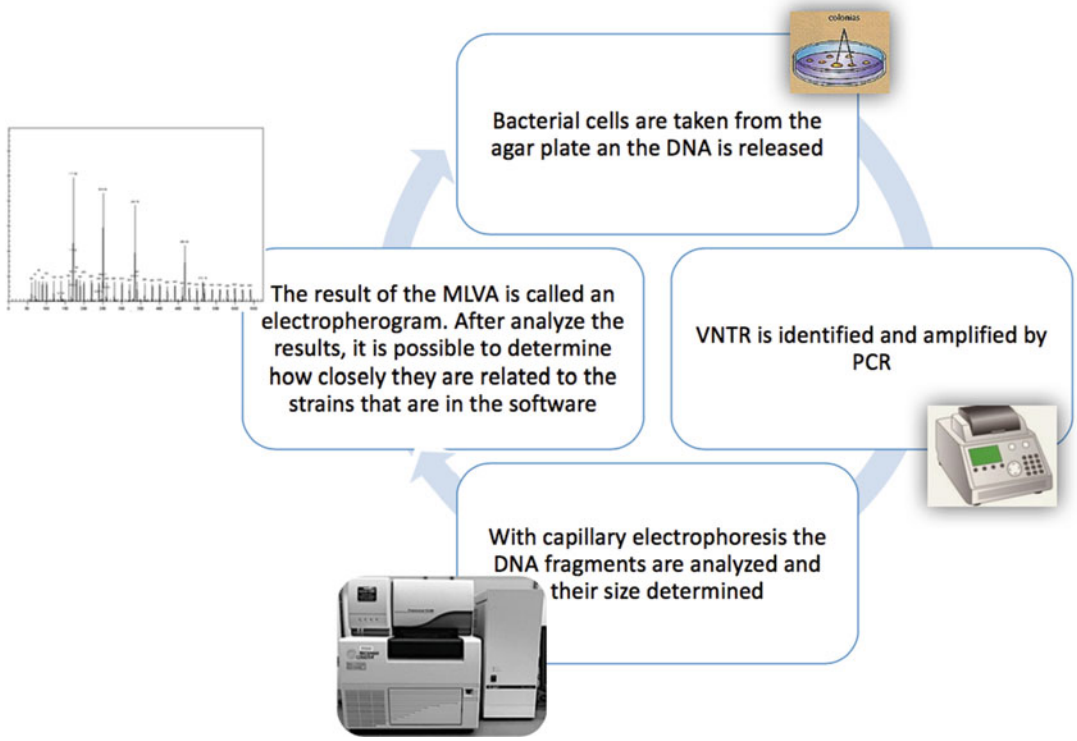

Fig. 3 How multiple locus variable-number tandem repeat analysis (MLVA) is performed

after their identification, multiplying these fragments using PCR. After performing the PCR, the DNA fragments can be analyzed and compared to the standards that are used as a guide to determine how much they are related (Fig. 3) [11].

The MLVA technique has been used in a very limited number of strains, which is why it must be subjected to new tests with several sporadic epidemiologically linked strains to determine whether in practical use it is a suitable and effective technique [11]. Some of the advantages and limitations of MLVA (https://www.cdc.gov/pulsenet/pathogens) are listed in Table 3.

5 *C*omparative *G*enomic *H*ybridization Microarray (CGH)

The main objective of this technique is to determine similarities of the strains that are being studied. It has been used to compare bacterial genomes that have not yet been sequenced. This technique takes account of characteristic genes, related to unique phenotypes, that are amplified by PCR. After sequencing, the analysis is performed according to those genes that are specific and conserved (Fig. 4) [12, 13].

Table 3
Advantages and limitations of the use of multiple locus variable-number tandem repeat analysis (MLVA)

ADVANTAGES	Limitations
It allows to observe with more detail the differences between bacteria with similar PFGE pattern	Requires trained personnel
It permits to identify rapidly bacterial strains during an outbreak	A specific protocol is needed for every microorganism
	Only the most common microorganisms can be subtyped

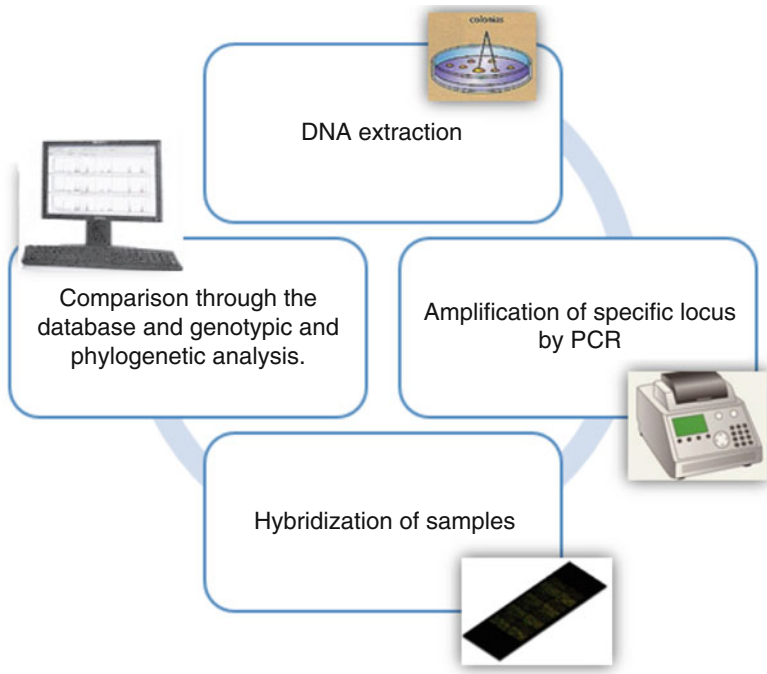

Fig. 4 Comparative genomic hybridization microarray (CGH)

The CGH permits the study of the intraspecies diversity. Thus, this comparative genomic hybridization is based on DNA arrays, identifying those genes that are absent or are very diverse.

There are two types of CGH: DNA microarrays and DNA macroarrays. They differ in the size and the number of oligonucleotides they contain (Table 4) [2, 13, 14].

This technique is useful for determining genes related to virulence and bacterial antibiotic resistance. Within the limitations of this technique is that mutations, deletions, reordering of genes and new genes are not detected [2, 15].

Table 4
Characteristics of DNA microarrays and macroarrays [2]

DNA macroarrays	DNA microarrays
Hybridization of <1000 probes	Hybridization of >1,000,000 probes
Fast, effective, and cost-effective	More expensive
Lower discriminatory power	High discriminatory power

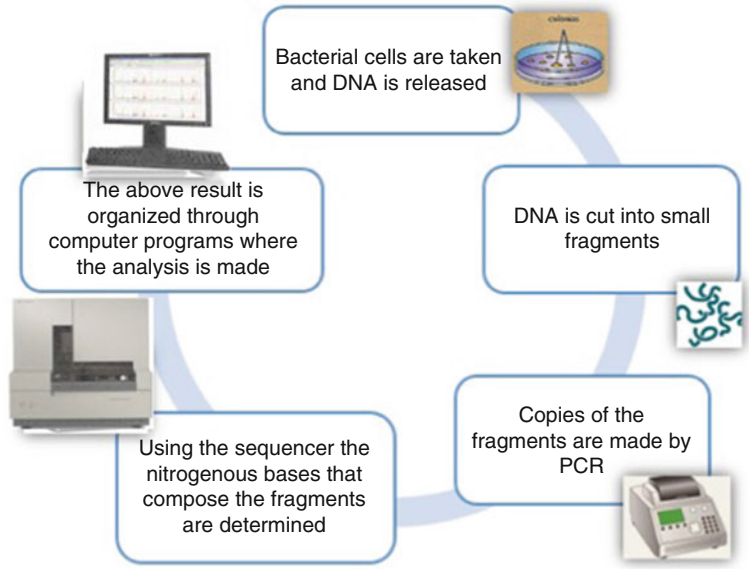

Fig. 5 Whole genome sequencing (WGS)

6 Whole Genome Sequencing (WGS)

Considering that each organism has a sequence of nitrogenous bases (adenine, guanine, cytosine, and thymine) that makes it unique, the aim of this process is to determine the genome sequence of the microorganism, and then identify which bacterial species is related to the health problem, for example, the one that is being studied. Using WGS valuable, very detailed, and accurate information is obtained. This is one of the most recent techniques, and it has been used for molecular diagnosis and also in clinical microbiology [16].

The DNA, composed of millions of nitrogenous bases, is initially cut into small fragments. Then it must be identified which bacterial species corresponds to each of them. For this purpose DNA tags, which serve as bar codes, are used. The last ones are identified by the sequencer, informing the specific order of the nitrogen bases that characterizes each pathogen. Finally, a

computerized analysis can compare the sequences of each bacterial species and identify their differences, which will allow us to have the result (Fig. 5) (https://www.cdc.gov/pulsenet/pathogens).

Whole Genome Sequencing has been used to monitor outbreaks in its initial stages and provides information on drug resistance, virulence determinants and genome evolution of the studied bacteria with advantages an limitations [16].

Among the advantages of WGS is remarkable that it is more detailed and precise, though still expensive.

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Part I

Animal-Pathogen Interaction Section

Chapter 3

Real-Time Reverse Transcription PCR as a Tool to Study Virulence Gene Regulation in Bacterial Pathogens

Gili Aviv and Ohad Gal-Mor

Abstract

Quantitative real-time PCR (qRT-PCR) is a highly sensitive and reliable method for detection and quantification of DNA. When combined with a prior stage of RNA reverse transcription to generate complementary DNA (cDNA), this is a powerful approach to determine and analyze gene transcriptional expression. Real-time quantitative reverse transcription PCR has become the gold standard method in studying genes expression and virulence regulation under various genetic backgrounds (e.g., in the absence of regulators) or environmental conditions. Here we demonstrate the utilization of this approach to study the transcriptional regulation of the conjugation pilus of the *Salmonella enterica* serovar Infantis virulence plasmid (pESI).

Key words Real-time PCR, Reverse transcription, cDNA, SYBR green dye, ROX, Transcription, Regulation, Gene expression

1 Introduction

Salmonella enterica is a Gram-negative highly diverse bacterial pathogen that is able to infect and cause a disease in a wide range of animal hosts including human. The single biological species *S. enterica* contains more than 2600 distinct biotypes known as serovars that can be associated with diverse animal hosts and may cause different clinical manifestations [1]. Hundreds of genes scattered over the *S. enterica* chromosome and different virulence plasmids are required for its pathogenicity. Such genes may encode secretion pathways, adhesion and invasion factors, host-translocated effectors and toxins, flagella and chemotaxis proteins and more [2]. Such diversity of virulence factors indicates that *Salmonella* pathogenicity is a multifactorial phenotype that is shaped by complex interactions between the pathogen and its host. In order to cause a disease, a pathogen has to tightly regulate multiple virulence pathways and orchestrate the expression of numerous virulence genes in timely and spatial manner in response

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to environmental signals [3]. Therefore, studying virulence gene regulation is essential to understand *Salmonella* biology and its unique pathogenicity.

Real-time quantitative reverse transcription PCR (real-time qRT-PCR) is a well-established technology that has become the method of choice for the detection and quantification of RNA targets [4] that has dramatically changed the way by which gene expression is being measured. Real-time qRT-PCR is based on the inherent quantifiable nature of PCR, making this technique a quantitative as well as a qualitative assay that provides a very wide dynamic range and allows comparison between RNAs with different abundance [5].

In real-time qRT-PCR, RNA isolated from the cells is reverse transcribed into the first strand of cDNA, which now can be used as the template for a subsequently real-time PCR analysis. This technique enables reliable detection and measurement of products generated during each cycle of the PCR process, by combining amplification and detection into a single step. Detection is achieved using different fluorescent chemistries that correlate amplicon (the PCR product) concentration to fluorescence intensity [6]. Thus, generation of the PCR amplicon can be identified at precise points over time and linked with a particular PCR cycle number. When the amplification of the target is first detected, this value is referred to as cycle threshold (C_T), indicating the number of the PCR cycle, at which fluorescence intensity of the generated amplicon is higher than the background. Therefore, the greater the quantity of target DNA in the reaction, the earlier an increase in fluorescent signal will be detected, resulting in a lower C_T.

Currently available fluorescent chemistries to detect the amplification of a target during the real-time PCR reaction can be classified into two main groups. The first group comprises fluorophore-linked oligonucleotides (primer probes) such as the TaqMan chemistry, enabling the detection of specific PCR products only. The second group includes double-stranded DNA (dsDNA) intercalating molecules, such as the SYBR Green 1 or EvaGreen dyes, allowing for nonspecific detection of amplified products due to their ability to bind dsDNA.

To study virulence genes regulation in *Salmonella* we employ the SYBR green 1 dye chemistry and ROX as the passive reference dye. The dsDNA-SYBR Green complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm). This way, the fluorescence that is measured after each PCR cycle is proportional to the accumulating dsDNA amplicon [7]. For data analysis we use the comparative C_T method ($\Delta\Delta C_T$) [8], which compares the C_T value of a target RNA of interest with a reference control. This may involve a comparison between two genetic backgrounds (e.g., wild type vs. regulatory mutant) or growth conditions (e.g., growth in rich LB broth vs. minimal media). The C_T

values of both the sample and the reference are normalized to the C_T of an endogenous housekeeping gene that presents a constant expression under the examined experimental conditions (we usually use the *rpoD* or 16S rRNA mRNA).

To demonstrate this approach we will show the transcription regulation of *pilV*, encoding the minor pilin subunit of the conjugative pilus of the virulence-resistance plasmid, pESI in *Salmonella enterica* serovar Infantis under different environmental conditions and genetic backgrounds (Figs. 1 and 2) [9, 10].

2 Materials

1. Luria–Bertani (LB) broth (Lennox) For 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl (pH 7.0).
2. Minimal media [e.g., N-minimal media containing 80 mM MES (pH 5.8) or 100 mM Tris–HCl (pH 7.0), 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 0.5 mM K_2SO_4, 337 μM K_2HPO_4/KH_2PO_4, 20 mM $MgCl_2$, 38 mM glycerol, and 0.1% Casamino acids].
3. RNA stabilization reagent (e.g., the QIAGEN RNAprotect Bacteria Reagent).
4. RNA purification kit (e.g., the QIAGEN RNeasy Mini Kit).
5. TE buffer (Tris–EDTA, 30 mM Tris, 1 mM EDTA, pH 8.0) containing 15 mg/mL lysozyme from chicken egg white and 20 mg/mL Proteinase K.
6. RLT buffer (from the RNeasy Mini Kit) containing β-mercaptoethanol (10 μL of β-ME per 1 mL of RLT buffer).
7. Analytical grade ethanol absolute.
8. 70% ethanol.
9. RNase-free DNase I.
10. Sodium acetate (3 M, pH 5.2).
11. DEPC-treated water.
12. Spectrophotometer (e.g., NanoDrop).
13. Reverse transcription kit for qRT-PCR.
14. FastStart Universal SYBR Green Master (ROX).
15. Real-time PCR system.

3 Methods

3.1 RNA Purification

RNA isolation is based on the QIAGEN RNeasy Mini Kit protocol [11] with various adjustments. Other established protocols (e.g., phenol–chloroform extraction or TRIzol reagent) or kits from other manufacturers can be used as well for this purpose.

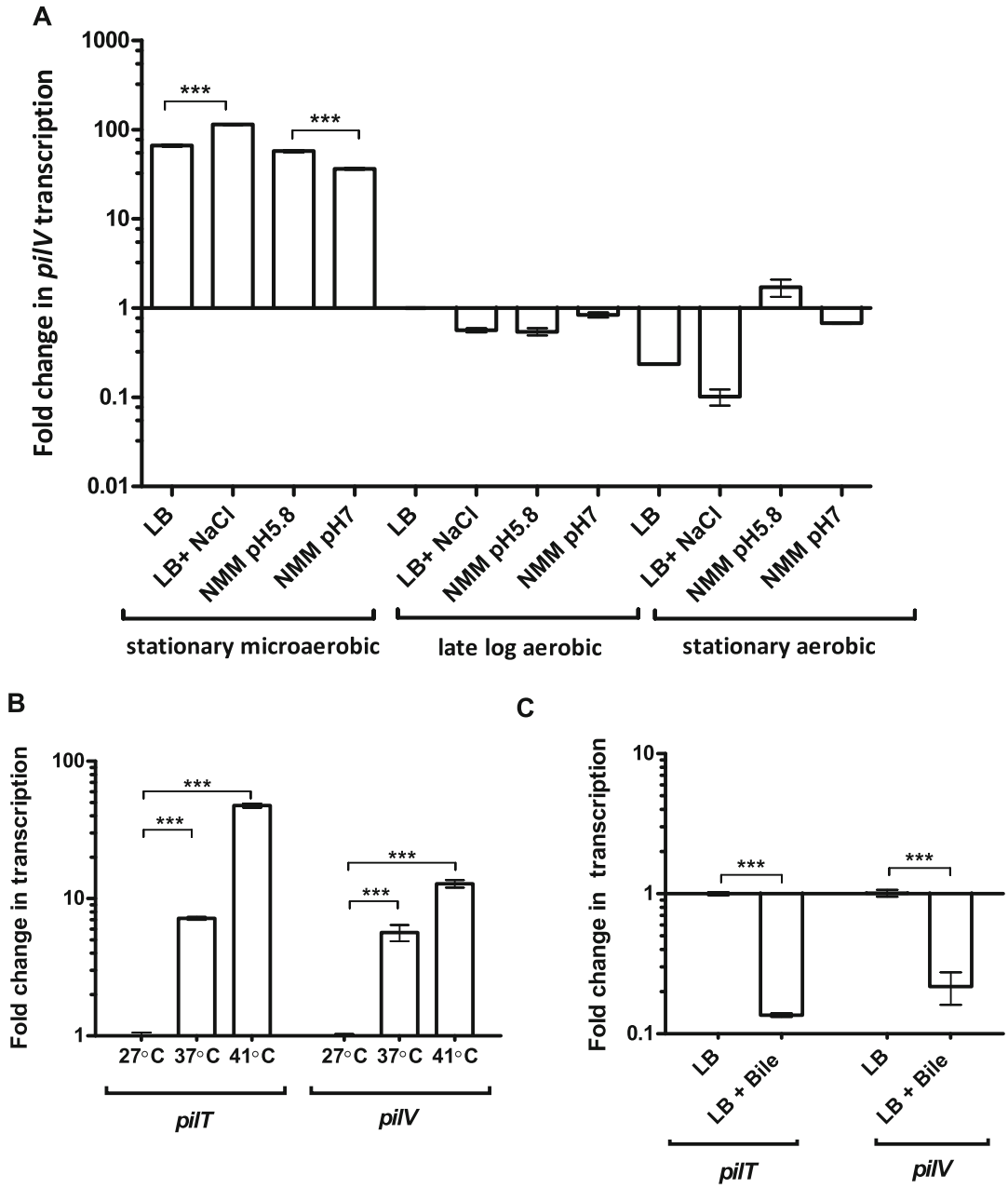

Fig. 1 The transcription of pESI pilus is induced in response to microaerobic, physiological temperature and repressed by bile. Real-time qRT-PCR was used to study the expression of *pilV*, encoding the minor pilin subunit of the conjugative pilus of the virulence-resistance plasmid, pESI in *Salmonella enterica* sv Infantis. (**a**) qRT-PCR shows the fold change in *pilV* transcription under different growth conditions. RNA was extracted from *S.* Infantis st. 119944 cultures grown in LB, LB supplemented with 0.3 M NaCl, N-minimal medium pH 5.8 and N-minimal medium pH 7.0. Differences in *pilV* expression are shown relative to the transcription of *pilV* in LB late-logarithmic culture grown under aerobic conditions. Induction of *pilV* under microaerobic growth conditions can be appreciated. (**b**) Fold change in the transcription of *pilV* and *pilT* grown in LB under microaerobic conditions at 37 °C and 41 °C relative to 27 °C. Induction of both genes at 41 °C can be seen. (**c**) Fold change in the transcription of *pilV* and *pilT* grown under microaerobic conditions at 37 °C in LB supplemented with 4% bile salts (sodium cholate) is shown. Repression of gene expression can be seen in the presence of bile. All RT-PCR results show the mean and standard deviation of three to six biological repeats. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. This figure is reproduced from ref. [9] with permission from the publisher

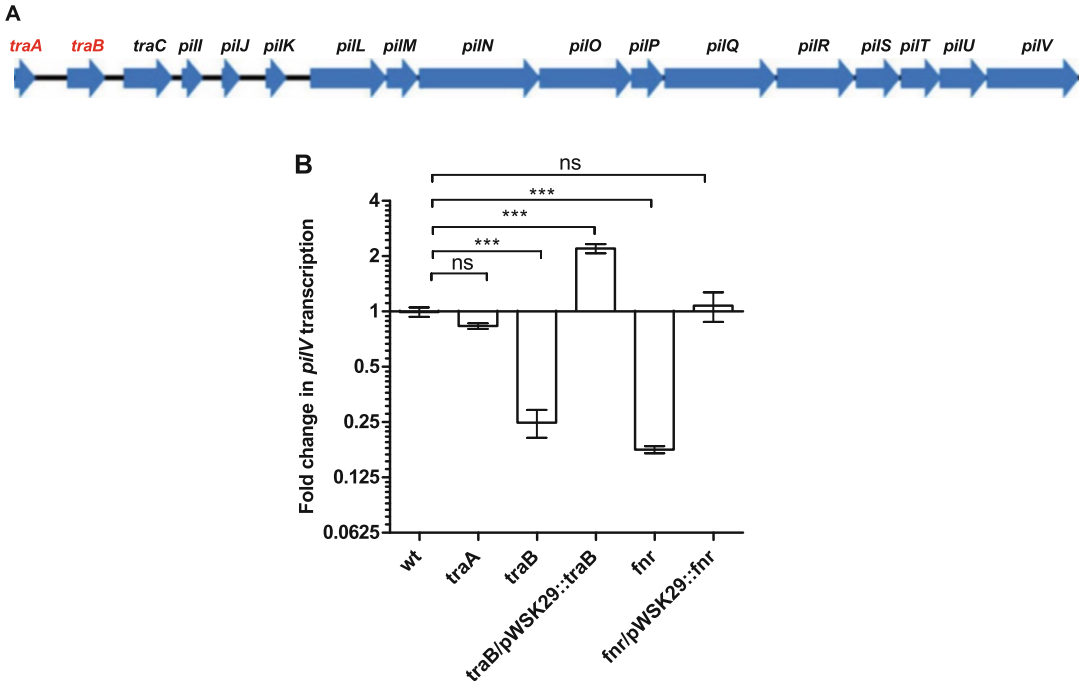

Fig. 2 pESI pilus transcription is regulated by TraB and FNR. (**a**) Gene organization of the *pil* cluster encoded on pESI. Arrowheads show location and orientation of the different ORFs. Putative regulatory genes are shown in red. (**b**) RNA was extracted from *S.* Infantis st. 119944 (wt), its derivative mutants (*traA*, *traB*, and *fnr*) and complemented strains *traB*/pWSK29::*traB* and *fnr*/pWSK29::*fnr* cultures grown in LB under microaerobic conditions at 37 °C. qRT-PCR analyses were conducted to determine the fold change in the transcription of *pilV* in the indicated backgrounds relative to the wild-type strain. One-way ANOVA with Dunnett's Multiple Comparison Test was implemented to determine statistical significance. *, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.0001$; ns, not significant. A positive regulatory role in controlling *pilV* expression was found for the regulators Fnr and TraB. This figure is reproduced from Aviv et al. 2016 [9] with permission from the publisher (ASM Journals)

1. Grow appropriate *Salmonella* cultures at the desired experimental growth conditions.
2. Measure the optical density (OD_{600}) of the cultures using a spectrophotometer and normalize (using the growth media) all cultures to $OD_{600} \sim 1$.
3. Transfer 0.5 mL (~4×10^8 CFU) from the normalized cultures into 1.5 mL microcentrifuge test tubes (*see* **Note 1**).
4. Immediately add 1 mL of RNAprotect Bacteria Reagent and mix well by vortex for 5 s (*see* **Note 2**).
5. Incubate for 5 min at room temperature (*see* **Note 3**).
6. Centrifuge for 10 min, 11,000 × *g* at room temperature using a microcentrifuge.

7. Remove the supernatant and add 115 μL of TE buffer containing 15 mg/mL lysozyme and 20 mg/mL Proteinase K and carefully resuspend the pellet by pipetting.
8. Incubate at room temperature for 10 min and gently mix by inverting the tube every 2 min (*see* **Note 4**).
9. Add 350 μL of RLT buffer containing β-mercaptoethanol and mix.
10. Add 250 μL of analytical grade absolute ethanol and mix (*see* **Note 5**).
11. Transfer the samples into the RNeasy Mini Spin column placed in a 2 mL collection tube and centrifuge for 1–2 min at 11,000 × *g* in a table top centrifuge. Make sure that all liquid has passed through the column before discarding the flow-through.
12. Add 350 μL of buffer RW1 to the column. Centrifuge for 1 min at 11,000 × *g* in a microcentrifuge.
13. Prepare DNase I RNAse-free Stock Solution by dissolving lyophilized DNase I in 550 μL of RNase-free water. Place on ice the required amount for the subsequent steps and store the rest in aliquots at −20 °C (*see* **Note 6**).
14. Prepare the DNase I Incubation Mix by adding 10 μL of DNase I Stock Solution to 70 μL RDD buffer (supplied with the RNeasy Mini Kit) and mix gently (*see* **Note 7**).
15. For on-column DNase I treatment, add 80 μL DNase I Incubation Mix directly to the RNeasy column membrane and incubate for 15 min at room temperature inside biosafety cabinet, to protect from potential contaminations (*see* **Note 8**).
16. Add 500 μL buffer RPE (supplied in the RNeasy Mini Kit) to the RNeasy spin column and centrifuge for 1 min at 11,000 × *g* in a microcentrifuge. Repeat this stage once more.
17. Place the RNeasy spin column in a new 1.5 mL collection tube and add 40 μL of RNase-free water directly to the spin column membrane.
18. Incubate for 10 min at room temperature and centrifuge for 1 min at 11,000 × *g* in a microcentrifuge to elute the RNA. Place the purified RNA on ice.
19. Measure the RNA concentration using spectrophotometer and take about 2 μg of RNA for a secondary DNase I treatment in solution (*see* **Note 9**).
20. Mix the following ingredients in a 1.5 mL test tube for a secondary DNAse I digestion: ~2 μg of the purified RNA, 10 μL buffer RDD, and 2.5 μL DNase I Stock Solution and top up to a final volume to 100 μL with RNase-free water.

21. Incubate at room temperature for 10 min. Avoid long incubation times, which may compromise RNA integrity.
22. Perform ethanol precipitation of the DNAse I-treated RNA by adding 10 μL of 3 M sodium acetate (pH 5.2) and 250 μL of ice-cold ethanol absolute. Mix well and incubate for ≥3 h at −20 °C.
23. Centrifuge at 11,000 × *g* for 20 min at 4 °C in a refrigerated microcentrifuge. Carefully remove the supernatant without disturbing the pellet (which may be invisible).
24. Add 750 μL of ethanol 70%, invert the tube a few times and centrifuge at maximal speed for 2 min at 4 °C.
25. Remove supernatant and leave the tube open inside a biosafety cabinet (not on ice) for ~15 min, until the entire ethanol has evaporated.
26. Add 40 μL of RNase-free water and resuspend the RNA by pipetting. Place the RNA samples on ice.
27. Measure RNA concentration using NanoDrop spectrophotometer or equivalent and store unused RNA at −80 °C. Avoid repetitive freezing and thawing of the RNA samples.

3.2 Reverse Transcription of the RNA (cDNA Synthesis)

1. Into a clean RNAse-free 0.2 mL PCR tube add 150–200 ng purified DNAse I-treated RNA, 4 μL of iScript Reverse Transcription Supermix (Bio-Rad Laboratories) or any other cDNA synthesis mix of choice (*see* **Note 10**), and RNase-free water to a final volume of 20 μL.
2. Insert the tubes in a Thermal Cycler and run the following program: priming for 5 min at 25 °C, reverse transcription for 30 min at 42 °C, reverse transcriptase inactivation for 5 min at 85 °C.
3. Store the cDNA at −20 °C for short periods or at −80 °C for long-term storage.

3.3 Preparation of the Samples for RT-PCR

1. Dilute each one of the PCR primers (forward and reverse) to a final concentration of 30 pmol/μL in a RT-PCR primers mix (*see* **Note 11**).
2. Place a 96-Well optical real time PCR plate or proper PCR tubes on ice. For each reaction add 10 μL of SYBR Green reagent, 0.8 μL of the primers mix, and 7.2 μL of RNase-free water.
3. To each reaction add 2 μL of the cDNA (made in 3.2) to make a total volume of 20 μL per reaction. We recommend including at least three replicates for each target.
4. In addition, set up a control reaction to test for DNA contamination. To prepare this control, dilute 150–200 ng purified RNA (before the reverse transcription stage) in 20 μL RNase-

free water that will be used as a control in the absence of a reverse transcription step. Take 2 μL from this RNA suspension into 18 μL of the real-time PCR mix (as in **step 2**).

5. Additional control should include PCR reaction with no template. Instead of cDNA add 2 μL of RNAse-free ddH_2O. Make sure to include this type of control for every amplified target. This reaction controls for the lack of DNA contamination in any of the used reagents.
6. If using 96-well plate, cover the top with an adhesive PCR plate foil and centrifuge briefly (~30 s) at 500 × *g* to collect residual liquid from the sides of the wells.

3.4 Real-Time PCR Reaction

1. Place the plate in the RT-PCR instrument.
2. Set the correct parameters according to the type of experiment performed, in this case Quantitation-Comparative C_T ($\Delta\Delta C_T$).
3. Real-time cycling conditions are as follow: 95 °C for 10 min; and 40 cycles of 95 °C for 15 s, 60 °C for 1 min.
4. Make sure to include a melt (dissociation) curve analysis of the amplified targets.
5. Examine the melting temperatures from the dissociation curve in order to assess the homogeneity of the PCR products and to determine the specificity of the PCR reaction (*see* **Note 12**).

3.5 Data Analysis

In order to analyze the results we use the comparative threshold method ($2^{-\Delta\Delta C_T}$). This involves comparing the C_T values of the sample of interest (a mutant or a certain growth condition, particular treatment, etc.) with a reference (e.g., the wild-type strain or standard growth conditions). The C_T values of both the sample and the reference are normalized to the C_T values of an appropriate endogenous housekeeping gene determined for each condition or background (*see* **Note 13**).

Steps for calculating the fold change of expression levels:

1. Calculate the average C_T of the endogenous housekeeping gene, target and the reference.
2. Calculate the delta C_T (ΔC_T) of the target gene by subtracting the average C_T of the endogenous housekeeping gene from the C_T value of the target.
3. Calculate the delta C_T (ΔC_T) of the reference by subtracting the delta C_T of the endogenous housekeeping gene from the C_T value of the reference.
4. Calculate the delta delta C_T ($\Delta\Delta C_T$) value by subtracting the delta C_T (ΔC_T) of the reference from the delta C_T (ΔC_T) of the target gene using the following formula: $\Delta\Delta C_T$ = [delta] $C_{T\ \text{target}}$ – [delta] $C_{T\ \text{reference}}$.
5. Calculate the fold change of expression as $2^{-\Delta\Delta C_T}$.

4 Notes

1. Applying higher amount of cells may block the column and result in a lower RNA yield.
2. RNAprotect Bacteria Reagent should be added to the bacteria culture in a 2:1 ratio (V/V).
3. At this stage, one can store the RNAprotect-treated culture at −80 °C or continue to **step 6**.
4. Mix gently by inverting up and down the tube. Do not use vortex at this stage.
5. From hereafter perform all the following steps in a biosafety cabinet (to prevent contamination of the samples) and use only RNase-free reagents. Use only filtered pipette tips and make sure to wear gloves and replace them frequently.
6. When preparing the DNase I stock solution, inject the RNase-free water into the vial using a needle and syringe. Mix gently by inverting the vial. Aliquot the DNase I solution and store at −20 °C.
7. This amount is sufficient for one sample. For more than one sample, multiply the amounts by the number of samples you need to treat.
8. Make sure to add the DNase I Incubation Mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix stays on the walls or the O-ring of the spin column.
9. In our hands, a second treatment of DNAse I in solution is required to eliminate all traces of DNA from *Salmonella* cultures grown in rich media.
10. If using other cDNA synthesis kit, the volume of the reagents may need to be adjusted according to the manufacture protocol.
11. Design the RT-PCR primers with an annealing temperature of 55–60 °C to amplify a150–200 bp fragment.
12. This step is very important especially when using the SYBR green 1 dye because it lacks sequence specificity. If a PCR product results in the no-template and no-reverse transcription controls, make sure that these amplicons have a different melting curve than the experimental samples. If they share the same Tm, it may indicate a genomic DNA contamination in the RNA samples. Primer-dimers may also give a detectable signal, but these will have a different melting curve than the target amplicon.
13. Select an endogenous housekeeping gene, which have similar C_T values under the studied growth conditions or genetic backgrounds (e.g., 16 s rRNA, *rpoD*, or *dnaK*).

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Chapter 4

Usage of a Bioluminescence Reporter System to Image Promoter Activity During Host Infection

Gili Aviv and Ohad Gal-Mor

Abstract

Bioluminescence is the process of production and emission of light by a living organism, usually as the by-product of the oxidative enzyme, luciferase. Currently available technology allows for the exploitation of a bioluminescent reporter system to study bacterial gene regulation during rodent infection, in real time, over a large dynamic range. Here we show how this imaging system can be used to study virulence gene regulation during *Salmonella enterica* infection in the mouse model. To demonstrate this technique we show the ex vivo expression pattern of the gene *dksA*, encoding a conserved and pleotropic regulator, which plays a key role in *Salmonella* pathogenicity [1].

Key words Luciferase, Bioluminescence, Reporter gene, In vivo imaging, Transcriptional regulation, Host infection

1 Introduction

Salmonella enterica is a gram-negative pathogen that is able to infect and cause disease in a wide range of animal hosts including human. Hundreds of genes scattered along its genome are involved in the virulent lifestyle of *S. enterica* and its complex interactions with diverse hosts. These virulence genes are tightly regulated and their expression is orchestrated according to a multifarious net of cellular and environmental signals. Thus, studying virulence gene regulation is essential to understand *Salmonella* biology and its pathogenic nature. The production of bioluminescence and the technology enables to quantity its signal can be now utilize to follow after pathogens gene expression during the infection.

Production and emission of light by a living organism is called bioluminescence. Different marine and terrestrial bacteria including the *Vibrio*, *Photobacterium*, and *Xenorhabdus* genera harbor the *lux* genes encoding for the oxidative enzyme luciferase (*luxAB*) and the fatty acid reductase system (*luxCDE*) required for the synthesis of the fatty aldehyde, which is used as the substrate for the

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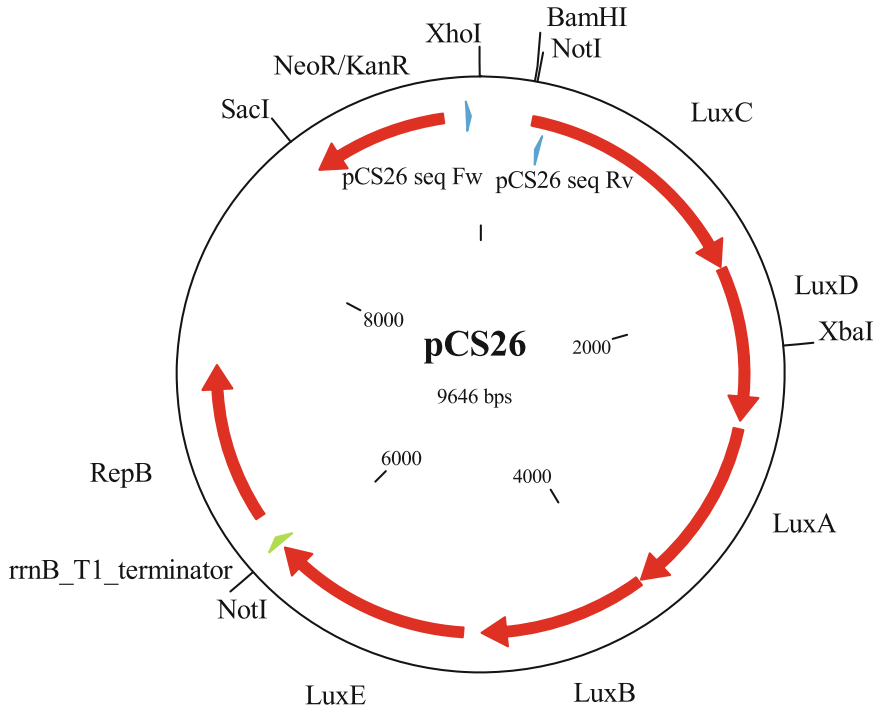

Fig. 1 pCS26 Map. The *lux* operon (*luxCDABE*) and the cloning sites upstream to the *lux* operon (XhoI, BamHI, and NotI) are indicated. Sequencing primers are shown as blue arrow heads. Construction of this vector was reported in reference [3]

luminescence chemical reaction [2]. A recently developed technology involves an ultra-sensitive CCD camera that can detect the light emission produced during bioluminescence and uses this signal for noninvasive imaging of small laboratory animals.

A self-sufficient bacterial luminescence-based reporter system has been developed by Bjarnason and colleagues [3]. This reporter plasmid (termed pCS26) contains a promoter cloning site upstream to the *luxCDABE* operon of the bacterium *Photorhabdus luminescens* [2] inserted into the vector pSC101 (Fig. 1). The advantage of this system is that it does not require any additional substrate to produce light when it is expressed from an active promoter. Thus, introducing a functional promoter of interest, (containing the upstream regulatory region and the RNA polymerase binding site of a target gene) results in light production when the promoter is turned on. This bioluminescent system generates light at a wavelength of 490 nm [4] and has a large dynamic range, enabling detection and quantification of a very weak to a very strong transcriptional activity. This approach was proved to be a very useful tool in studying promoter activity in vivo, during mouse infection of different bacterial pathogens [1, 5–7] (*see* **Note 1**).

To study the expression pattern of *dksA* we cloned its native regulatory region upstream to the *luxCDABE* operon and

introduced this construct (pCS26::p*dksA*) into wildtype *S.* Typhimurium. The empty vector was used as a negative control. C57BL/6 mice were orally infected with these *S.* Typhimurium reporter strains and 24 h postinfection they were sacrificed and imaged for bioluminescence along their gastrointestinal tract (Fig. 2) [1].

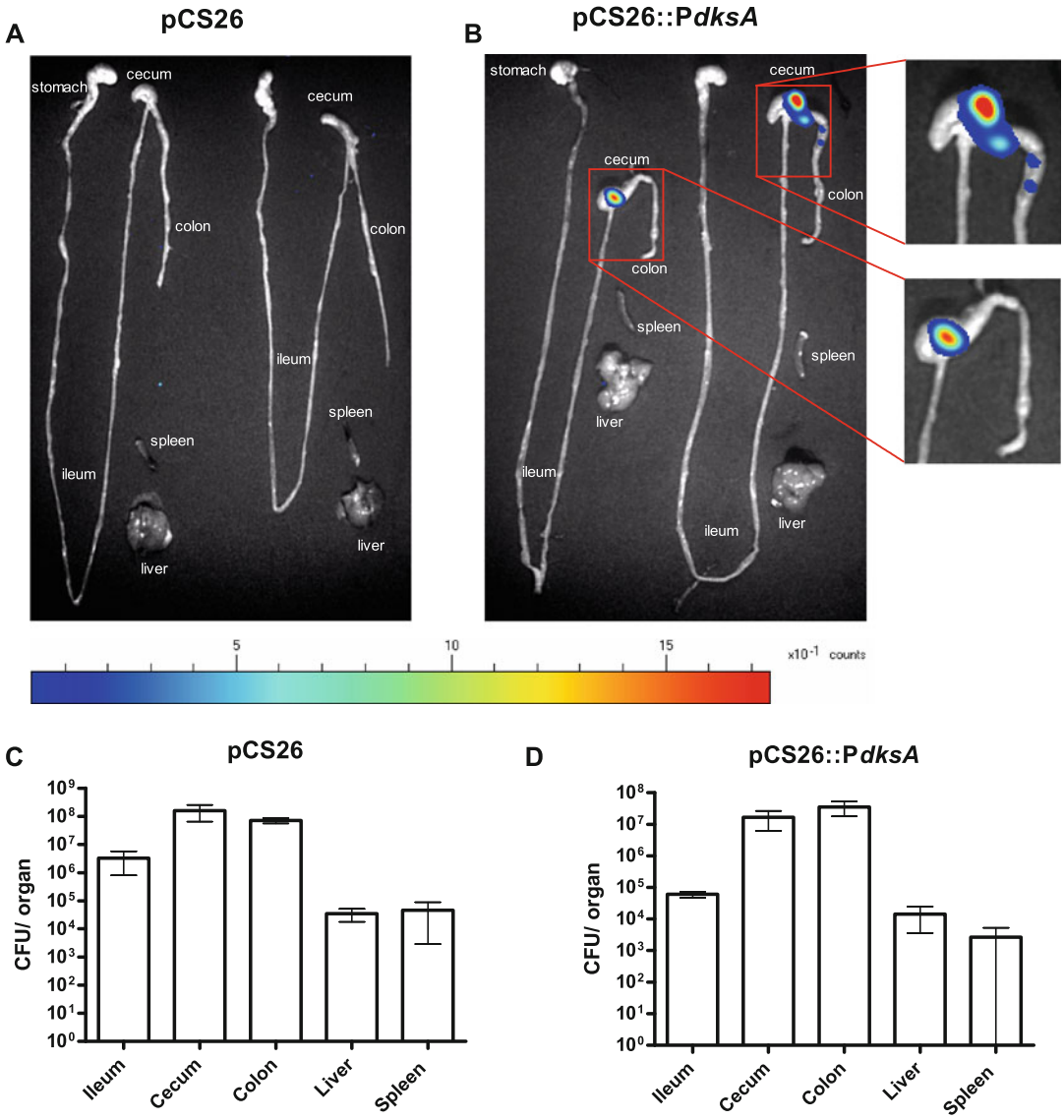

Fig. 2 *dksA* is expressed at the mid-cecum during intestinal colonization in vivo. Streptomycin-pretreated C57BL/6 mice were infected with 5–8 $\times$ 10^6 CFU of *S.* Typhimurium harboring pCS26 (**a**) or pCS26::p*dksA* (**b**) 24 h.p.i. the intestinal tract and systemic sites (liver and spleen) were removed and bioluminescence was imaged using a photon-counting system. Organs from two mice are shown from each infection. To determine the total numbers of colonizing *Salmonella* (CFU), organs were homogenized in saline, diluted and spread plated on XLD agar supplemented with kanamycin. Bars represent the mean bacterial loads and SEM in three mice infected with *S.* Typhimurium carrying pCS26 (**c**) or pCS26::p*dksA* (**d**). This figure is reproduced from Azriel et al. 2016 [1] with permission from the publisher (ASM Journals)

2 Materials

1. Luria–Bertani (LB) broth (Lennox) For 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl (pH 7.0).
2. Bacterial genomic DNA purification kit.
3. Plasmid mini extraction kit.
4. Hot start high fidelity DNA polymerase.
5. Appropriate restriction enzymes.
6. DNA gel extraction kit.
7. T4 DNA ligase.
8. Oligonucleotides for sequencing:
   pCS26 seq Forward: 5′- CCGACGTCTAAGAAACCATTATTATC-3′.
   pCS26 seq Reverse: 5′- CACTAAATCATCACTTTCGGGAAAG-3′
9. 6–8 week old C57BL/6 female mice (*see* **Note 2**).
10. Stainless feeding needles (24G-1″ straight 1.25 mm ball).
11. Surgical scissors and tweezers.
12. Streptomycin and kanamycin.
13. HEPES buffer.
14. Saline (0.9% w/v of NaCl in dH_2O).
15. In vivo imaging instrument suitable for bioluminescence imaging [e.g., PhotonIMAGER (BIOSPACE LAB) or IVIS Spectrum In Vivo Imaging System (PerkinElmer)].
16. BeadBlaster 24 microtube homogenizer or any other microtube homogenizer machine.
17. Xylose lysine deoxycholate (XLD) agar plates.

3 Methods

3.1 Cloning the Target Promoter in the Bioluminescence Reporter System

1. Extract the appropriate bacterial genomic DNA that will be used as the template for the PCR by bacterial genomic DNA purification kit or any other standard method.
2. Isolate the reporter vector pCS26 [3] (Fig. 1) DNA using a plasmid mini extraction kit.
3. Conduct a PCR to amplify the regulatory region controlling the expression of the target gene (*see* **Note 3**). Make sure to include appropriate restriction sites (e.g., XhoI and BamHI) at the 5′- and 3′-ends of the amplified DNA.
4. Clean the resulted PCR product from an agarose gel using a gel extraction kit.

5. Cut the pCS26 and the PCR product with XhoI and BamHI.
6. Apply a DNA purification step to remove uncut DNA, enzymes and salts that were left from the restriction reaction (*see* **Note 4**).
7. Ligate the insert into the cut pCS26 using T4 DNA ligase at 16 °C overnight.
8. Transform the ligated plasmid into competent *Escherichia coli* DH5α cells.
9. Confirm the presence of the target insert by restriction analysis and DNA sequencing using the oligonucleotides "pCS26 seq Forward" and "pCS26 seq Reverse."
10. Isolate the reporter plasmid from the surrogate *E. coli* and introduce the plasmid by electroporation into an appropriate electrocompetent *Salmonella* cells.

3.2 Mice Infection and Imaging

1. Twenty-four hours prior to the infection administrate the mice with streptomycin (20 mg per mouse) by oral gavage in 100 μL HEPES buffer (100 mM, pH 8.0) (*see* **Note 5**).
2. Grow the *Salmonella* reporter strains in LB supplemented with kanamycin (50 μg/mL) at 37 °C with aeration for overnight.
3. Infect the mice orally with ~5–7 × 10^6 CFU of the reporter strains suspended in 0.2 mL of saline.
4. Twenty-four hours postinfection (or at later time points if needed) sacrifice the mice and remove their intact gastrointestinal tract as well as systemic organs (e.g., liver and spleen) and place them on a black nonreflective cardboard sheet inside the imaging machine in order to record the luminescence signal (Fig. 2).
5. After imaging, isolate the gastrointestinal tract organs (e.g., ileum, cecum and colon) and homogenize them in 700 μL of saline using a microtube homogenizer.
6. Prepare serial dilutions (0–10^{-6}) in saline and plate them on XLD agar plates supplemented with kanamycin (50 μg/mL) for CFU count.
7. Count the colonies and calculate the bacterial load per organ (*see* **Note 6**).

4 Notes

1. This assay can be used not only for in vivo detection of gene expression, but also for examining gene expression under different environmental conditions and growth phases in culture using a luminometer. In this case gene expression can be

expressed as the luminescence normalized to optical density at OD_{600}.

2. For each reporter strain use at least 4–5 mice as some variation in *Salmonella* colonization is not uncommon.
3. For positive control, clone the promoter of a constitutively expressed gene such as *rpoD* and for negative control use the empty (promoter-less) vector.
4. It is recommended to desalt the cut inserts by ethanol precipitation (2.5 volume of ice-cold ethanol absolute; 0.1 volume of 3 M sodium acetate pH 5.2; and 5 μg of yeast tRNA). The cloning vector needs to be purified by a gel extraction kit in order to remove the uncut vector molecules.
5. Pretreating the mice with streptomycin prior to infection diminishes the mouse microflora and allows better *Salmonella* colonization in the gut.
6. CFU count in organs is important to show that low or no luminescence, is not due to poor colonization, but instead reflects the expression pattern of the target promoter.

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Chapter 5

lacZ Reporter System as a Tool to Study Virulence Gene Regulation in Bacterial Pathogens

Gili Aviv and Ohad Gal-Mor

Abstract

β-galactosidase assay has been established as one of the most widely used reporters and can be effectually exploited to study promoter activity of *Salmonella* and other pathogens under various conditions. This method includes a preliminary stage of fusing the target promoter to a promoter-less *lacZ* gene encoding for the enzyme β-galactosidase. Supplementation of the synthetic ONPG substrate results in the accumulation of a chromogenic product proportionally to the activity of the fused promoter. Here we demonstrate the usage of this reporter system to study the regulation of the *Salmonella* Type three secretion system effector gene *sseL* in *S.* Typhimurium [1].

Key words Beta-galactosidase (β-gal), *lacZ*, Lactose operon, *O*-nitrophenyl-β-D-galactoside (ONPG), Reporter, Gene regulation

1 Introduction

The *lacZ* gene from *Escherichia coli* encodes the enzyme β-galactosidase that cleaves the carbohydrate lactose to form glucose and galactose. Nevertheless, β-galactosidase can also recognize and hydrolyze a variety of chromogenic and fluorogenic synthetic substrates. One example is the colorless substrate *o*-nitrophenyl-β-D-galactoside (ONPG) that is hydrolyzed by β-galactosidase to yield galactose and o-nitrophenol. The byproduct o-nitrophenol has a yellow color, allowing quantitative spectrophotometric measurement. Since the production of o-nitrophenol is proportional to the concentration of β-galactosidase, the production of the yellow color can be used to determine indirectly the levels *lacZ* expression. This chemical reaction has been long exploited to create a reporter system that can monitor gene expression in an assay developed by Jeffrey Miller at 1972 [2]. This assay has been established as one of the most widely used reporters to study gene expression in molecular biology both in prokaryotes and eukaryotes. Basically, when a promoter-less *lacZ* gene is joined to a target promoter, the activity

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of the β-galactosidase can be used as a readout for the promoter activity. In this assay the synthetic substrate ONPG is added to the bacterial cells harboring the *lacZ* under the control of a promoter of interest that can be examined under various growth conditions (medium composition, environmental signals, stresses, etc.) and genetic backgrounds (e.g., in the absence of regulators). The amount of o-nitrophenol produced is dependent on the reaction time, and the number of cells generating a specific enzyme activity value expressed in Miller units [2].

Although this assay provides indirect measurements of promoter activity (as oppose to real-time qRT-PCR, for example), it is highly reproducible, straightforward, time-saving and does not require any expensive reagents and equipment, which may not be available to all labs. The *lacZ* fusion with the promoter of interest can be cloned into a low-copy number vector such as pMC1403 [3] or replace the chromosomal locus of the target gene. Here we demonstrate this reporter system to study the regulation of the *Salmonella* pathogenicity island (SPI) 2 effector gene *sseL* in *S.* Typhimurium and its regulation by the transcriptional regulator PhoP [1].

2 Materials

1. Luria–Bertani (LB) broth (Lennox) For 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl (pH 7.0).
2. Low phosphate low magnesium minimal (LPM) medium: 80 mM MES (pH 5.8), 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 0.5 mM K_2SO_4, 337 μM K_2HPO_4/KH_2PO_4 (pH 7.4), 20 mM $MgCl_2$, 38 mM glycerol, and 0.1% Casamino acids.
3. Bacterial genomic DNA purification kit.
4. Plasmid pMC1403 [3] (illustrated in Fig. 1) and plasmid extraction kit.
5. Hot start high fidelity DNA polymerase.
6. Primers to amplify the target promoter.
7. DNA gel extraction kit.
8. Restriction enzymes.
9. T4 DNA ligase.
10. X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside).
11. Sequencing primers: lacZ forward (5′-CCC CGA AAA GTG CCA CCT G-3′) and lacZ reverse (5′-GGA AGG GCG ATC GGT GCG GG-3′).

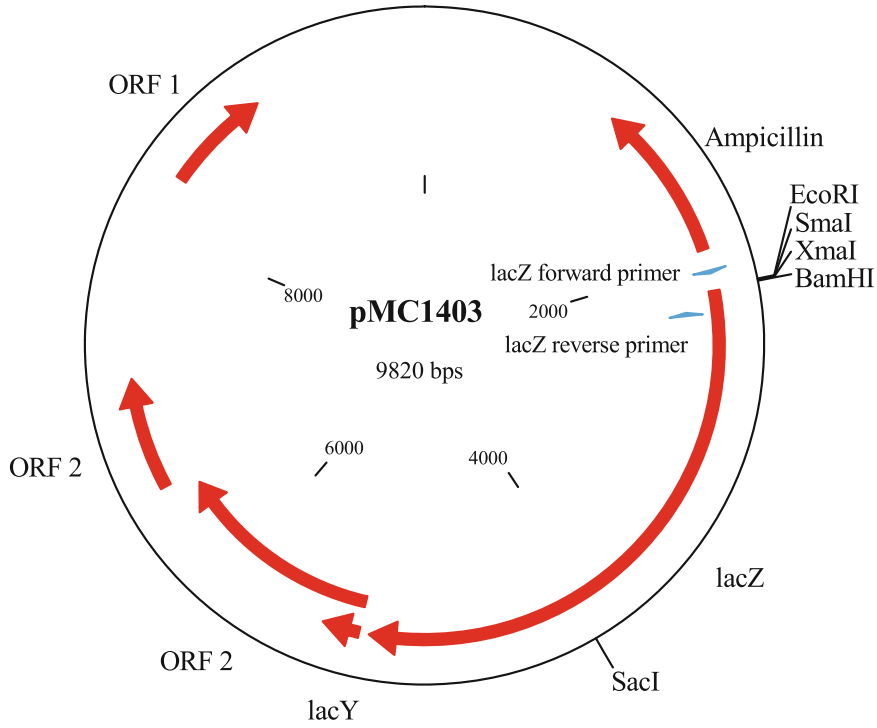

Fig. 1 Map of the *lacZ* reporter system pMC1403. A multiple cloning site upstream to the promoter-less *lacZ* contains the EcoRI, SmaI, XmaI, and BamHI sites. The position of *lacZ* forward and reverse primers is indicated as blue arrowheads

12. Ampicillin.
13. Z-buffer (for 1 L): 16.1 g of $Na_2HPO_4{\cdot}7H_2O$ (0.06 M), 5.5 g of $NaH_2PO_4{\cdot}H_2O$ (0.04 M), 0.75 g of KCl (0.01 M), 0.246 g of $MgSO_4{\cdot}7H_2O$ (0.001 M) and 2.7 mL β-mercaptoethanol (0.05 M). Prepare without β-mercaptoethanol, adjust pH to 7.0 and filter-sterilize (*see* **Note 1**).
14. P-buffer (for 200 mL): 2.136 g of $Na_2HPO_4{\cdot}2H_2O$ (0.06 M) and 1.248 g of $NaH_2PO_4{\cdot}2H_2O$ (0.04 M). Adjust to pH 7.0 and autoclave. Add 4 mg/mL ONPG before use for the amount of P-buffer needed.
15. *O*-nitrophenyl-β-D-galactoside (ONPG), 4 mg/mL in 0.1 M phosphate buffer, pH 7.0.
16. Toluene.
17. Stop solution: 1 M Na_2CO_3 (5.3 g in 50 mL).
18. Count up timer.

3 Methods

3.1 Construction of lacZ::Reporter Gene Fusion

1. Purify the bacterial genome of interest that will be used as the template for the PCR by bacterial genomic DNA purification kit or any other standard method.
2. Isolate the plasmid pMC1403 [3] using a plasmid mini extraction kit.
3. Amplify by PCR the target regulatory region using primers containing appropriate restriction sites (*see* **Note 2**).
4. Purify the PCR product using a gel extraction kit.
5. Cut plasmid pMC1403 and the PCR product with the same two restriction enzymes (we normally use EcoRI and BamHI; *see* Fig. 1) and purify the digested plasmid and the insert using DNA gel extraction kit and ethanol precipitation, respectively.
6. Ligate the insert into the plasmid using T4 DNA ligase at 16 °C overnight.
7. Transform the ligated construct into competent *Escherichia coli* cells (*see* **Note 3**) and plate the transformed bacteria onto LB plates supplemented with 100 μg/mL ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside), if the target promoter is active in-vitro you can expect to see the transformants as blue colonies.
8. Verify the cloning by restriction analysis and sequencing using the sequencing primers "*lacZ* forward" and "*lacZ* reverse."
9. Transform the plasmid into the appropriate *Salmonella* background strain.

3.2 Culture Growth and β-Galactosidase Assay

1. Grow *Salmonella* overnight with aeration at 37 °C.
2. The next day subculture by diluting the overnight culture 1:100 in fresh medium. Grow with aeration at 37 °C until the culture reaches 2–5 × 10^8 cells/mL (OD_{600} of 0.28–0.70), unless a different growth stage is wanted for the gene expression studies.
3. Place the culture on ice for 2 min to stop culture growth.
4. Measure and record the OD_{600}.
5. Into 2 mL test tubes prefilled with 0.9 mL of Z-buffer add 0.1 mL of the culture to a final volume of 1 mL. For low-activity promoters, one may add up to 0.5 mL of the culture, while complementing the total volume of the reaction to 1 mL with Z-buffer.
6. Add one drop of toluene to each tube and immediately vortex for 10 s (*see* **Note 4**).
7. Open the tube's caps and incubate the reaction tubes in a water bath set to 37 °C in a fume hood. This step is required to evaporate all the toluene from the solution.

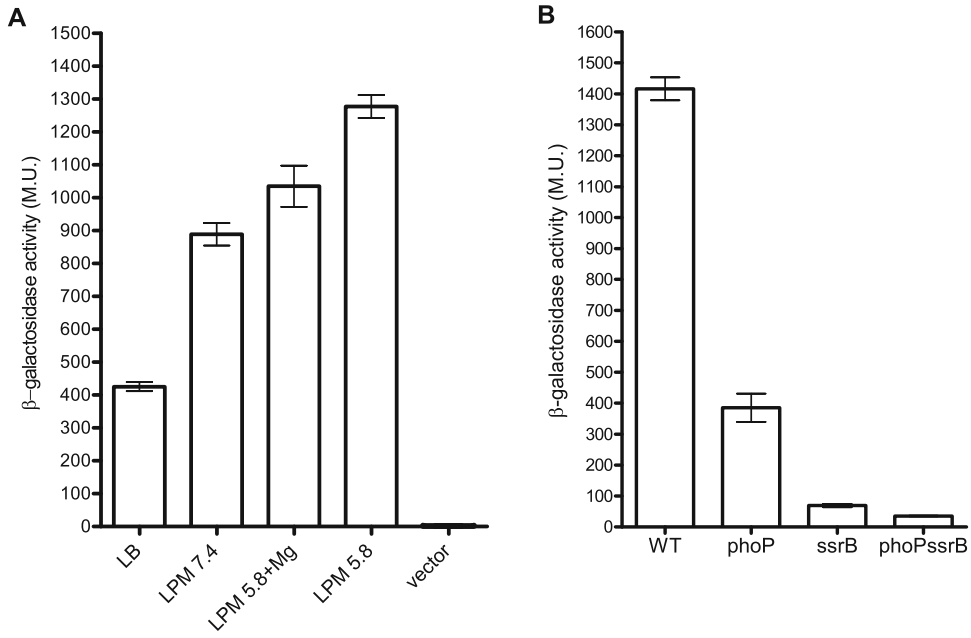

Fig. 2 *sseL::lacZ* is induced in response to low phosphate, low magnesium, and acidic pH environmental cues and is activated by PhoP and SsrB. (**a**) *S.* Typhimurium strains carrying *sseL:lacZ* were grown for 16 h at 37 °C in LB, LPM (pH 7.4) LPM (pH 5.8) supplemented with 10 mM $MgCl_2$, and LPM (pH 5.8), and were assayed for β-galactosidase activity presented in Miller units (M.U.). Basal *lacZ* expression of *S.* Typhimurium harboring pMC1403 (vector) that was grown in LPM (pH 5.8) is also shown. The induction of *sseL::lacZ* in LPM (pH 5.8) vs. LB can be appreciated. **(b)** β-galactosidase expression of *S.* Typhimurium wild-type (WT), *phoP*, *ssrB* and *phoP ssrB* double mutant strains harboring *sseL::lacZ* grown in LPM medium (pH 5.8). The positive regulatory role of PhoP and SsrB in *sseL::lacZ* expression is demonstrated. This figure is reproduced from Gal-Mor et al. (2011) [1] with permission from the publisher (PLOS Journals)

8. Equilibrate the tubes in a water bath at 28 °C for 5 min.
9. Add 0.2 mL of ONPG (4 mg/mL) to each tube and gently mix by inverting the tubes a few times. This is starting time of the reaction. Start running the timer.
10. Incubate the reaction at 28 °C until yellow color is clearly seen.
11. When a sufficient yellow color has been developed, stop the reaction by adding 0.5 mL of 1 M Na_2CO_3.
12. Record the stopping time of the reaction.
13. Centrifuge the tubes in a microfuge at 11,000 × *g* for 5 min to pellet cell debris (*see* **Note 5**).
14. Transfer 1 mL of the supernatant into cuvettes and measure the optical density at 420 nm for each reaction. The OD_{420} nm should be between 0.02–1.5 (*see* **Note 6**).
15. Calculate the β-galactosidase specific activity (Fig. 2) using the following formula:

$$\text{Miller units} = 1000 \times \frac{OD_{420}}{t \times v \times OD_{600}}$$

Where t = time of the reaction in minutes, v = volume of culture used in the assay (in mL; usually 0.1–0.5 mL).

4 Notes

1. Store the Z-buffer at 4 °C and add the required β-mercaptoethanol amount just before use to the appropriate volume of Z-buffer needed for the experiment.
2. We normally amplify a PCR fragment containing the entire intergenic region upstream to the target gene (or at least 500 bp upstream from the first methionine if the intergenic region is smaller than 500 bp) as well as the first seven amino acid codons of the target gene. If the target gene is organized in a polycistronic operon, and is not the first ORF in the operon, make sure that all the relevant regulatory sequence is cloned.
3. We recommend to use the *E. coli* K-12 strain MC1061, containing a null deletion of the entire *lac* operon.
4. This step is required to permeabilize the bacteria envelope allowing ONPG to penetrate into the cytoplasm where the β-galactosidase is expressed.
5. This step is important to minimize light scattering by cell debris that can affect the absorbance at OD_{420}.
6. The absorbance at OD_{420} should be between 0.02 and 1.5 to be within the linear range of the assay. If a reading is above 1.5, dilute the reaction with Z-buffer and multiply the newly measured OD_{420} by the dilution factor.

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Chapter 6

Western Blotting Against Tagged Virulence Determinants to Study Bacterial Pathogenicity

Gili Aviv and Ohad Gal-Mor

Abstract

Western blotting is a common approach to detect the presence of a target protein in biological samples or proteins mixture using specific antibodies. This method is also useful to study regulation of virulence determinants by analyzing changes in protein expression between different genetic backgrounds or under varying environmental conditions. To avoid the need to raise specific antibodies for each studied protein, commercial antibody against commonly used peptidic epitopes can be utilized if the right target tagged version is constructed. Here we describe a C-terminal fusion between a protein of interest and the two hemagglutinin A (2HA) tag. The tagged protein is cloned into a low-copy number vector and expressed under its native promoter in *Salmonella enterica*. Then, the expression of the tagged protein can be analyzed by Western blotting and commercially available anti-2HA antibodies.

Key words Western blotting, Gel electrophoresis, Protein tagging, Immunoblotting, Hemagglutinin, Antibodies

1 Introduction

Western blotting (also known as immunoblotting) is a well-established analytical technique used to detect a particular protein in a complex mixture or biological samples (cell lysate, cellular fractionation, conditional media, etc.) based on antigen–antibody specific recognition [1]. Western blotting involves a polyacrylamide gel electrophoresis (PAGE) and transfer of the separated proteins onto a special membrane [typically nitrocellulose or polyvinylidene difluoride (PVDF)] in a process called blotting. The membrane-transferred proteins are accessible to a primary antibody binding, followed by a secondary antibody conjugated with a catalytic enzyme (e.g., horseradish peroxidase) that is used in a detection reaction.

Nevertheless, since raising and using a specific primary antibody against individual proteins of interest is time-consuming and expensive, one may use commercially available antibodies against

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common peptide epitopes (tags) such as FLAG, glutathione-S-transferase (GST), histidine (His), or hemagglutinin A (HA). The tag of selection is fused to the protein of interest, usually at the C- or the N-terminus. This approach provides high specificity and versatility, but requires a prior step of cloning and expression of the tagged target in the appropriate bacterial host under its native promoter. The recombinant tagged version of the protein can be cloned into a low-copy number vector under its native promoter or replace the chromosomal locus of the target gene (*see* **Note 1**).

The commonly used HA tag (YPYDVPDYA) is derived from the binding domain of the *Influenza* hemagglutinin protein and contains a high composition of charged residues comprising a strong antibody recognition site. Here, we describe a protocol to construct a C-terminal fusion between a target protein of interest and a 2HA tag and determine the expression of this tagged protein in different backgrounds of *S. enterica*. To demonstrate this protocol, we show the construction of the *Salmonella* flagellin subunit FliC tagged with a 2HA epitope.

2 Materials

2.1 2HA-Tagged Protein Fusion

1. Luria–Bertani (LB) broth (Lennox) For 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl (pH 7.0).
2. Bacterial genomic DNA purification kit.
3. Plasmid mini extraction kit.
4. Hot start high fidelity DNA polymerase.
5. Oligonucleotide primers.
6. DNA Gel Extraction Kit.
7. Appropriate restriction enzymes.
8. Low-copy number cloning vector (*see* **Note 2**).
9. T4 DNA ligase.
10. Competent *Escherichia coli* DH5α cells.
11. Oligonucleotides for sequencing:

For pWSK29/pWSK129:

'M13/Puc forward' (5'-GTTTTCCCAGTCACGACGTTG-3')

'M13/Puc primer reverse' (5'-AGCGGATAACAATTTCACACAGGA-3')

For pACYC184:

'pACYC184 forward' (5'-CACCGGAAGGAGCTGACTG-3')

'pACYC184 reverse' (5'-GTAGCACCTGAAGTCAGCCC-3')

Table 1
Reagents required for casting the polyacrylamide separation gel

% gel	6%	8%	10%	12%	15%
Separation range (kDa)	60–200	40–100	20–70	20–60	10–40
Acrylamide–Bis (mL)	2.3	3.0	4.0	4.5	5.6
ddH_2O (mL)	8.7	7.9	6.9	6.3	5.2
TEMED (μL)	12	9	6	6	6

2.2 SDS-Polyacrylamide Gel

1. SDS-PAGE running buffer (For 1 L 10×): 10 g SDS, 30.3 g Tris, 144.1 g Glycine.
2. SDS-PAGE sample buffer (2×): 1.5 mL of 1 M Tris pH 6.8, 1.2 mL of 10% SDS, 6 mL of 50% glycerol, 1.5 mL of β-mercaptoethanol, 0.18 mg of bromophenol blue. Aliquot into 1 mL portions and store at −20 °C.
3. SDS-PAGE resolving gel (15 mL for 2 mini gels): 40% Acrylamide–Bis solution (37.5:1; according to Table 1), ddH_2O (according to Table 1), 3.8 mL of 1.5 M pH 8.8 Tris–HCl, 150 μL of 10% SDS, 150 μL of 10% ammonium persulfate (APS) and N, N, N′, N′-tetramethylethylenediamine (TEMED, according to Table 1 and *see* **Notes 3** and **4**).
4. 5% stacking gel (6 mL for 2 mini gels): 0.75 mL of 40% Acrylamide–Bis solution (37.5:1), 4.4 mL of ddH_2O, 0.75 mL of 1 M pH 6.8 Tris–HCl, 60 μL of 10% SDS, 60 μL of 10% APS, and 6 μL of TEMED (*see* **Notes 3** and **5**).

2.3 Immunobloting

1. Transfer buffer: 3.03 g of Tris, 14.4 g of glycine, 0.375 g of SDS. Dissolve all three reagents in 500 mL of dH_2O. Add 200 mL of methanol (*see* **Note 6**) and adjust to 1 L with dH_2O.
2. TBS (10×): dissolve 80 g NaCl, 2 g KCl, and 30 g Tris in 700 mL dH_2O. Adjust the pH to 7.4 with concentrated HCl and complete the volume to 1 L.
3. Washing buffer (TBST): 250 μL of Tween 20 in 1 L of 1× TBS.
4. Blocking buffer: 5% skim milk powder in TBST.
5. Detection reagent: ECL Western Blotting Detection Reagent.
6. Transparency film sheets.

2.4 Antibodies

1. Mouse monoclonal antibody [HA.C5] against HA tag. Add 20 μL of the antibody in 20 mL of blocking solution.
2. Mouse monoclonal [8E2/2] against Dnak. Add 3 μL of the antibody in 30 mL of blocking solution.
3. Goat polyclonal secondary antibody against mouse IgG- H&L (HRP). Add 3 μL of the antibody in 15 mL of blocking solution.

3 Methods

3.1 Construction of 2HA-Tagged Proteins Fusion

1. Isolate the relevant bacterial DNA, which will be used as the PCR template by a bacterial genomic DNA purification kit.
2. Isolate the cloning plasmid (e.g., pWSK29, pWSK129, or pACYC184) using a plasmid mini extraction kit.
3. Amplify the 2HA DNA sequence using a PCR and primers, which include appropriate restriction sites at the 5′ and 3′ ends of the sequence (*see* **Note 7**). Make sure to also introduce a stop codon (e.g., TAA) downstream to the 2HA tag if a C-terminus tag is constructed.
4. Clone the 2HA DNA sequence into the cloning vector of choice, using the flanked restriction sites.
5. Amplify by PCR the target gene of interest (without the stop codon) including the upstream regulatory region, using primers that include appropriate restriction sites. The restriction site at the 3′-end should be the same as the restriction site, which is located upstream from the cloned tag in a way that the cloning will result in an in-frame C-terminus tag fusion (*see* **Note 8**).
6. Separate the PCR products in an agarose gel and purify the PCR products using DNA gel extraction kit.
7. Digest the cloning plasmid (containing the 2HA tag) and the insert (target gene) with the same pair of restriction enzymes. Purify the digested DNA after the restriction reaction (*see* **Note 9**).
8. Ligate the insert into the cloning plasmid using T4 DNA ligase at 16 °C for overnight.
9. Transform the ligation product into competent *Escherichia coli* DH5α cells.
10. Isolate the recombinant clone after plating on selective LB agar plates.
11. Verify the clone by restriction analysis followed by sequencing to make sure that no mutation has been introduced during PCR amplification and that the tag is inserted in-frame.
12. Transform the recombinant construct into the appropriate *Salmonella* background.

3.2 Preparation of Protein Fractions

1. Grow *Salmonella* culture under the desired growth conditions under appropriate selection.
2. Measure the cultures OD_{600} and normalize all cultures to the same optical density (*see* **Note 10**).
3. Centrifuge the cultures at 11,000 × *g* for 2 min at room temperature in a microcentrifuge and remove the supernatant carefully using a pipette. Resuspend the pellets in 150–200 μL of 1× SDS-PAGE sample buffer. Boil the samples for 5 min and place on ice immediately.

3.3 Electrophoretic Wet Transfer and Blotting

1. Load the samples on the SDS-PAGE gel including molecular weight markers and run the gel at constant current of 30 mA in running buffer until the loading dye reaches the bottom of the gel.
2. Gently, separate the glass cast, take out the acrylamide gel and equilibrate in transfer buffer to remove any remnants of electrophoresis buffer salts.
3. Place the electrophoretic wet transfer tank onto a magnetic stir plate (*see* **Note 11**).
4. For each gel, cut one piece of polyvinylidene fluoride (PVDF) membrane and two pieces of Whatman filter paper to the dimensions of the gel (e.g., 7.5 × 8.5 cm for mini gels).
5. Place the PVDF membrane in methanol for 1 min, then soak the membrane for 2 min in ddH_2O and then place it in a transfer buffer.
6. Build the gel and membrane sandwich inside the gel holder cassette using two filter papers (7.5 × 8.5 cm) and two fiber pads (sponges) soaked with transfer buffer.
7. Place a wet fiber pad on top of the black side of the cassette immersed in transfer buffer.
8. Place the following components between the two wings of the gel holder from the anode to the cathode: fiber pad, Whatman filter paper, PVDF membrane, gel, filter paper, and fiber pad (Fig. 1). Use a clean glass test tubes to remove any air bubbles caught between the layers of the sandwich and to ensure proper contact between the gel and membrane by rolling it over the sandwich layers.
9. Close and lock the cassette, insert it into the transfer tank with the latch side up and with the black wing facing the black electrode plate (cathode).
10. Place the cassette inside the chamber and fill it with cold transfer buffer until the gels and membranes are submerged under transfer buffer (~1.5 L).
11. Put a stirrer magnet at the bottom of the chamber, and turn on the stirrer. Set the power to100 V and transfer the proteins for 1 h.
12. After the blotting is completed, disassemble the tank transfer system and gently open the gel and membrane sandwich. Rinse the membrane briefly in ddH_2O to ensure that no residual gel shards adhere to the membrane and incubate the membrane in fresh blocking buffer for 1 h with gentle shaking at room temperature.
13. Incubate the membrane with the primary antibody solution, e.g., anti-2HA tag or anti-DnaK as a loading control for 1 h at room temperature or for overnight at 4 °C.

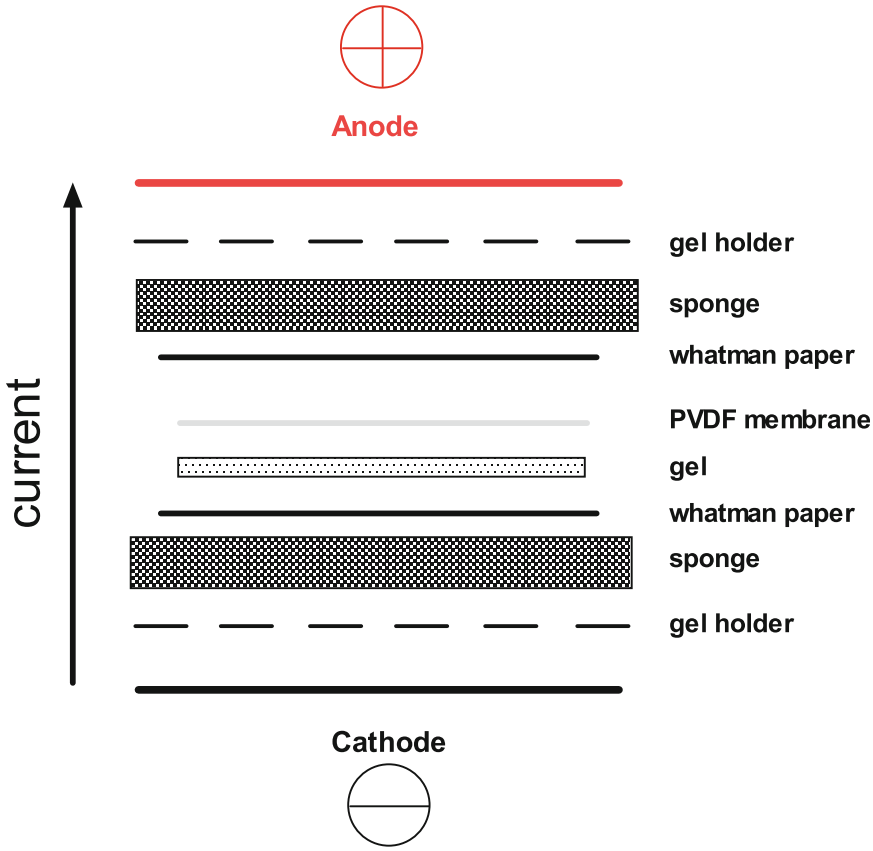

Fig. 1 The assembly of the transfer sandwich. A schematic representation of Western blotting "sandwich," used for proteins blotting from an acrylamide gel to the PVDF membrane during a wet transfer

14. Wash the membrane for 5 min with the washing buffer under gentle shaking and repeat this washing step four times.
15. Incubate the membrane with the secondary antibody, e.g., goat anti-mouse conjugated with peroxidase diluted in blocking buffer for 1 h at room temperature, with gentle shaking.
16. Wash the membrane with washing buffer four times (5 min each time).
17. Prepare 2 mL of the enhanced chemiluminescence (ECL) Western Blotting Substrate (used for peroxidase substrate, by mixing 1 mL of each reagent) in a tube. Place the membrane onto one sheet of transparency film (transferred proteins facing up) and pour the 2 mL of the ECL substrate on top of the membrane. Place immediately the second plastic film to cover, make sure that the ECL substrate evenly covers the entire membrane surface and incubate for 1 min at room temperature.
18. Place the plastic films wrapping the membrane on a paper towel and squeeze out excess of the ECL reagent using Kimwipes disposable wipers and place the membrane inside an X-ray cassette for the detection of the signals using X-ray film (Fig. 2).

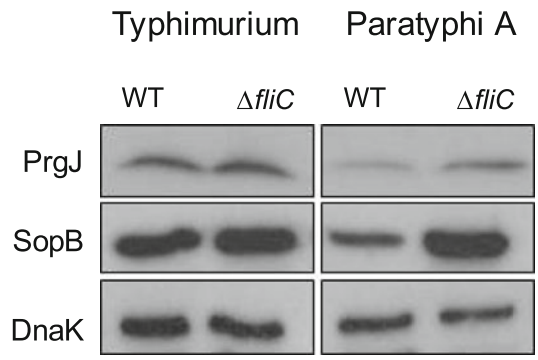

Fig. 2 The absence of flagellin induces SPI-1 gene expression in *S.* Paratyphi A. SDS-PAGE Western blot analysis of bacterial cell lysate from *S.* Typhimurium and *S.* Paratyphi A wild type (WT) and a *fliC* mutant (Δ*fliC*) expressing SopB-2HA or PrgJ-2HA (two SPI-1 effector proteins). Protein fractions were probed using anti-HA antibodies and anti-DnaK as a loading control. This analysis showed higher expression of SPI-1 effectors in the absence of *fliC* in *S.* Paratyphi A compared to *S.* Typhimurium. This figure is reproduced from Elhadad et al. [2] with permission from the publisher (ASM Journals).

4 Notes

1. For most purposes expressing the tagged protein under the native regulatory region from a low-copy number vector will provide reliable data. However, one can also use the λ-red recombinase method and replace the gene of interest in the chromosome with a tagged version as described in reference [3].
2. For *S. enterica* or *E. coli*, we normally use the pWSK29, pWSK129 [4], or pACYC184 [5] vectors.
3. When preparing the SDS-PAGE resolving and stacking gel add the APS and the TEMED last, because together they catalyze the polymerization of acrylamide and bisacrylamide. Mix well and quickly pipet the gel solution into the gap between the glass plates.
4. When pipetting the resolving gel, do not forget to allow space for the stacking gel (~0.5 cm lower from the end of the gel comb) and then add water onto the top of the gel until it overflows. This will create a smooth interface between the stacking and resolving layers and protect the gel from ambient oxygen, which inhibits acrylamide polymerization.
5. When the polymerization of the resolving gel is completed (about 30 min), pipet the stacking gel solution until it overflows and insert a gel comb immediately without trapping air bubbles under the teeth of the comb.
6. Use high-quality analytical grade methanol.

7. Add 4 nucleotides, (we usually add AAAA) upstream and downstream to the restriction sites in the forward and reverse primers, respectively. This allows optimal digest of the restriction sites in the obtained amplicon.
8. In certain cases the C-terminus of a protein may be important for its function and a C-terminal fusion cannot be constructed. In such cases an N-terminus tag can be considered. Under such circumstances, make sure the protein is not secreted and that the N-terminus is not cleaved by the Sec system.
9. It is recommended to purify the cut inserts by standard ethanol precipitation (we use 2.5 volume of ice-cold ethanol absolute; 0.1 volume of 3 M sodium acetate pH 5.2; and 5 μg of yeast tRNA). The digested cloning vector has to be separated in an agarose gel and purified by a gel extraction kit in order to exclude uncut molecules.
10. Normalization of total proteins amounts in each sample can also be determined using standard protein concentration assays (e.g., Bradford).
11. Electrophoretic wet transfer of the proteins from the gel to a polyvinylidene fluoride (PVDF) membrane should be done under cold temperature (standard cold room is not cold enough), if you do not use a cooling system for the tank transfer system, one may fill a large box (at least 30 × 30 cm) with crushed ice and put the transfer chamber inside.

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Chapter 7

Molecular Methods to Analyze the Effect of Proteins Expressed by *Salmonella* During Its Intracellular Stage

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Abstract

Salmonella is probably the intracellular pathogen most extensively studied. Once inside the cell, this bacterium produces different proteins involved in the infection process known as effectors that translocate through its own secretion systems to the eukaryotic cytosol exerting diverse effects on the cell. Additionally, *Salmonella* can be engineered to include a protein expression system that, upon the addition of an inducer molecule, can produce heterologous proteins at a specific time during the course of the infection. The effect of such proteins on the eukaryotic (i.e., tumoral) cells can be detected following distinct approaches, which converts *Salmonella* in an effective tool to produce proteins inside eukaryotic cells with different purposes, such as killing tumoral cells. Here, we present diverse technics currently used to produce proteins by *Salmonella* inside tumoral cells and analyze its cytotoxic effect.

Key words *Salmonella*, Protein expression, Therapeutic proteins, Antitumoral drugs, Bacterial lysis, Salicylate, Drug delivery

1 Introduction

The idea of treating tumors with living bacteria was conceived during the nineteenth century, when several cancer patients were infected with diverse bacteria and a considerable tumoral regression was observed in some of them (reviewed in [1]). The main trouble associated with this treatment is the inherent toxicity of bacteria, which produce lethal infections while inducing tumor regression. In the last decades, the interest of using living bacteria as antitumoral agents is rising due to the advances in biotechnology and synthetic biology, which allows exceeding the above mentioned limitation, extending the potential of the treatment. *Salmonella* has been further studied as antitumor vector due to its intrinsic properties. As a facultative anaerobe, *Salmonella* can grow in the hypoxic area of large solid tumors additionally to aerobic areas, in contrast to strict anaerobes as *Clostridium*, which only grows in

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necrotic areas [2, 3]. Furthermore, *Salmonella* proliferates in tumors at ratios 1000/1 compared to normal target organs as liver or spleen, which converts these bacteria in an "intelligent bomb" that can preferentially kill tumoral cells hosting them more frequently than healthy tissues.

Genetic engineering allows the rational design of different bacterial strains with selective peculiarities to behave appropriately in each scenario. Good examples are the mutation on receptors TAR or TRG, which respectively detect aspartate secreted by living cells and ribose of a necrotic tissue [4]. Deleting TAR or TRG genes, bacteria can preferably accumulate in the proliferative zone or the necrotic area of the tumor, respectively.

Due to the difficulties to study the behavior of *Salmonella* in murine models, an alternative approach to evaluate the possibilities of *Salmonella* as antitumoral vector relies on the infection of monolayer tumoral cell cultures, which combines the feasibility of an in vitro system with the battery of technics that permits monitoring the course of the infection and analyze the effect or localization of determined proteins in eukaryotic cells.

The intrinsic bacterial cytotoxicity can be increased by engineering bacteria to express therapeutic proteins [1, 5–13]. Another advantage of using bacteria in this kind of therapies is the availability of different protein production systems based in regulated promoters whose activation can be controlled by the external addition of different inducer molecules (i.e., salicylate, arabinose, tetracycline) [13–15].

We have developed a protein production system inducible by salicylate that is composed by regulatory elements from soil bacteria. It consists on a regulatory module composed by two divergent promoters, Pnah and Psal, which control the expression of two regulators NahR and XylS2 respectively. NahR, constitutively expressed, binds salicylate promoting its own expression from Pnah, and XylS2 expression from Psal, which simultaneously is activated by salicylate and triggers transcription from a third promoter named Pm. The system is completed with an expression module that contains a gene of interest (i.e., for the production of therapeutic molecules) cloned downstream the Pm promoter that, upon salicylate addition, produces a high amount of protein due to the synergic actuation of regulators [15, 16]. Additionally, we have included an attenuator element (*nasF* attenuator) between the Pm promoter and the gene of interest that reduces basal expression levels to minimize spurious transcription. Such attenuator is relaxed by the effect of the antiterminator protein NasR, which is placed downstream XylS2 and therefore, is also expressed from Psal [17]. In summary, the attenuator silences the system until the addition of salicylate, which switches on a cascade of regulators that conclude in the production of the protein of interest. Finally, the regulatory module contains a fluorescence protein gene to track the spatiotemporal location of bacteria during the infection process.

Additionally, we have designed an anhydrotetracycline (AHT) inducible releasing system, based on lambda phage lysis mechanism, to lyse bacteria and deliver the cytoplasmic content directly into the tumoral cell cytosol [18]. Following internalization, *Salmonella* replicates inside a vacuole known as *Salmonella*-containing vacuole (SCV). The SifA protein is necessary to maintain the integrity of the SCV and, therefore, bacteria carrying a mutation in this gene are released into the host cell cytosol several hours after internalization [19]. Briefly, we have combined the lysis system, triggered by anhydrotetracycline, with the salicylate induced expression system and with a *sifA* mutation with the aim of massively producing a therapeutic agent and discharging it directly into the eukaryotic cytosol.

An excessive replication of *Salmonella* inside the eukaryotic cell induces apoptosis, which hinders the real effect of the therapeutic drug. To measure the effect of the therapeutic protein per se, we take advantage of *purD* mutants, which are unable to replicate unless purines are added to the medium [20], controlling this way, independently, drug production and bacterial replication. This combined control of protein production by salicylate, and bacterial growth by adenine concentration, offers the possibility to study the role of heterologous proteins and also of *Salmonella* effectors during eukaryotic cells infection [20].

The rational combination of all these implements allows the engineering of *Salmonella* as a powerful tool to test the efficiency of particular antitumoral molecules to efficiently kill specific tumoral cells lines in vitro, what is a previous requirement for a personalized antitumoral therapy.

In this chapter, we detail the technics that we used to (1) infect eukaryotic cells and follow the progression of the infection of distinct mutants; (2) detect the localization of cytotoxic proteins produced and released by intracellular *Salmonella,* and (3) analyze the different effects produced by cell cycle progression or apoptosis markers production (Fig. 1).

2 Materials

2.1 Bacteria and Cell Handling, and General Infection

1. Salmonella strains and tumoral cell lines (HeLa, MCF-7, . . .).
2. Luria–Bertani (LB) medium (For 1 L): Bacto tryptone 10 g, yeast extract 5 g, and NaCl 5 g. Add 15 g of agar for solid media.
3. Commercial Dubelcco's modified Eagle's medium (DMEM): This medium is supplemented with 10% fetal calf serum (FCS; complement previously heat-inactivated), 2 mM L-glutamine and a commercial mixture of antibiotics penicillin and streptomycin 100× (*see* **Note 1**).

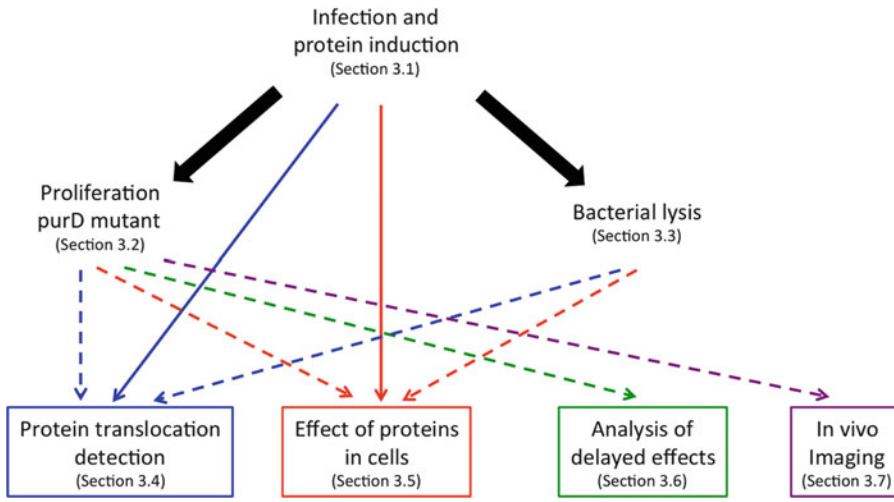

Fig. 1 Workflow of methods to analyze location and/or effect of proteins produced by *Salmonella*

Table 1
Cell density used in different applications

	Number of cells	Petri/Well size	Incubation hpi
Protein extraction	3×10^6	10 cm Ø	24
Microscopy	2×10^5	1 cm Ø	4–24
	10^5	1 cm Ø	48
	1.5×10^5	3.5 cm Ø	24
Cell cycle analysis	2×10^5	4 cm Ø	24
	10^5	4 cm Ø	48
	5×10^4	4 cm Ø	72
Cytosine deaminase	2×10^5	10 cm Ø	144
LDH assay	5×10^4	1 cm Ø	24

hpi hours post infection. The volume of media used in each dish or well is proportional to the diameter (100 µL per 1 cm Ø)

4. Cells should be cultivated in a determined plate size that allows the growth of the appropriate amount of cells for each technic. Table 1 summarizes this information.
5. Phosphate-saline buffer (PBS 1×) (For 1 L): NaCl 8 g, KCl 0.201 g, Na_2HPO_4 1.42 g, $KH_2PO_4$0.272 g. Adjust pH = 7.4.
6. Earle's balanced salt solution (EBSS commercial).
7. Gentamicin sulfate solutions: Dilute a commercial stock of 10 mg/mL in DMEM to prepare 100 µg/mL and 16 µg/mL solutions.

8. Expression induction solution: 0.1 M sodium salicylate (16.01 mg/mL in bidistilled water) and sterilize by filtration. Keep aliquots protected from light at 4 °C.
9. Adenine hemisulfate solution: 73.30 mM (13.5 mg/mL in 10 mL HCl 0.1 N). Sterilize by filtration and store at 4 °C (*see* **Note 2**).
10. Anhydrotetracycline solution (AHT): Commercially acquired at 2 mg/mL. Dilute to 20 μg/mL in ultrapure ethanol to achieve a 100× stock.
11. Trypsin: Dilute commercial trypsin (2.5×) tenfold in PBS 1× and store in 10 mL aliquots. In some applications, trypsin can be supplemented with EDTA (20 μL EDTA 0.5 M in 10 mL 0.25× trypsin) to disaggregate efficiently cells clamps (*see* **Note 3**).
12. Triton X-100 0.1%.

2.2 Protein Immunodetection

1. Formalin 3.7%: Dilute commercial formalin (usually at 37%) 10× in PBS 1×.
2. PBT: 0.1% Triton X-100 in PBS 1×.
3. Blocking buffer: PBT complemented with 3–7% FCS (*see* **Note 4**).
4. Antibodies anti-DNAK and anti-HA. For antibodies use follow manufacturers' recommendations (*see* **Note 5**).
5. Nuclei staining solution: 1 mg/mL DAPI dye (4′,6-Diamidino-2-phenylindole) (or Hoechst bisbenzimide) in distilled water and dilute 1:1000 directly into the sample for nuclei staining.
6. Lysis buffer: 20 mM Tris–HCl pH 7.6, 1 mM EDTA pH 8, 150 mM NaCl, 1% Triton X-100 in distilled water. Complement with a protease inhibitor cocktail containing 50 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (*see* **Note 6**).
7. Mounting media: 50% glycerol, 50% PBS 1×.

2.3 Cell Cycle Analysis

1. Ethanol solution: 80% ethanol analytical grade in PBS 1×.
2. PBS-BSA solution: 0.1% of bovine serum albumin into PBS 1×.
3. Extraction solution: Dissolve 0.21 g citric acid in 10 mL H_2O. Separately, dissolve 14.32 g $Na_2HPO_4.12\ H_2O$ in 200 mL distilled water. To prepare properly, remove 8 mL of Na_2HPO_4 solution and replace with 8 mL of citric acid solution and adjust pH to 7.8 (*see* **Note 7**).
4. Staining solution:100 μg/mL RNAse, 0.1% Triton X-100, 0.1 mM EDTA pH 8, 40 μg/mL dissolved in PBS 1×. Protect from light and discard after use.
5. 70-μm nylon filters.

2.4 Lactate Dehydrogenase Measurement (LDH)

1. 9% (v/v) Triton X-100 in water.
2. Any commercial kit for cytotoxicity measurement in 96 wells plate. Follow the manufacturer's instructions to prepare the reconstitutive substrate mix.
3. Staurosporine (STS): 1 mM in 1 mL of DMSO and keep the solution at −20 °C protected from light.

2.5 Cytosine Deaminase Activity

1. 5-FC:13 mg/mL in 10 mL of distilled water (Stock 100 mM), and sterilize by filtration (0.20 μm filters). Keep at 4 °C protected from light. It is convenient to make 1 and 10 mM diluted stocks in distilled water.
2. 5-FU: 2 mg/mL in 10 mL of DMSO (Stock 15.37 mM). Keep at 4 °C protected from light.

2.6 Live Imaging

1. HeLa Kyoto cells stably expressing H2B-mCherry and mEGFP-α-tubulin.
2. Leibovitz's (L-15) commercial medium.
3. Poly-D-lysine 35-mm glass bottom culture dishes.
4. Image J software.

3 Methods

The optimum growing temperature for *Salmonella* is 37 °C and they are usually cultivated aerobically at 180 rpm. Most tumoral cells are also incubated at 37 °C in an atmosphere of 5% CO_2. Therefore, carry out all experiments under these conditions.

3.1 Infection and Protein Induction Conditions

1. In order to properly infect the eukaryotic cells, *Salmonella* must be added to the culture at early stationary phase. The day previous to the infection, prepare cultures of *Salmonella* strains and grow overnight in LB medium supplemented with antibiotics when necessary.
2. The same day, detach previously cultured cells with trypsin and plate the appropriate number of cells depending on the surface of the petri dish or the well to be used in supplemented DMEM medium containing antibiotics. Cells must be plated around 20 h before the infection to avoid division and maintain its number constant at the time of the infection. In different applications, the density of the cell culture in the plate and the duration of the experiment are variable. Table 1 shows our recommendations for distinct experiments.
3. Dilute *Salmonella* culture (1:33) in 3 mL of the LB medium and incubate for 3 h 30 min.

   The eukaryotic cells must be washed twice with PBS and equilibrated with EBSS buffer 30 min before the infection.

4. Add bacteria to the plate containing cells at a desired multiplicity of infection (M.O.I.). In most applications, it is used a 100:1 M.O.I., which can be increased to 250–500:1, or reduced to 50:1 depending on the experiment. A higher M.O.I. leads to a greater number of infected cells, what is suitable in applications where the expected effect of the protein produced is not easy to detect (*see* **Note 8**).
5. Incubate cells with bacteria for 20 min to allow bacterial invasion. Remove the medium by aspiration and wash twice with PBS to eliminate extracellular bacteria (*see* **Note 9**).
6. Remove PBS, substitute it by medium containing 100 μg/mL gentamicin to kill extracellular bacteria and incubate for 1 h. Remove media by aspiration, wash twice with PBS and add DMEM containing 16 μg/mL gentamicin. The infected culture must be incubated as much as necessary for every application.
7. Add 2 mM salicylate per well/plate to induce the cascade system and incubate the time required depending on the experiment. Salicylate must be added to the cell culture once the infection is established (usually at the same time when the amount of gentamicin is reduced to 16 μg/mL) (*see* **Note 10**).

3.2 Proliferation of ΔpurD Mutants Inside the Cell

1. Continue from the **step 5** in Subheading 3.1.
2. Add 366 μM of adenine hemisulfate to DMEM media containing 100 μg/mL gentamicin and incubate for 1 h.
3. Keep such adenine concentration when reducing gentamicin to 16 μg/mL in the medium and incubate for 1 additional hour (with 2 mM salicylate when needed). In these conditions, Δ*purD* mutant is allowed to grow as wild type strain during the first infection stages.
4. Replace medium reducing adenine concentration to 9.15 μM while maintaining gentamicin concentration (and salicylate when needed) to avoid bacterial proliferation.
5. Incubate cells in this medium until analysis.

3.3 Controlled Autolysis of Intracellular Bacteria

1. Proceed following **step 7** in Subheading 3.1.
2. Allow the protein production as long as necessary, and subsequently induce bacterial lysis adding 0.2 μg/mL of AHT.
3. Incubate for a minimum of 10 h to ensure that most bacteria are lysed.
4. If quantification of survivor intracellular bacteria (gentamicin-protected) is needed, wash three times the eukaryotic cells with PBS and gently lyse with 0.1% Triton X-100 for 10 min. Different dilution series (10^1–10^5) should be plated on LB agar and count the number of colonies after 24 h incubation at 37 °C.

5. Cells can alternatively be treated for immunofluorescence (*see* Subheading 3.4.1), western blot analysis (*see* Subheading 3.4.2), cell cycle analysis (*see* Subheading 3.5.1) or LDH detection (Subheading 3.5.2).

3.4 Detection of Secreted Proteins

The proteins can be detected in the cytosol of eukaryotic cell either by immunofluorescence or by western blotting (*see* **Note 11**). Continue the protocol behind the **step 7** (Subheading 3.1), **step 5** (Subheading 3.2) or **step 5** (Subheading 3.3).

3.4.1 Detection by Immunofluorescence

1. Around 4 h after the induction, cells are washed with PBS and fixed with formalin 3.7% for 30 min at R.T. (*see* **Note 12**).
2. Permeabilize membranes washing 5 min with PBT and block membrane receptors with blocking buffer 45 min at 37 °C.
3. Incubate the sample with primary antibody diluted in blocking buffer (following the manufacturers' recommendations) for 90 min at R.T.
4. Wash three times with PBT 10 min each time.
5. Add fluorescent secondary antibody diluted in PBS (following manufacturers' recommendations) and incubate 90 min at R.T. in darkness.
6. Stain cellular nuclei with DAPI staining solution for 15 min at R.T. keeping sample protected from the light.
7. Wash four times with PBT at R.T. and place the coverslip face down in a slide containing mounting medium. Translocation is considered positive when the fluorochrome-conjugated secondary antibody is detected in the eukaryotic cytoplasm (Fig. 2).

3.4.2 Immunodetection by Western Blot

Alternatively to direct visualization, it is possible to check if a protein has been translocated from the bacteria to the cellular cytosol by separating each fraction and hybridizing with a probe.

1. To get sufficient amount of protein, the protocol should be carried out with 5–6 million cells infected with a M.O.I. of 1:250. Remove the media with a pipette and keep it in a tube to do not lose floating cells (*see* **Note 13**).
2. Treat with trypsin-EDTA for 10 min at 37 °C and add the previously removed medium to neutralize the reaction (*see* **Note 14**).
3. Wash twice with 1 mL PBS by centrifugation (10 min at 0.5 × *g*) followed by PBS resuspension. After the last washing step, carefully remove all the liquid.
4. Resuspend on 100 μL of lysis buffer and keep on ice for 30 min, shacking in a vortex every 5 min.

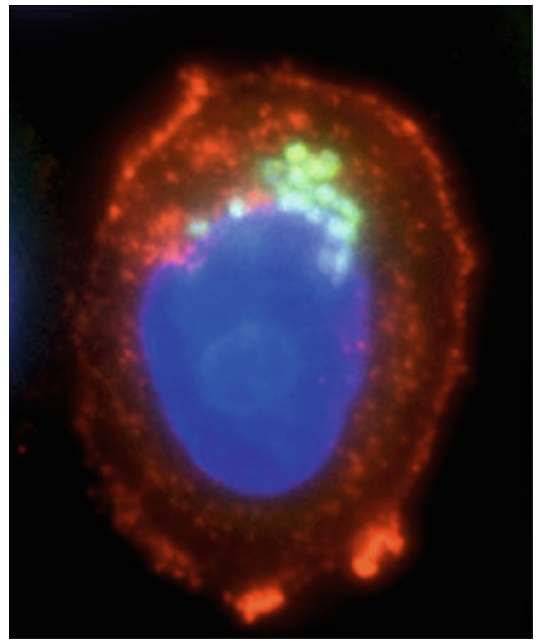

Fig. 2 Secretion of the HA epitope fused to *ssph2* signal peptide. The HA-tagged protein is spread in eukaryotic cytosol confirming its translocation. *Salmonella* strain constitutively express GFP (green), HeLa cell nuclei are stained with Hoechst (blue) and anti-HA epitope is detected by antibody staining (red)

5. Centrifuge the cell lysate at 13,000 rpm in a microcentrifuge for 10 min. The supernatant is transferred to a clean tube, while the pellet is additionally washed with 1 mL PBS by centrifugation as above and finally, resuspended in the original volume of lysis buffer (100 μL).
6. Protein concentration can be determined with conventional methods, and to detect enough amounts of proteins, 20 μg of protein from the pellet and an equivalent volume of supernatant must be loaded for SDS-PAGE.
7. Gel electrophoresis, western blotting and immunodetection can be done following conventional user's protocol.
8. Translocation is considered positive when the peroxidase-conjugated secondary antibody is visible mostly in the supernatant fraction, whereas it is negative if the signal appears in the pellet fraction (Fig. 3) (*see* **Note 15**).

3.5 Effect of Cytotoxic Proteins in the Cell

3.5.1 Cell Cycle Analysis in Flow Cytometer

1. Continue the protocol behind **step7** (Subheading 3.1), **step 5** (Subheading 3.2) or **step 5** (Subheading 3.3).
2. Recover the DMEM medium with floating cells and transfer it to a clean 10 mL tube (*see* **Note 16**).
3. Wash plates once with 500 μL of PBS, recover PBS and mix it with the medium recovered in **step 2**.

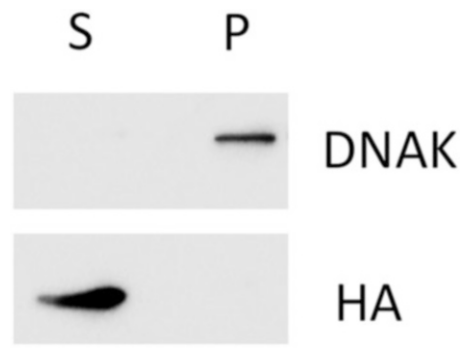

Fig. 3 Immunodetection of secreted proteins by Western blot. Supernatant (S) and pellet (P) fractions of HeLa cell cultures infected with *Salmonella* bearing the HA-epitope fused to *ssph2* signal peptide. The HA epitope and the bacterial chaperone DnaK were detected with their corresponding antibodies

4. Add 500 μL of trypsin 1×–EDTA to harvest cells and incubate 4–5 min depending on the cell type (*see* **Note 14**) at 37 °C.
5. Neutralize the reaction with the medium plus PBS mix previously recovered (**steps 2** and **3**) and homogenize cells pipetting carefully.
6. Centrifuge tubes at 0.5 × *g* for 5 min aspirating supernatant afterward.
7. Carefully resuspend cells by flicking the tubes a few times and wash with cold PBS centrifuging as above.
8. Resuspend pellet in 100 μL of cold PBS and fix with 900 μL of cold ethanol solution drop by drop while shacking in vortex at 1500 rpm. Keep at −20 °C at least 24 h before analyzing.
9. Transfer the content of the tube to a clean 1.5 mL tube and use a refrigerated microcentrifuge for the following steps (*see* **Note 17**).
10. Centrifuge 5×10^5 cells of the cell suspension at 0.5 × *g* for 5 min and remove ethanol of the supernatant by aspiration (*see* **Note 18**).
11. Wash twice with 500 μL of cold PBS-BSA solution by centrifugation and aspiration.
12. Incubate with 400 μL of extraction solution for 5 min at R.T. (*see* **Note 19**).
13. Remove liquid by aspiration and incubate sample with 700 μL of staining solution for 30 min at 37 °C protecting it from light.
14. Homogenize and filtrate sample trough a 70 μm nylon filter directly over cytometer tubes.
15. Proceed to the flow cytometry keeping low the acquisition velocity (200 events/sec) (*see* **Note 20**) (Fig. 4).

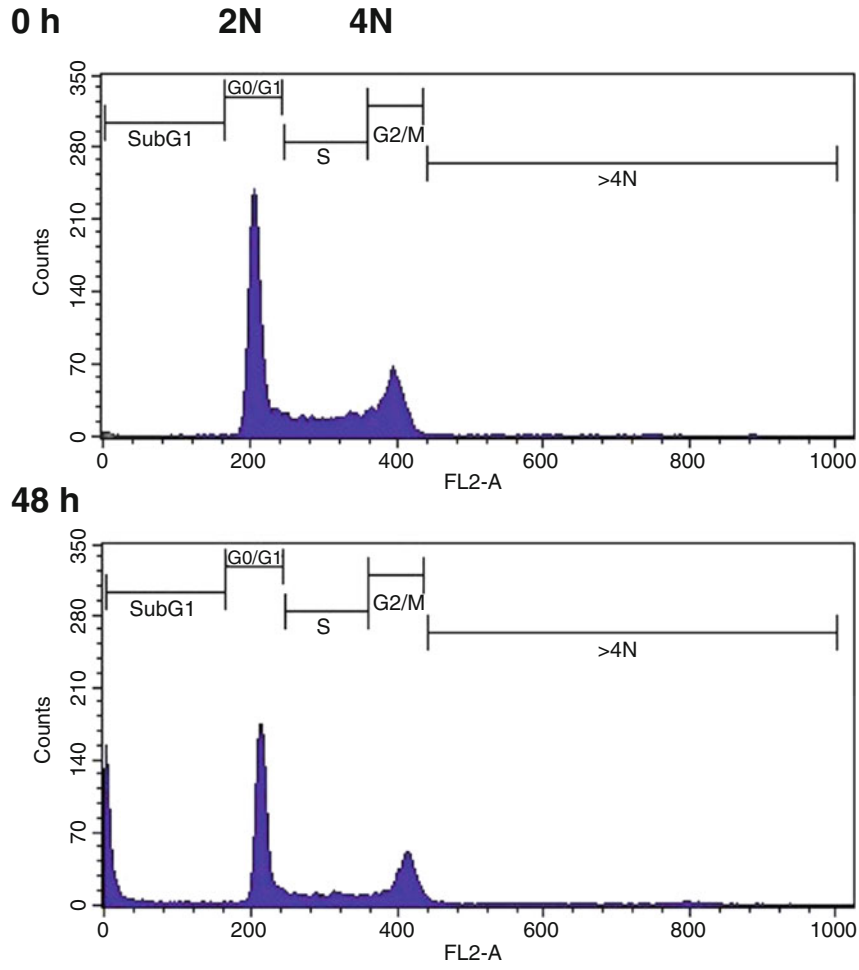

Fig. 4 Cell cycle distribution of HeLa cells infected with SpvB producing *Salmonella*. Appearance of proapoptotic cells is detected by the increment of subG1 peak 48 h after the treatment. DNA content is represented on the x-axis and the number of cells counted is represented on the y-axis

3.5.2 Cell Death Measure by Lactate Dehydrogenase (LDH) Activity

Use any assay kit and follow manufacturer's recommendations to measure cell death by LDH release in confluent infected cultures.

1. Continue the protocol behind the **step 7** (Subheading 3.1) or **step 5** (Subheading 3.2) (*see* **Note 21**).
2. Collect the supernatant after 24 h of salicylate induction (experimental LDH release) (*see* **Note 22**).
3. Add 1 mL of the medium to cells. Then, add 100 μL of 9% Triton X-100 to each well. Incubate for 60 min at 37 °C (experimental LDH released by cell lysis) (*see* **Note 23**).
4. Centrifuge the samples, including the controls, at 4 °C for 5 min and 0.5 × *g*.

5. Transfer 50 μL of each sample by triplicates to a 96-well plate (*see* **Note 21**).
6. Add 50 μL of reconstitutive substrate mix according to the manufacturer's recommendations and incubate for 30 min at R.T. protected from light. Then, add 50 μL of stop solution (solution provided with the kit).
7. Measure the absorbance at 490 nm of the samples treated with Triton X-100 and non-treated separately (*see* **Note 24**).
8. The percentage of cytotoxicity is calculated as 100 × [(experimental LDH release − spontaneous LDH release)/(total LDH release − spontaneous LDH release)], in which spontaneous LDH release is the level detected in the supernatant of an uninfected nonconfluent cell culture. Total release is the activity in infected cell lysates (experimental LDH release by cell lysis) including the experimental release in the supernatants. The experimental LDH release corresponds to the cell death (*see* **Note 25**).

3.6 Analysis of the Delayed Cytotoxic Effects of Cytotoxins

For some cytotoxins, it is essential to check the evolution of cell cultures for several days after the exposition to these compounds, as is the case of 5-FU. Here, we detail the protocol followed for 5-FU analysis that can be used with modifications for similar delayed effect cytotoxins.

3.6.1 Cytotoxic Effect of 5-FU-Producing Salmonella *in Tumor Cell Cultures*

1. Continue the protocol at the **step 3** of Subheading 3.2 (*see* **Note 26**). Incubate cells in DMEM containing 366 μM of adenine, gentamicin 16 μg/mL, and salicylate 2 mM for 5 h.
2. Substitute the medium reducing adenine concentration to 9.15 μM while maintaining gentamicin concentration and salicylate. Add 50 μM of 5-FC and incubate for 6 days (*see* **Note 27**).
3. Continue the protocol at the **step 2** (Subheading 3.5.1) to analyze the cell cycle distribution.

3.6.2 Bystander Effect of Cytotoxins

5-FU produced by *Salmonella* can freely diffuse across the cell membrane and affect neighboring cells, which is known as the bystander effect [21].

1. Continue the protocol at the **step 3** of Subheading 3.2 (*see* **Note 28**). Keep the infected cells in DMEM with 366 μM of adenine hemisulfate, gentamicin 16 μg/mL, and salicylate 2 mM for 5 h.
2. Substitute the medium keeping the adenine, gentamicin and salicylate concentration. Add 250 μM of 5-FC. Incubate for 40 h (*see* **Note 27**).
3. Centrifuge the infected cells at 0.5 × *g* for 5 min, collect the supernatants and pass the samples trough a 0.20 μm filter.

4. Add 2 mL of filtered supernatant and 8 mL of DMEM containing gentamicin 16 μg/mL to the uninfected cells previously seeded. Incubate for 6 days.
5. Continue the protocol at the **step 2** (Subheading 3.5.1) to analyze the cell cycle distribution.

3.7 Live-Infected Cell Imaging

1. 1.5×10^5 HeLa Kyoto cells stably expressing H2B-mCherry and mEGFP-α-tubulin are plated on poly-D-lysine 35-mm glass bottom culture dishes and infected at M.O.I 100:1 as described in Subheading 3.2. Continue the protocol behind the **step 2** (Subheading 3.2).
2. Replace the culture medium by 3 mL of Leibovitz's medium supplemented with 2 mM L-glutamine and 10% FBS, and containing 16 μg/mL gentamicin, a reduced adenine concentration of 9.15 μM, and 2 mM sodium salicylate (*see* **Note 29**).
3. Each sample is analyzed separately under DeltaVision widefield microscope system equipped with a thermostat at 37 °C. Images are acquired using a 60× objective every 30 min for 20 h as an image stack of 16- X 1-μm z-planes with 2 × 2 binning and analyzed using ImageJ software.

4 Notes

1. During the infection process, penicillin–streptomycin must not be used.
2. The stock solution should be heated at 50 °C previously to its use since it might precipitate when stored at 4 °C.
3. Commercial trypsin is supplied at 2.5× and when diluted tenfold, its final concentration is 0.25×. This diluted stock is usually named as trypsin 1× in most laboratories.
4. The percentage of FCS included in the blocking buffer depends on the cell line and the specificity of the antibody. For HeLa, it is recommended to use at 3%, but for MCF-7 the concentration should be increased to 5%.
5. As a general approach, primary antibodies are usually diluted ranging from 1:50 to 1:1000 in the best case. They can be recovered and frozen to be reutilized up to 4–5 times. Secondary antibody should be diluted ranging from 1:300 to 1:1000.
6. This cocktail can be substituted by any other commercial one adding 1–100 μL sample.
7. We recommend preparing this way, since the experiments have shown to be more repetitive. The extraction solution can be conserved up to 1 week at 4 °C.

8. An elevated M.O.I. can induce early apoptosis itself and therefore, it is important to adjust the M.O.I. for every application.
9. Every medium change involves two PBS washing steps as this one, but to avoid being repetitive we do not have state it in every step entailing such changes.
10. Each protein requires a different expression time. SpvB effect was visible around 3–4 h after the induction, but in contrast, the effect of CD and 5–FC was only detectable 5 days after the induction. Thus, the user must monitor the effect of other proteins.
11. If users need to use confocal microscopy, introduce sterilized coverslips into the wells. To sterilize it immerse it in 70% ethanol for 5 min and rinse with abundant sterile water.
12. This is a standard protocol; the induction timing can be different for each application, as controlled bacterial lysis.
13. Following medium recovery, add around 1–5 mL PBS 1× to the plate and put it back into the same tube. This step ensures cell retrieval and guarantees the correct trypsin treatment.
14. The duration of the trypsin-EDTA treatment depends on the cell line. For some cell lines, 3 min could be enough to avoid cell damage.
15. It is recommendable to include a specific antibody against bacteria to distinguish the bacterial and cytoplasmic fractions. We use the bacterial chaperone DNAK.
16. The volumes of this protocol are adapted to 6 well plates. If done in other plates, scale the volumes as necessary.
17. Points 1–8 are usually carried out in 10 mL plastic tubes and, to facilitate further 4 °C washing steps, it is better to transfer the content to a 1.5 mL tubes (points 9–14).
18. It is not recommended to aspirate all the ethanol to avoid losing cells, subsequent washing steps will dilute it. From 5×10^5 to 6×10^5 cells should be stained, keeping frozen the remaining sample.
19. The original protocol indicates that this incubation should be done on ice, but we have noticed that the extraction solution crystalizes after this step hindering the aspiration.
20. The instrument setting depends on each cytometer. We propose the next settings to simplify the analysis of samples for following detectors respectively: (FCS, SSC, FL2A, FL2W); Voltage (E-1, 295, not applicable, not applicable); AmpGain (4.48, 1, 1.09, 1.6).
21. A triplicated control of noninfected cells 2.5×10^4 (spontaneous LDH release) must be included. Noninfected cells multiply

faster than the infected cells. An amount of 2.5×10^4 instead of 5×10^4 will be used to avoid the confluence in the well.

22. The same culture medium will be used by triplicate to correct the LDH activity contributed by serum and other compounds in culture medium and the varying amounts of phenol red in the medium.
23. The same culture medium with triton X-100 should be used to remove the background in these samples by serum and other compounds in culture medium and the varying amounts of phenol red in the medium.
24. Remove the bubbles in the wells before measuring the absorbance and up to 1 h after incubation in a plate reader. Samples should be measured separately since blanks are different.
25. Use 1 μM staurosporine (STS) as a control of cell death.
26. For long-term cell incubations as this one, use 2×10^5 cells per plate at a M.O.I of 50:1.
27. Treat 2×10^5 and 2×10^6 uninfected cells with 50 μM of 5-FC and 10 μM of 5-FU, respectively as controls.
28. Prepare 2×10^5 cells separately in two plates: (1) for 5-FU production by infection (infected cells, M.O.I 50:1) and (2) bystander effect assay: non infected cells.
29. Leibovitz's medium is designed for supporting cell growth in environments without CO_2 equilibration. Do not keep the cells in the incubator after adding this medium.

Acknowledgments

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Chapter 8

Organoids as a Model to Study Infectious Disease

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Abstract

The advent of the gastric organoid culture system has provided a new model to emulate native epithelial tissue in vitro. Gastric organoids grow from isolated epithelial stem cells and develop into three dimensional structures that can be used to study host physiology. Here we describe current laboratory protocols for growing gastric organoids and the microinjection of pathogens such as *Helicobacter pylori* into the lumen of gastric organoids in order to study the cellular response following infection.

Key words Gastric organoids, Microinjection, Pathogens, *Helicobacter pylori*, Fluorescent dye

1 Introduction

In 2009, Sato et al. established long-term epithelial primary culture methods under which isolated gastrointestinal (GI) stem cells grow to form three dimensional structures [1]. These three dimensional epithelial structures, referred to as "organoids," have proved useful in studying biological processes that occur in native tissue. In the past, GI disease studies have chiefly been limited to in vivo animal models or in vitro immortalized cancer cell lines. Whereas in vivo models have the cellular diversity that is necessary to support tissue function, approaches for experimental manipulation are limited. Conversely, in vitro cancer cell lines are simple to work with, but have pathologic genetics and function in a more homogeneous cell population. GI organoids represent a significant advancement in our ability to replicate the gastrointestinal environment in terms of cellular diversity of normal epithelial cells, and can be an applicable model to bridge the gap between human and mouse research. Human and mouse gastric organoids have value in studying gastric physiology and disease, especially in examining *Helicobacter pylori* (*H. pylori*) infection [2–4]. Injection of bacteria into human and mouse intestinal organoids results in a similar pathogenic response as with in vivo tissue [5–7]. Thus the use of GI organoids expands

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our current understanding of bacterial–host interactions and allows for a deeper understanding of toxic factors or pathogens that mediate disease [3, 4].

Here we describe our current protocol to inject *H. pylori* into organoids derived from the mouse gastric corpus, as an example of microinjection to study organoid epithelial cell-pathogen interaction.

2 Materials

Prepare all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations.

2.1 Generation and Maintenance of Gastric Organoid Cultures Derived from Mouse Stomach Tissue

1. Microdissecting curved scissors.
2. #7 fine point curved forceps.
3. Silicon dish (Sylgard® 184 Silicone Elastomer Kit prepared in a glass culture dish).
4. Dissection microscope.
5. Matrigel Matrix (Corning), basement membrane, growth factor reduced, phenol red free.
6. Sterile Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium, phenol red free, (Corning). Store at 4 °C.
7. 250 mM EDTA stock in ultrapure water.
8. Dissociation buffer: 43.3 mM sucrose (Fisher), 54.9 mM D-sorbitol (Sigma), in DPBS.
9. Sterile 24-well cell culture plate with lid.
10. Sterile 2-well chambered cover glass slides.
11. Sterile 5 mL round bottom polystyrene test tube, with snap cap.
12. Sterile 15 mL centrifuge tubes.
13. Sterile 1 mL Tuberculin syringe with 26G × 3/8 in.
14. Mouse gastric organoid culture growth medium: Advanced Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12, Invitrogen), 2 mM L-glutamine (Invitrogen), 10 mM HEPES (Sigma), 100 U/mL penicillin/100 mg/mL streptomycin (Thermo Scientific), 1× N2 medium supplement (Invitrogen), 1× B27 medium supplement (Invitrogen), 10% R-spondin conditioned medium, 10% Noggin conditioned medium, 50% Wnt3a conditioned medium, 50 ng/mL epidermal growth factor (EGF) (PeproTech), 10 nM Human [Leu15]-Gastrin I (Sigma) (*see* **Note 1**).
15. 70% Ethanol.

2.2 Helicobacter pylori Culture

1. Columbia Agar Base (Thermo Scientific): 0.0425 g/mL in ultrapure water.
2. Defibrinated horse blood (Colorado Serum Company).
3. β-cyclodextrin (Sigma).
4. Cycloheximide (Sigma), stock solution 50 mg/mL in 95% ethanol.
5. Vancomycin (Sigma), stock solution 30 mg/mL in ultrapure water.
6. Trimethoprim (Sigma), stock solution 40 mg/mL in DMSO.
7. Sterile petri dishes 100 × 15 mm.
8. 2 L Erlenmeyer flask.
9. *H. pylori* Sydney Strain 1 (SS1).
10. Microaerophilic chamber (BD Diagnostic Systems).
11. BD GasPak EZ (BD Diagnostic Systems).
12. Brucella broth (BD Diagnostic Systems).
13. Fetal bovine serum (HyClone).
14. *H. pylori* plate preparation: Suspend 21.25 g Columbia Agar Base into 475 mL of ultrapure water, in a 2 L Erlenmeyer flask. Add 1 g β-cyclodextrin. Use a stir bar to mix. Autoclave agar solution at 121 °C for 20 min. Cool agar to 55 °C in a water bath for at least 1 h. In a biosafety hood, add to cooled agar 500 μL cycloheximide (Final: 50 μg/mL), 83 μL vancomycin (Final: 5 μg/mL), 125 μL trimethoprim (Final: 10 μg/mL) and 25 mL defibrinated horse blood (Final: 5%). Mix by swirling the flask by hand and with a serological pipet add 25 mL of blood agar medium per plate, avoiding bubbles (this protocol makes approximately 20 plates). Wait until agar is set before storing at 4 °C, upside down in a plastic sleeve. Plates can be stored for 2 weeks.
15. 50% Glycerol.

2.3 Microinjection

1. Parafilm.
2. Nanoject II microinjector apparatus (Drummond Scientific).
3. P-2000 Micropipette Puller (Sutter Instruments).
4. Replacement Capillaries for Nanojet II Injectors (Drummond Scientific).
5. Micro scissors, straight, 7 cm long, 0.1 mm tips, 3 mm blades.
6. Mineral Oil.
7. Stereoscope.
8. Lucifer Yellow: a fluorescent dye of M.W. 457.24.
9. Long needle syringe.

3 Methods

3.1 Generation and Maintenance of Gastric Organoid Cultures Derived from Mouse Stomach Tissue

3.1.1 Culturing of Mouse Gastric Glands

1. Euthanize mice using a method approved by the institution where the research is to be conducted. We have euthanized mice with isoflurane inhalation to deep anesthesia or CO_2 inhalation, immediately followed by cervical dislocation.
2. Disinfect the exterior abdomen of the mouse with 70% ethanol. Make an incision into the abdominal cavity. Extend the incision to the rib cage by cutting the abdominal musculature on both sides. Grasp the forestomach and cut the esophagus and the proximal duodenum. Remove whole stomach from the abdominal cavity, rinse whole stomach in cold DPBS, then open stomach along the greater curvature. Rinse the opened stomach in cold DPBS to remove food debris.
3. Pin the opened stomach (luminal side down) on a silicon dish, add ice cold DPBS to cover the tissue to prevent the tissue from drying. Quickly but carefully, remove the serosal muscle layer and fat using surgical scissors and forceps under a dissection microscope.
4. Cut out the corpus from the opened stomach and mince into <5 mm pieces.
5. Place minced tissue into a 15 mL centrifuge tube with 5 mL cold DPBS.
6. Add EDTA (5–10 mM final concentration) to the tissue in cold DPBS and place on a rocker at 4 °C for 2 h.
7. At this point:

   Thaw the required amount of 500 μL Matrigel aliquots in 4 °C or on ice.

   Prepare mouse gastric organoid culture growth medium.
8. After rocking, remove EDTA solution and add 5 mL solution of dissociation buffer.
9. Vigorously shake by hand for 1–2 min to dissociate whole glands from tissue. From this point work in a biosafety hood.
10. Under a microscope, pipette approximately 50 μL of tissue solution to visualize the isolated glands. Isolated glands should be visible and approximately 50% solution area should be glands. Otherwise repeat **step 9**.
11. Transfer solution containing the glands to 5 mL round-bottom tube, avoiding nondissociated tissue.
12. Centrifuge for 5 min in 4 °C at a speed of 150 × *g*. Remove all of the solution, avoiding the gland pellet at the bottom of the tube.
13. Add Matrigel to tube and gently mix, avoid the introduction of bubbles.

14. Pipette 30 μL of Matrigel containing glands per 24-well plate.
15. Transfer 24-well plate into a CO_2 incubator (5% CO_2, 37 °C) for 15–20 min to allow Matrigel to polymerize.
16. Overlay 500 μL of organoid culture medium per well.
17. Incubate the organoids in a CO_2 incubator (5% CO_2, 37 °C).
18. Refresh medium every 3–4 days. If cultures are healthy, approximately 30–40% or greater of glands should grow out into organoids. Evaluate growth daily.
19. Organoids should be passaged every 7–10 days, when organoids attain a size of >500 μm.

3.1.2 Passaging of Gastric Organoids for Microinjection

1. Remove medium and add approximately 1 mL cold DPBS per well.
2. Use a sterile pipette tip to help break up Matrigel, then transfer suspension to 5 mL round-bottom tube.
3. Centrifuge at 150 × *g* for 5 min at 4 °C.
4. Aspirate supernatant.
5. Add 1 mL cold DPBS. Using 26 G syringe, gently draw up the organoids and DPBS. Eject quickly from syringe once to break up the organoids.
6. Refill the 5 mL round-bottom tube with cold DPBS.
7. Centrifuge at 150 × *g* for 5 min at 4 °C.
8. Remove supernatant. Add fresh Matrigel and gently mix, avoid introduction of bubbles.
9. Pipette 50 μL of Matrigel containing fractions of organoid per chambered cover glass well for experiments. Remainder of the Matrigel/organoid fraction mixture can be returned to a 24-well plate for regrowth (*see* Subheading 3.1.1, **steps 14–19**).
10. Transfer chambered cover glass into a CO_2 incubator (5% CO_2, 37 °C) for 15–20 min to allow Matrigel to polymerize.
11. Overlay 1 mL of organoid culture medium per well.
12. Incubate the organoids in a CO_2 incubator (5% CO_2, 37 °C).
13. Change medium every 3–4 days.
14. Grow organoids 3–5 days before experiment.

3.2 Helicobacter pylori Culture

1. In a biosafety cabinet, warm plates to room temperature.
2. Remove *H. pylori* frozen stock from a storage tube with a sterilized loop and dilute into Brucella broth at 2 × 10^6 *H. pylori* per 100 μL. Pipette solution onto a blood agar plate and spread with loop around the plate.

3. Incubate *H. pylori* inoculated plates for 3–4 days upside down at 37 °C in a humidified microaerophilic chamber with a CO_2 GasPak. Check growth and incubate as needed.
4. Use a sterilized loop to harvest *H. pylori* from plates and suspend in Brucella broth supplemented with 10% fetal bovine serum and CO_2 GasPak in a humidified microaerophilic chamber in an incubator at 37 °C for 16–18 h (*see* **Notes 2** and **3**).
5. Collect bacteria by centrifugation at 2000 × *g* for 5 min and resuspend in Brucella broth without serum.
6. Dilute *H. pylori* in 50% glycerol (1:100 dilution) to calculate density in hemocytometer.

3.3 Microinjection

To make the following procedures clear to the naked eye, a high concentration of Lucifer yellow was used in this section, in addition to *H. pylori*. Lower LY concentrations can be used once procedures are established, or other dyes of interest substituted (*see* **Note 4**).

1. At least 1 day before *H. pylori* injection, replace organoid culture medium with 1 mL of the culture medium without penicillin/streptomycin.
2. Use a micropipette puller to pull replacement glass capillaries. Setting for pulling replacement glass capillaries is as follows:

Heat	Filament	Velocity	Delay	Pull
400	4	50	250	200

3. Trim pulled glass capillaries with micro scissors (glass capillary needle) to approximately 2–3 mm for LY [Fig. 1A (b)] or 5–6 mm for *H. pylori* [(Fig. 1A (c)] from tip. Inner capillary size on tip can be 5–8 μm [(Fig. 1B (b)] or 15–20 μm [(Fig. 1B (c)], respectively (*see* **Notes 5** and **6**).
4. Before attaching glass capillary needle to the Nanoject II, capillary is filled with mineral oil by using a long needle syringe, avoid air bubble contamination (*see* **Note 7**).
5. Gently slide capillary into place in Nanoject II.
6. Click "EMPTY" to squeeze mineral oil from the capillary to make sure no air is in the tip (*see* **Note 7**).
7. Set injection volume in NanojectII microinjector apparatus (instrument volume range 2.3–69.0 nL), i.e., 2.3 nL/slow for LY or 23 nL/slow for *H. pylori*.
8. Onto a sheet of Parafilm, pipette approximately 2 μL of LY (Fig. 2) or Brucella broth containing *H. pylori* and orient Nanoject II with attached capillary to the droplet under the stereoscope.

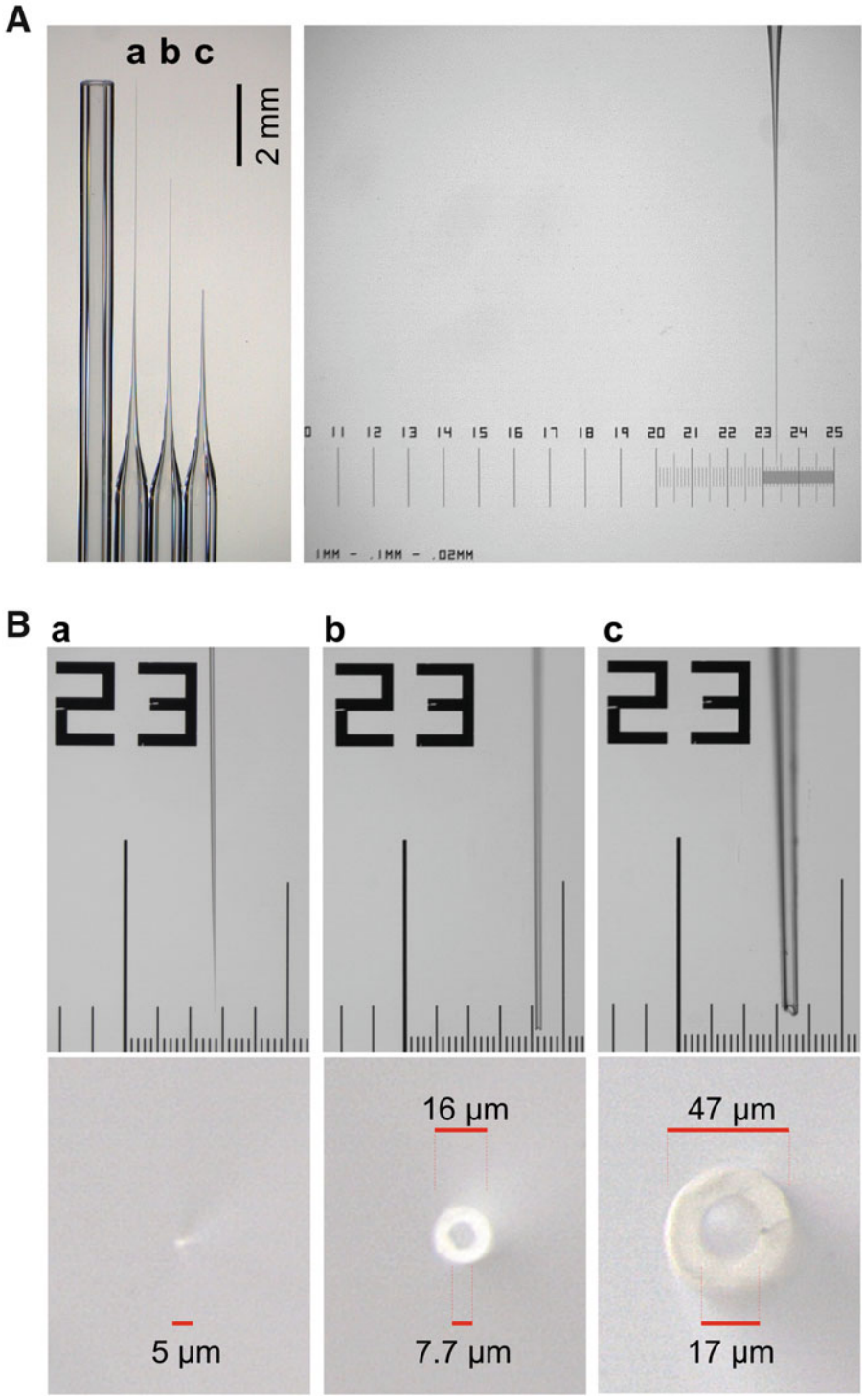

Fig. 1 Glass capillary needle. **A**. Stereoscopic image of original glass capillary (left), untrimmed pulled capillary (a), 2–3 mm trimmed capillary (b) and 5–6 trimmed capillary (c) in the left panel. Right panel shows low magnification image of 2–3 mm trimmed capillary on the stage micrometer (Microscope World, 25 mm KR812). **B**. High magnification of stereoscopic images of a pulled capillary (a), 2–3 mm trimmed capillary (b), and 5–6 trimmed capillary (c) in the left panel. The approximate diameters of the original capillary are inner: 500 μm and outer: 1100 μm

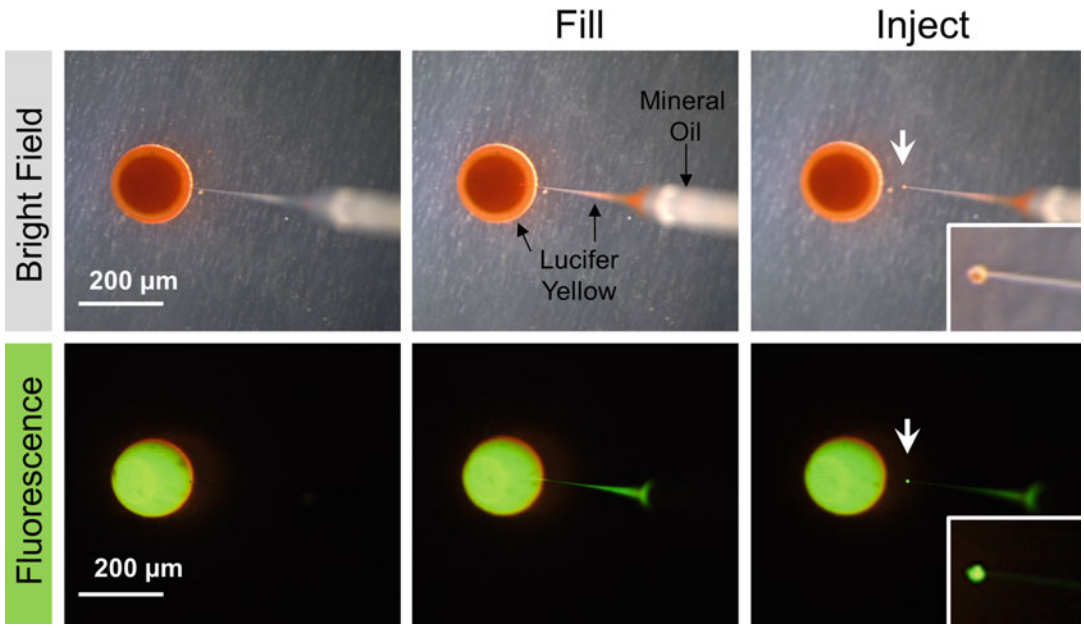

Fig. 2 Filling solution of interest into a glass capillary needle. Upper panel shows 2 μL of Lucifer yellow on Parafilm under bright-field light microscopy before, during and after filling glass capillary needle using Nanoject II, while lower panel shows same events under fluorescence (GFP filter)

9. Press "FILL" to take up solution on Parafilm (Fig. 2).
10. Remove the tip from the solution, press "INJECT" to make sure solution comes out from tip (Fig. 2).
11. Place organoids, in 2-well chambered cover glass, on the stage of the stereoscope, and insert the glass capillary needle into an organoid (Figs. 3 and 4 "Before").
12. Use Nanoject II microinjector apparatus, click "INJECT" once to inject 2.3 nL LY (20 mM) or 23 nL of Brucella broth containing 5×10^9/mL *H. pylori* into an organoid with a diameter of approximately 500 μm (Figs. 3 and 4 "Injection"). This yields approximately 1 mM fluorescence or 1×10^5 bacteria injected per organoid, respectively (*see* **Notes 5**, **6** and **8**).
13. Slowly and gently remove Nanoject II microinjector from the organoid.
14. Incubate the organoids in a CO_2 incubator (5% CO_2, 37 °C).
15. Perform proposed experiments (*see* **Note 9**).

4 Notes

1. Wnt3a, Noggin and R-spondin conditioned medium is used in the protocol. While these proteins are commercially available as recombinant proteins, we recommend using cell lines that have

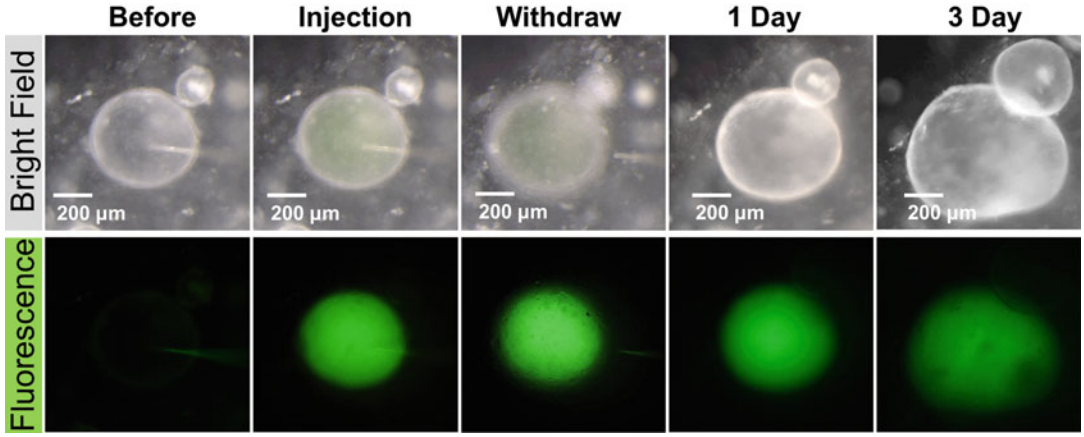

Fig. 3 Microinjection of Lucifer yellow into a gastric organoid. Stereoscopic images during Lucifer yellow (LY) injection in bright-field (upper panel) or fluorescence (lower panel). Images taken before, during, and immediate, 1 or 3 days following microinjection of 2.3 nL LY using 2–3 mm trimmed capillary needle in Nanoject II. Retention of microinjected LY over 3 days confirmed maintenance of barrier integrity of gastric organoid

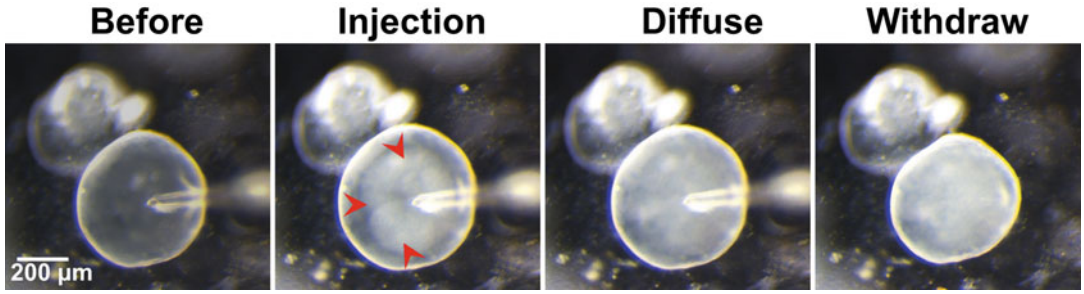

Fig. 4 Microinjection of *H. pylori* into a gastric organoid. Stereoscopic images of a gastric organoid before, during, and after microinjection of *H. pylori* into the lumen. The bacteria are visible as a cloud (red arrows) inside the organoid and then spread out (diffuse) in the lumen

been engineered to produce and secrete these proteins. In our experience with Wnt3a, the bioactivity (TopFlash) of the conditioned medium made in the lab from such cell lines is several hundred-fold higher than the commercially available recombinant protein. We advise consulting with investigators to decide on the best source of such cell lines.

2. We recommend to culture bacteria in a liquid medium before injection, since contamination with agar often clogs the tip of the glass capillary needle.
3. *H. pylori* can be cultured in the liquid directly from frozen stock. If there is enough *H. pylori*, start from Subheading 3.2, **step 4**. If *H. pylori* concentration needs to be increased, suspend *H. pylori* in Brucella broth supplemented with 10% fetal

bovine serum and a CO_2GasPak in a humidified microaerophilic chamber in an incubator at 37 °C for 16–18 h with shaking, followed by replacement with fresh Brucella broth with 10% fetal bovine serum and CO_2 GasPak in a humidified microaerophilic chamber in an incubator at 37 °C for 16–18 h WITHOUT shaking. This lack of shaking will stimulate *H. pylori* motility.

4. Fluorescent dyes should be readily visible in the μM concentration range in the confocal microscope. This microinjection technique may also be utilized for fluorescent measurement, including pH and calcium sensitive dyes. We also succeeded to monitor luminal pH using SNARF, a pH sensitive dye microinjected into the gastric organoid [2].
5. It would be better to set up tip of glass capillary needle as small as possible to avoid organoid injury, although the damaged area is quickly sealed by neighboring cells next to damaged cells [2]. Figure 3 shows that there is no major leakage of LY after ejection from the glass capillary needle and LY stays within the organoid both 1 and 3 days after injection. Therefore, microinjection can be applied for injecting pharmacological agents, biological substances, bacteria, viruses, parasites or toxins.
6. Using a bigger glass capillary needle, it is sometimes hard to insert into the organoid. In this case, tap the end of the tail edge of Nanoject II pipet holder in which the glass capillary needle was placed.
7. Air bubbles in the mineral oil within the glass capillary needle leads to injection of an inaccurate volume. Remove air bubbles by clicking "EMPTY" or make a new glass capillary needle.
8. The Nanoject II technique was originally developed for *Xenopus* oocytes [8, 9] and has also been utilized for small intestinal organoid studies [5]. For best results, inject organoids that are at least 300 μm in diameter. Some gastric organoid studies inject approximately 200 nL into organoids [3], however it is recommended to calculate the organoid volume based upon diameter. For an organoid with a 500 μm diameter, the volume is approximately 65 nL, and it is recommended not to exceed this amount.
9. *H. pylori* colonized on the epithelium of gastric organoids cause epithelial responses which replicate in vivo studies, confirmed at 1 or 4 days after infection [3, 4]. Western blot, immunofluorescent, RT-PCR can be applied to study cellular response to infection. As seen in Subheading 3.1.2, **step 14**, organoids will be an adequate size to introduce microinjection

after about 3–5 days of culture. Organoid sustain health for 10–12 days without passage. Therefore, experiments should be conducted within 1 week after infection.

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Chapter 9

Surface Proteome Biotinylation Combined with Bioinformatic Tools as a Strategy for Predicting Pathogen Interacting Proteins

Anita Horvatić, Josipa Kuleš, Nicolas Guillemin, Franjo Martinković, Iva Štimac, Vladimir Mrljak, and Mangesh Bhide

Abstract

Constant advancements in methodology and mass spectrometry instrumentation, genome sequencing and bioinformatic tools have enabled the identification of numerous pathogen proteomes. Identifying the pathogen interacting proteins by means of high-throughput techniques is key for understanding pathogen invasion and survival mechanisms and in such a way proposing specific proteins as pharmaceutical targets. Herein we describe the methodology for the enrichment and identification of pathogen surface proteome using cell surface protein biotinylation followed by LC-MS/MS and bioinformatic analyses of such data. This strategy is to be employed for the determination of protein subcellular localization and prediction of potential pathogen interacting proteins.

Key words Biotinylation, LC-MS, Surface proteome, Bioinformatics, Subcellular localization, Interacting proteins, DAVID, CELLO

1 Introduction

Defining the cell surface proteome has profound importance for understanding host-pathogen interactions. Pathogen plasma membrane proteins (PM) that reside on the cell surface regulate and directly interact with host cells proteins during recognition and invasion process influencing on immune response of host organism [1]. Furthermore, as PMs are involved in ion transport, cell signaling and communication, this makes them ideal targets for various therapeutics and promising vaccine candidates [2]. Owing to their hydrophobic nature, plasma membrane proteins pose analytical challenges and, despite efforts to overcome difficulties, remain under-represented in proteomic studies. The most critical component of the experimental approach is the enrichment and purification of plasma membrane proteins [3]. The most commonly used

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techniques for enrichment and extraction of membrane proteins are protein shaving, biotinylation followed by (strept)avidin affinity chromatography, and ultracentrifugation. The availability of novel -omics technologies coupled to high-throughput protein expression and purification, and bioinformatic tools together with -omics databases availability enables more rational and faster identification of antigens among large number of pathogen proteins [4]. Antigen identification represents the most important bottleneck in vaccine development against any pathogen, as this was usually achieved through rather empirical, time-consuming, and labor-intensive in vivo and in vitro experiments [5].

Chemical labeling of cell surface proteins is an emerging technology for the isolation of target proteins containing specific residues which can subsequently be resolved from untagged proteins using affinity purification. Biotinylation of cell surface proteins is a method of choice for the selective capture of plasma membrane proteins, but it is limited to pathogens that can be cultivated in protein-free media. The procedure involves selective, covalent labeling of proteins with a biotinylation reagent followed by capture of biotin-conjugated proteins/peptides via an avidin/streptavidin-coated solid support (i.e., resins, magnetic beads, microtiter plates and chips). Unbound components (nontagged proteins) are washed away and captured proteins are eluted or detached under various conditions.

Chemical derivatization of reactive groups in proteins with a biotin moiety is one of the most widely used techniques in protein biochemistry. Biotinylation reagents typically consist of three components: the biotin moiety, a spacer—possibly containing a cleavable linker unit—and a reactive moiety that interacts with the proteins of interest [6]. Selection of the most suitable reagent should consider the following factors: water solubility and membrane impermeability, presence of a cleavable linker, size of the spacer, target functional group on the protein and binding characteristics of the biotin moiety. The highly stable interaction between biotin and avidin ($Kd = 10^5$ M) presents a drawback for this method, as elution of biotin-labeled proteins from the avidin support is difficult. In an attempt to resolve this problem, a disulfide bridge in the linker region of the biotinylation reagent has been introduced (sulfo-NHS-SS-biotin). Under reducing conditions, the disulfide bridge is cleaved, thus removing the biotin label and releasing the captured proteins/peptides.

Low membrane protein concentration, low yield of biotinylation, as well as molecular weight and hydrophobicity of membrane proteins requires very sensitive and high resolution instrumentation. For that reason, nanoLC-MS/MS, as a high-throughput analysis technique, using a bottom-up proteomic approach is the method of choice for the analysis of biotinylated surface proteins. Both strategies, shotgun and gel-based proteomic approach, can be

employed, having in mind protein amount and detergent (originating from lysis buffer) removal prior to LC-MS analysis. Commonly used detergents for the extraction of membrane proteins are Triton X-100, CHAPS, SDS, sodium deoxycholate, NP-40, etc., which cause interferences during LC-MS analysis resulting in low number of identified proteins. Depending on detergent type, methods such as dialysis, ultrafiltration, strong cation exchange and/or reverse phase chromatography, or detergent removal resins can be applied for detergent removal [7]. The other gel-based approach, mostly for detergent removal, includes tube gels or SDS-PAGE followed by in gel digestion and LC-MS analysis [8]. The quality of proteomic data due to the low abundance of biotinylated proteins, inadequate sample preparation or processing can result in false positive or negative results.

High-throughput methodologies, such as LC-MS, produce big datasets and identified proteins might differ in confidence. Among that, due to nonspecific binding not all enriched proteins are actually surface membrane proteins. For that reason, bioinformatics is inevitable for in silico data validation, filtering and database mining. There are different computational programs available for subcellular localization prediction, such as CELLO, BaCeILo, TargetP, and PSORTb, using various algorithms based on a decision tree of several support vector machines (SVMs), protein functional domains and/or the amino acid compositional differences in proteins from different subcellular locations [9–11]. Gene ontology (GO) analysis, interaction prediction and enrichment, as well as pathway analysis can be performed using open access platforms such as Cytoscape and its plugins or DAVID, depending on organism of interest and availability of its databases. Currently available computational approaches for predicting interacting proteins are based on genomic and structural information, use of network topology, literature mining/database search and machine learning algorithms utilizing heterogeneous -omics features [4]. Except for biotinylated proteins, bioinformatic tools can be also applied for the data analysis of any kind of proteomic results (identified proteins from cell lysates, enriched membrane proteins, etc.) in order to predict subcellular localization and interacting proteins (domains).

The isolation of surface membrane proteins of *Leishmania infantum* will be used as an example of how cell surface protein biotinylation with streptavidin affinity separation can be used for assessing pathogen interacting proteins. After subsequent tryptic digestion and LC-MS acquisitions, data can be processed using Proteome Discoverer. Further bioinformatic data filtering by CELLO and DAVID can be employed to determine subcellular localization, gene ontology, and potential interaction proteins using domain prediction.

2 Materials

2.1 Equipment

1. Cooling centrifuge.
2. Dry incubator shaker for small tubes.
3. NanoDrop spectrophotometer.
4. nanoLC-MS system (Dionex Ultimate 3000 RSLS nano flow system; Thermo Scientific Orbitrap Q Exactive Plus mass spectrometer).
5. Rotator.
6. Sonicator.
7. Vacuum concentrator.
8. Vortex.
9. −80 °C freezer.
10. Microscope.

2.2 Chemicals and Consumables

1. Acetonitrile (LC-MS grade).
2. Ammonium bicarbonate.
3. Ammonium hydroxide.
4. Dithiothreitol (DTT).
5. EZ-Link^{TM}Sulfo-NHS-SS-Biotin.
6. Formic acid (LC-MS grade).
7. Iodoacetamide.
8. Water (LC-MS grade).
9. Methanol.
10. NeutrAvidin agarose resin.
11. Spin columns (empty 800 μL spin columns).
12. Trypsin gold, porcine.
13. ZipTips (SCX, RP C18).

2.3 Solutions

1. Phosphate-saline buffer (PBS 1×; for 1 L): 8 g NaCl, 0.201 g KCl, 1.42 g Na_2HPO_4, 0.272 g KH_2PO_4. Adjust pH = 7.4.
2. Lysis buffer: Commercial RIPA buffer.
3. Quenching solution: 100 mM glycine in PBS.
4. Elution buffer: 50 mM DTT in ammonium bicarbonate.
5. Mobile phases for LC-MS (A—0.1% formic acid in water; B—80% acetonitrile/0.1% formic acid in water).
6. Solutions for strong cation exchange (SCX) chromatography: W1—0.1% formic acid in water; W2—50% methanol in water; E1—5% ammonium hydroxide–30% methanol in water.

2.4 Bioinformatic Tools

1. Proteome Discoverer (Thermo Scientific).
2. CELLO: sub**CEL**lular **LO**calization predictor (http://cello.life.nctu.edu.tw/).
3. **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**) (https://david.ncifcrf.gov/home.jsp).

3 Methods

3.1 Cell Surface Protein Biotinylation Protocol

Cell surface biotinylation was performed on purified promastigotes from stationary phase culture of *Leishmania infantum*, but can be performed on any other cell type by optimizing cell concentration and lysis buffer/conditions.

1. Wash cells three times with PBS (pH = 7.4) and centrifuge at 1000 × *g* for 1 min to remove any contaminating proteins.
2. Suspend cells at a concentration of 10^6–10^7 cells/mL in PBS.
3. Immediately before use, prepare a 10 mM solution of Sulfo-NHS-SS-Biotin. Add the appropriate volume of the Sulfo-NHS-SS-Biotin solution to the cells suspension (*see* **Note 1**).
4. Incubate reaction mixture at room temperature for 40 min with gently rotation on the rotator or rocking on the orbital shaker (*see* **Note 2**).
5. Quench the reaction by adding 100 μL of 100 mM glycine solution in PBS. Wash cells two more times with ice-cold PBS to remove nonreacted biotinylation reagent (*see* **Note 3**).
6. Centrifuge cells in a benchtop centrifuge 1 min at 500 × *g*, discard the supernatant, and add the lysis buffer of choice to the cell pellet (*see* **Note 4**).
7. Lyse cells by two cycles of freezing at −80 °C and thawing at room temperature, followed by 10 cycles of sonication at maximum amplitude. Check the degree of cell lysis microscopically.
8. Centrifuge cells at 16,000 × *g* for 10 min at 4 °C.
9. Transfer clarified supernatant to a new tube. The cell surface proteins are now biotinylated on exposed lysine residues.

3.2 Affinity Purification of Biotinylated Proteins

1. Measure protein concentration in sample solution (*see* **Note 5**).
2. Pack the NeutrAvidin Agarose Resin into a column (*see* **Note 6**). Place column into a collection tube. Centrifuge at 500 × *g* for 1 min to remove storage solution.
3. Wash the resin with 100 μL of PBS by centrifugation at 500 × *g* for 1 min and discard buffer from collection tube. Repeat this-step three times.

4. Place column in a new collection tube and add biotinylated sample to the column allowing sample to enter the resin bed. Incubate the mixture 1 h at room temperature with gently rotation.
5. Centrifuge for 1 min at 500 × *g* and collect flow-through.
6. Add 100 μL of lysis buffer to the column, centrifuge for 1 min at 500 × *g* and discard. Repeat twice.
7. Add 100 μL of PBS to the column, centrifuge for 1 min at 500 × *g* and discard. Repeat twice.
8. Place column in a new collection tube and add elution buffer. Incubate 30 min at 55 °C with shaking.
9. Centrifuge for 1 min at 500 × *g* and collect the eluate. Sample can be used for downstream proteomic investigations or stored at −20 °C if not used immediately.

3.3 LC-MS/MS Analysis of Biotinylated Proteins

1. Perform alkylation and tryptic digestion of eluted proteins (*see* **Note 7**).
2. Depending on digestion type and detergents used for cell lysis, apply suitable peptide purification (*see* **Note 8**).
3. Analyze peptides on suitable nanoLC-MS system (*see* **Note 9**).

3.4 Data Analysis

The LC-MS raw data can be analyzed using different programs, such as Proteome Discoverer, MaxQuant, Progenesis LC-MS, and Protein Pilot. In our proteomic workflow we use Proteome Discoverer and database search using SEQUEST, followed by Percolator validation (FDR based confidence scoring) in order to obtain confident protein identities (*see* **Note 10**). Each identified protein has its Protein card (Fig. 1) in Proteome Discoverer containing information about gene ontology, pathways and diseases involved, as well as links to available external data resources for that specific protein, such as STRING, NCBI map, KEGG, UniGene, and SNPs. Except SEQUEST search, Proteome Discoverer enables MASCOT and MS Amanda database searches.

3.5 Prediction of Protein Subcellular Locations

Identified protein usually contain remain of some cellular or other nonspecifically bound proteins. Prediction of subcellular localization can be also performed using CELLO [9] which uses the relationship between sequence similarity (sequence alignment) and identity in subcellular localization to predict subcellular localization, and it is based on multiclass SVM classification system.

1. Go to CELLO: subCELlularLOcalization predictor.
2. Load FASTA file(s) and chose suitable organism (eukaryotes) and sequence (proteins). For each subcellular localization software calculates the reliability (Fig. 2). List outer membrane proteins.

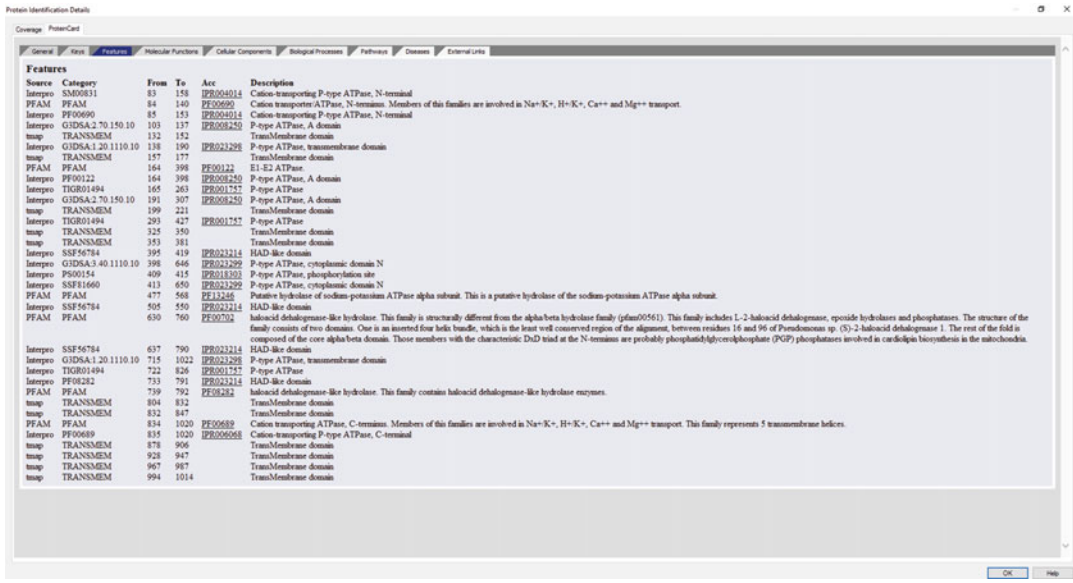


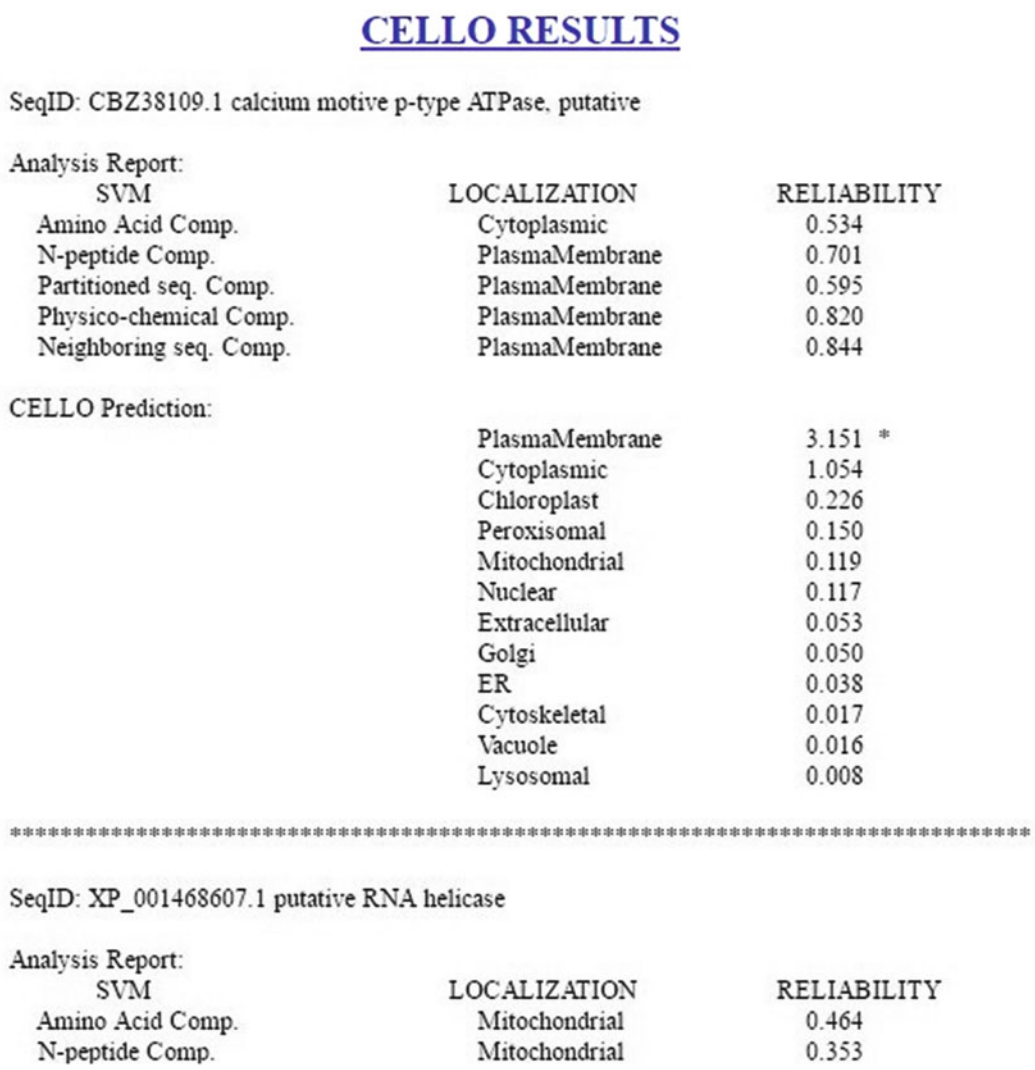
Fig. 1 Example of protein card of identified membrane protein in Proteome Discoverer

Other available databases and computational programs for subcellular localization (together with belonging links) can be found at http://www.geneinfinity.org/sp/sp_proteinloc.html.

3.6 Filtering Data Trough Bioinformatics to Identify Potential Interacting Proteins

List of identified proteins can be applied to The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**) [12, 13] database in order to obtain GO data and filter database to obtain gene ontology data and the list of potential interacting proteins. DAVID represents a set of data-mining and visualization tools that enable functional classification, biochemical pathway maps, and conserved protein domain architectures [14].

1. Copy the list of EntrezGeneID to a new Spread sheet.
2. Go to DAVID Bioinformatics Resources.
3. Go to "Start analysis" tab.
4. Paste the list of EntrezGeneID under the A section (Fig. 3) (**step 1**).
5. In **step 2**, choose the ENTREZ_GENE_ID as identifier.
6. Check "Gene list" in **step 3** and click on "Submit" to start the analysis.
7. Specify the targeted species, or all the proposed species for low information species (Fig. 4).
8. Click on "Functional analysis tool" on the right panel.
9. Click on "Clear all" to deactivate all analysis.



CELLO RESULTS

SeqID: CBZ38109.1 calcium motive p-type ATPase, putative

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Cytoplasmic	0.534
N-peptide Comp.	PlasmaMembrane	0.701
Partitioned seq. Comp.	PlasmaMembrane	0.595
Physico-chemical Comp.	PlasmaMembrane	0.820
Neighboring seq. Comp.	PlasmaMembrane	0.844

CELLO Prediction:

	PlasmaMembrane	3.151 *
	Cytoplasmic	1.054
	Chloroplast	0.226
	Peroxisomal	0.150
	Mitochondrial	0.119
	Nuclear	0.117
	Extracellular	0.053
	Golgi	0.050
	ER	0.038
	Cytoskeletal	0.017
	Vacuole	0.016
	Lysosomal	0.008

****

SeqID: XP_001468607.1 putative RNA helicase

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Mitochondrial	0.464
N-peptide Comp.	Mitochondrial	0.353

Fig. 2 Prediction of protein subcellular localization obtained as CELLO result

10. Select "GOTERM_BP_DIRECT", "GOTERM_CC_DIRECT", "GOTERM_MP_DIRECT" from Gene_ontology tab, and "Interpro", "Pfam", and "Prosite" from the "Protein_Domain" tab (Fig. 5).
11. Click on "Functional annotation table".
12. On the pop-up windows, select "Download the file" and save it as text.
13. Open the file with a spread sheet editor, with "Tab delimited" option.
14. Remove all protein/gene entries (rows) which are not concerned by GO Cell location terms related with "membrane" (column GOTERM_CC_DIRECT).

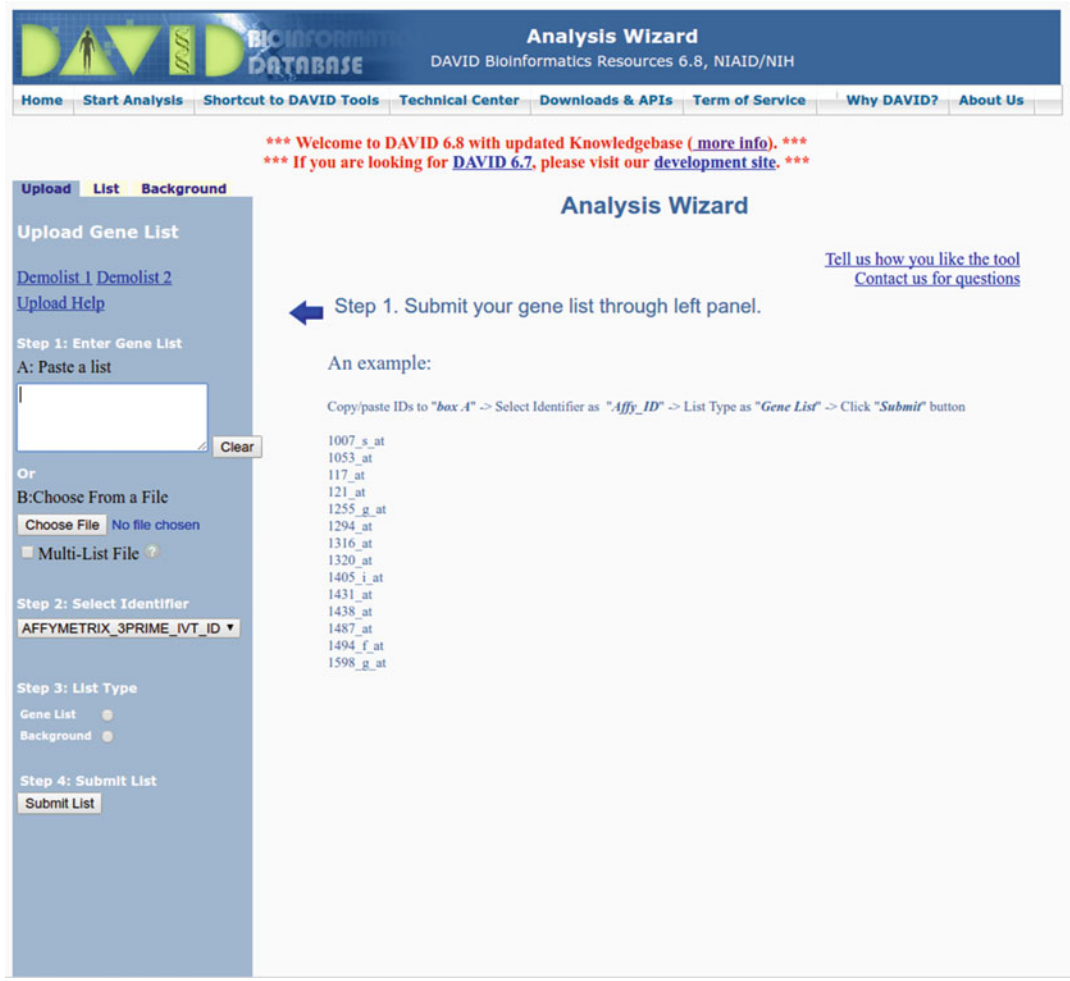


Fig. 3 The Start Analysis tab in DAVID

15. Remove all protein/gene entries (rows) which are implied in known not-related membrane process by GO Biological Process (column GOTERM_BP_DIRECT), like for example "translation" or "protein folding".
16. Remove all protein/gene entries (rows) which are implied in known not-related membrane functions by GO Molecular Functions (column GOTERM_MF_DIRECT), like for example "structural constituent of ribosome" or "DNA binding".
17. For all steps of removal, GO terms can be checked on http://www.geneontology.org/.
18. Using the PFAM, PROSITE and INTERPRO columns, proteins which have domains not related with protein–protein interaction can be removed, like "PF00166:Chaperonin 10 Kd subunit". Every protein domains can be checked on

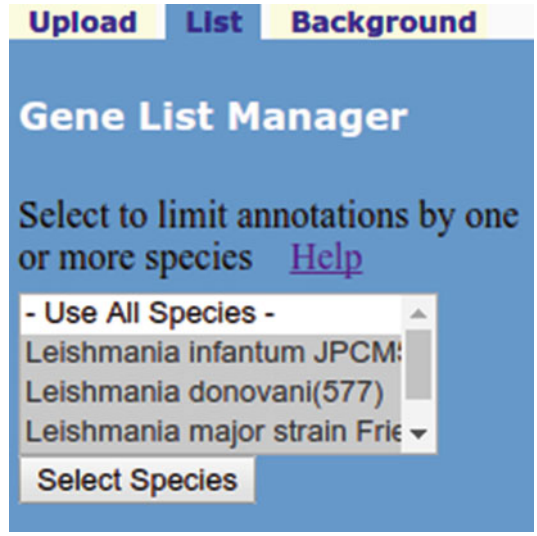


Fig. 4 Species selection screen

PFAM (http://pfam.xfam.org/), PROSITE (http://prosite.expasy.org/) and INTERPRO (https://www.ebi.ac.uk/interpro/) websites.

19. After this step, remaining proteins can be blasted to have more information if available. Manual screening of remaining proteins can be done using both protein domain analysis and BLAST results.
20. Proteins which have passed all those steps of selection are potentially membrane proteins which can interact with other proteins. Protein domain analysis can indicate if such proteins could have interspecies interaction, especially if the domain identified is find in target organism, like SAM domain.

4 Notes

1. Scale the concentration of biotinylation reagent up or down based on cell concentration, size or type. By using the appropriate molar ratio of biotin to the protein, the extent of labeling can be controlled. When labeling diluted protein solutions, a greater molar fold excess of biotin is used compared to a concentrated protein solution. A 100-fold molar excess of biotinylating reagents over the protein amount yields a better degree of cell surface proteins biotinylation as compared to other ratios.
2. Operating at 4 °C throughout the entire procedure helps reduce uptake of biotinylating reagents into the cell.

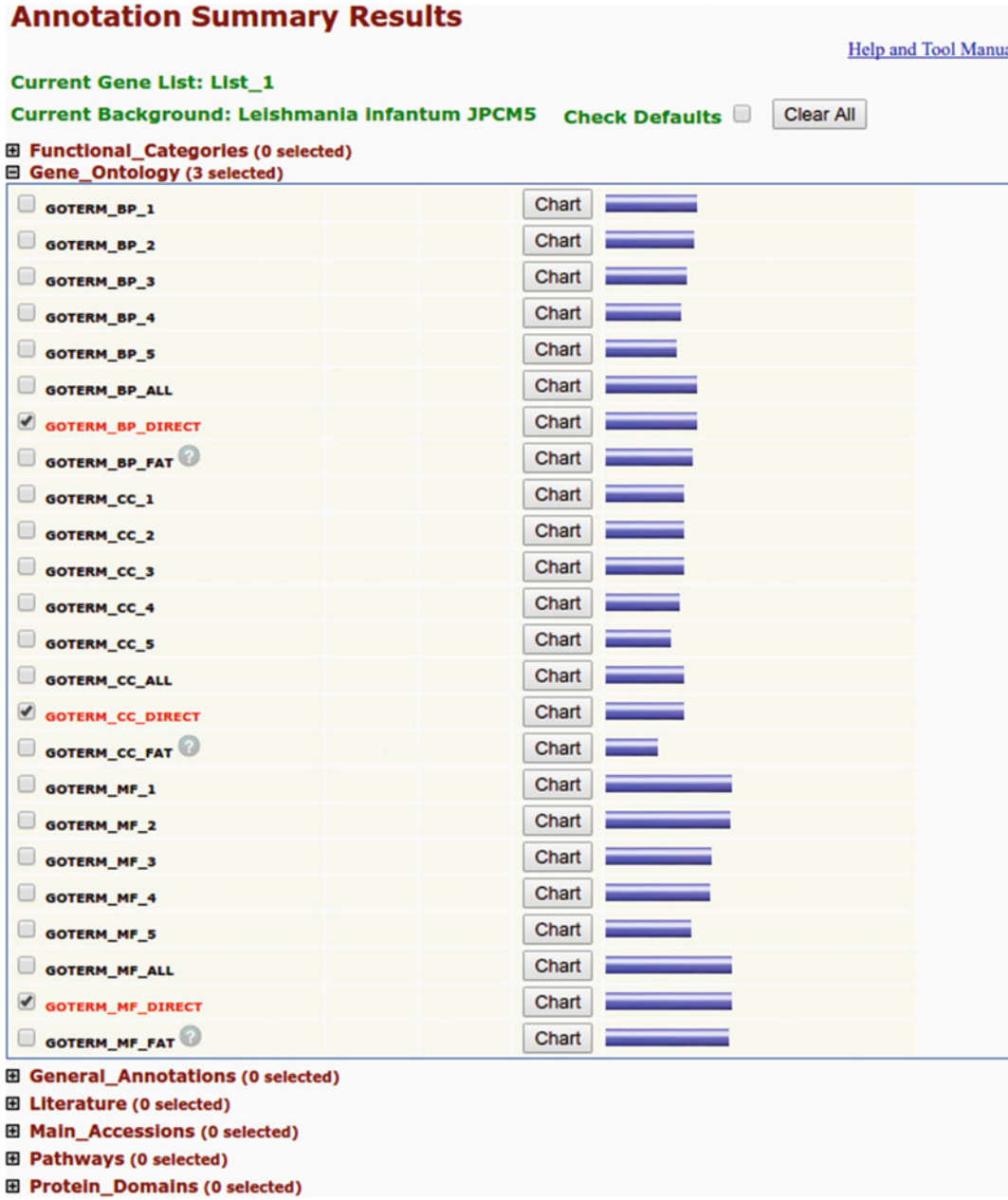


Fig. 5 Gene ontology options

3. A primary amine containing buffer solution as Tris-Cl, ammonium salts, or sodium azide is also commonly used to quench unreacted biotinylating reagent.
4. The choice of lysis buffer depends on the aim of the experiment and specific protocol applied, but also upon considerations bound to the downstream application. Adapt cell lysis buffer and protocol to specific cell type.

5. For protein concentration determination, use Bradford, BCA assay, NanoDrop, or other method compatible with your protein mixture.
6. Based on the protein concentration in the biotinylated sample, calculate the amount of sample and resin needed for affinity purification.
7. Digestion can be performed using different strategies. We recommend FASP protocol [15] using flat bottom filters with 10 kDa cutoff membranes that can be used for up to 200 μg of total protein containing detergents for alkylation (with iodoacetamide) and digestion using trypsin gold (in ratio 1:30). No reduction is needed since DTT is used for elution of biotinylated proteins. Although Triton X-100 cannot be removed by FASP, it does not interfere with FASP digestion. Samples can be also alkylated and digested in solution. Because of low protein amount, overnight ice cold acetone precipitation (four volumes of acetone) can be used. After that pellets should be dissolved in 8 M urea and diluted to 2 M urea with 50 mM ammonium bicarbonate buffer pH 7.6 to final concentration prior alkylation and digestion. Pellets can be also dissolved in sample loading buffer and loaded onto SDS-PAGE gel. Electrophoresis should be performed for approximately 10 min, just to ensure that proteins enter the gel and accumulate into one protein band for salt and detergent removal. Furthermore, standard in gel digestion [16] should be performed, having in mind the yield of tryptic peptide extraction from the gel.
8. If you use Triton X-100 based buffers (such as RIPA), we devised a strategy using strong cation exchange (SCX) chromatography to successfully remove detergents from the peptide sample prior to LC-MS analysis. For the purification up to 10 μg of proteins/peptides, strong cation exchange ZipTips can be used according to following procedure: wash with solution W1 and then load sample diluted in 0.1% formic acid onto SCX ZipTips by aspirating the sample ten times. Wash three times with solution W1, wash five times with solution W2, and elute in 10 μL of elution solvent E1. Finally dry out ammonia and methanol in a Speed-Vac centrifuge and resuspend the sample in 10 μL of 0.1% formic acid. For SDS or CHAPS detergents removal after FASP digestion or in gel digestion, purification with RP C18 ZipTips can be used according to manufacturer procedure. Although high concentrations of CHAPS can interfere with LC-MS analysis, low concentrations can be detected in MS spectrum that do not significantly influence the analysis result and can be easily removed from the nanoLC-MS system after a few sample loop washes and water injections.

9. For LC-MS analysis we usually inject 1 μg of proteins/peptides onto 15 cm nano RP C18 column. Peptides are separated through 3 h gradient from 5–40% mobile phase B followed by gradient increase to 90% B for 5 min. Gradient can be adjusted according to obtained chromatogram.
10. For protein identification in Proteome Discoverer we use SEQUEST to search FASTA files downloaded from NCBI database. As criteria for the search, among standard modifications (oxidation of methionine and carbamidomethylation of cysteine) we use thioacyl (K) as variable modification. The false discovery rate values in Percolator node were set to 1% (strict) and 5% (medium), respectively.

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Chapter 10

Systems Biology Modeling to Study Pathogen–Host Interactions

Müberra Fatma Cesur and Saliha Durmuş

Abstract

Pathogen–host interactions (PHIs) underlie the process of infection. The systems biology view of the whole PHI system is superior to the investigation of the pathogen or host separately in understanding the infection mechanisms. Especially, the identification of host-oriented drug targets for the next-generation anti-infection therapeutics requires the properties of the host factors targeted by pathogens. Here, we provide an outline of computational analysis of PHI networks, focusing on the properties of the pathogen-targeted host proteins. We also provide information about the available PHI data and the related Web-based resources.

Key words Pathogen–host interaction, Network analysis, Comparative interactomics, Infection mechanism, Drug target

1 Introduction

Infectious diseases remain to be one of the major health problems worldwide despite great advances in medicine. Detailed information about the infection mechanisms through the pathogen–host molecular interactions is required to contribute to the identification of novel efficient therapeutics against infections. Over the past decade, researchers direct their attention to the analysis of whole systems instead of studying individual molecules as a result of related biotechnological developments. The popularity of omics technologies supported by various high-throughput data producing methods has highly increased in comparison to traditional methods. Systems biology modeling to study PHIs is possible with genome-wide molecular profiling using high-throughput technologies to generate omics data, in this post-genomic era [1–5].

The detection of interacting molecules is notably significant to enlighten the infectious diseases since pathogenic microorganisms must interact with host cellular factors for initiating and sustaining infection. These interspecies molecular interactions initiate a battle

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between pathogen and host which is mediated by host receptors and conserved pathogen-specific chemical motifs [6–8]. The receptors including pattern recognition receptors, members of the C-type lectin receptors family, complement receptors, scavenger receptors, and integrins can trigger various downstream signaling pathways via recognition of pathogen molecules such as carbohydrates, proteins, glycolipids, nucleic acids, and proteolipids [7]. PHIs firstly initiate innate immune response within hours after infection by means of recruitment and activation of some cell types compromising macrophages, dendritic cells, and natural killer (NK) cells [6, 7]. This first line of host defense provides clearance of the pathogens, activation of the inflammatory response and recruitment of leukocytes to the sites of infection via release of chemokines and cytokines. Adaptive immune response is also activated within several days [7]. On the other hand, PHIs also determine the pathogen behavior within the host organism. Pathogens target components of the host systems in order to invade the host cells by breaking down the primary barriers, evasion of the host defense, regulation, and utilization of host cellular mechanisms to survive and proliferate [4, 8]. Therefore, the identification of these pathogen-targeted host factors and their properties is crucial to unravel the molecular mechanisms of the infectious diseases.

Pathogen–host interactions may be between proteins, nucleotide sequences, and small ligands. However, protein–protein interactions (PPI) of pathogen–host systems have been identified as the most important in determining the infection mechanisms [9–11]. Here we provide an outline of computational analysis of pathogen–host PPI networks with a special focus on the properties of the host proteins. The first step is the extraction of experimentally verified PHI data from the corresponding Web-based resources. The second step is the topological analysis of the PHI networks to identify pathogen and human proteins with critical graph properties, i.e., highly interacted proteins. The third step is the preparation of the host protein sets to be investigated. Finally, the fourth step is the analysis of host proteins based on their functional properties within the host's cellular systems to get insights on the pathogen-targeted mechanisms during infections.

2 Materials

2.1 Computationally Predicted PHI Data

Due to insufficient experimental PHI data, many computational PHI prediction methods such as homology-based, structure-based, and domain/motif interaction-based methods were developed [12–18]. Homology-based approach is a simple way for detection of probable PHIs and it assumes that an interaction between a pair of proteins is conserved in related species. In other words, it uses the logic that if a host protein interacts with a pathogen protein, the

homologs of these proteins in related species can also interact with each other [8, 19]. However, this method needs to be supported by other prediction methods or additional information like subcellular localization, function, expression levels and so on [19, 20]. Computational framework of the structure-based approaches based on known interacting proteins and a pair of proteins with similar structures to these known proteins. The method proposes these structurally similar proteins as putative interacting partners [8, 19]. The domain/motif interaction-based prediction methods are among the intriguing approaches to reveal PPI data. In this method, firstly, functional domains from the interacting intra-species PPI data are determined. Then, the probability of two proteins including a specific pair of domain that interact with each other is calculated [19]. Nourani et al. comprehensively reviews the computational PHI prediction studies [21].

2.2 Experimentally Verified PHI Data

The first large scale experimental PHI networks have been generated for commonly observed and human-threatening viruses and bacteria, within the last decade (Table 1). Currently, researchers are producing these data at an accelerated rate. In the near future, more complete PHI networks will be available for a wider range of pathogen and host organisms.

2.3 Web-Based PHI Data Resources

Following the initial efforts on the production of large-scale experimental PHI data, the PHI-specific databases have emerged on the Web (Table 2). These PHI data resources aim to integrate and present pathogen–host molecular interactions and related data available in the literature.

3 Methods

3.1 Extraction of PHI Data

Collection of PHI data for the pathogen–host systems under investigation is possible through the Web-based resources (Table 2) (*see* **Notes 1–3**). As an example, here we provide the steps for PHI data extraction from PHISTO which is one of the most comprehensive PHI database for the human host. PHISTO includes only experimentally found PPI data for pathogen-human systems [43].

1. Go to PHISTO user interface at www.phisto.org
2. Use “Browse” option to get the PHI data using taxonomic filtering functionality. If you need PHI data corresponding to any specific pathogen strain/species/family, “Browse” option would be a good choice to reach the required data easily.
3. Alternatively, use “Quick Search” or “Advanced Search” to get the PHI data. “Quick Search” can perform a query without a specified identifier, whereas “Advanced Search” option is used to search based on any selected subset of identifiers.

Table 1
The first large-scale experimental pathogen-human PPI networks (Adapted from Durmuş et al. [4])

Pathogen name	Pathogen type	Number of PHIs	Number of pathogen proteins	Number of human proteins	Experimental method	Reference
Epstein-Barr Virus	DNA virus	173	40	112	Yeast two hybrid	[22]
Hepatitis C Virus	RNA virus	481	11	421	Yeast two hybrid	[23]
Epstein-Barr Virus	DNA virus	147	1	147	TAP	[24]
Human Immunodeficiency Virus-1	Retrovirus	183	1	183	GST pull-down combined with LC-MS/MS	[25]
Influenza A virus (H1N1 A/PR/8/34)	RNA virus	135	10	87	Yeast two hybrid	[26]
Influenza A virus (H3N2 A/Udorn/72)	RNA virus	81	10	66	Yeast two hybrid	[26]
Bacillus anthracis	Gram+ bacteria	3073	943	1748	Yeast two hybrid	[27]
Yersinia pestis	Gram− bacteria	4059	1218	2108	Yeast two hybrid	[27]
Francisella tularensis	Gram− bacteria	1383	349	999	Yeast two hybrid	[27]
Hepatitis C Virus	RNA virus	56	2	56	Yeast two hybrid	[28]
Dengue Virus	RNA virus	139	10	105	Yeast two hybrid	[29]
Measles Virus	RNA virus	245	1	245	Modified tandem affinity chromatography with MS	[30]

Y. pestis	Gram+ bacteria	204	66	109	Yeast two hybrid	[31]
Human Immunodeficiency Virus-1	Retrovirus	497	16	435	Affinity tagging and MS	[32]
30 viral species	DNA and RNA viruses	1681	70	579	TAP	[33]
Human Respiratory Syncytial Virus	RNA virus	221	1	221	Quantitative proteomics in combination with green fluorescent protein GFP-trap immunoprecipitation coupled to SILAC-based LC-MS/MS	[34]
Hepatitis C Virus	RNA virus	112	7	94	Yeast two hybrid	[35]
Hepatitis C Virus	RNA virus	103	1	103	Protein microarray assays	[36]

Table 2
Web-based PHI data resources (Adapted from Durmuş et al. [4])

Database	Pathogen	Host	URL	Reference
HCVPro	HCV	Human	http://www.cbrc.kaust.edu.sa/hcvpro/	[37]
Human Immunodeficiency Virus-1 Human at NCBI	HIV-1	Human	http://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions	[38]
HoPaCI-DB	*Pseudomonas aeruginosa* and *Coxiella burnetii*	Mammalia, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*	http://mips.helmholtz-muenchen.de/HoPaCI/	[39]
HPIDB	Bacteria, Fungi, Viruses	Animal, Human, Plant	http://www.agbase.msstate.edu/hpi/main.htmL	[40]
PATRIC	Bacteria	Actinopterygii, Arachnida, Chromadorea, Insecta, Mammalia	https://www.patricbrc.org/	[41]
PHI-base	Bacteria, Fungi, Oomycete	Animal, Human, Insect, Fish, Fungi, Plant	http://www.phi-base.org/	[42]
PHISTO	Bacteria, Fungi, Protozoa, Viruses	Human	http://www.phisto.org/	[43]
Proteopathogen	*Candida albicans*	Mammalia	http://proteopathogen2.cnb.csic.es/	[44]
VirHostNet	Viruses	Human	http://virhostnet.prabi.fr/	[45]
VirusMentha	Viruses	all Hosts	http://virusmentha.uniroma2.it	[46]

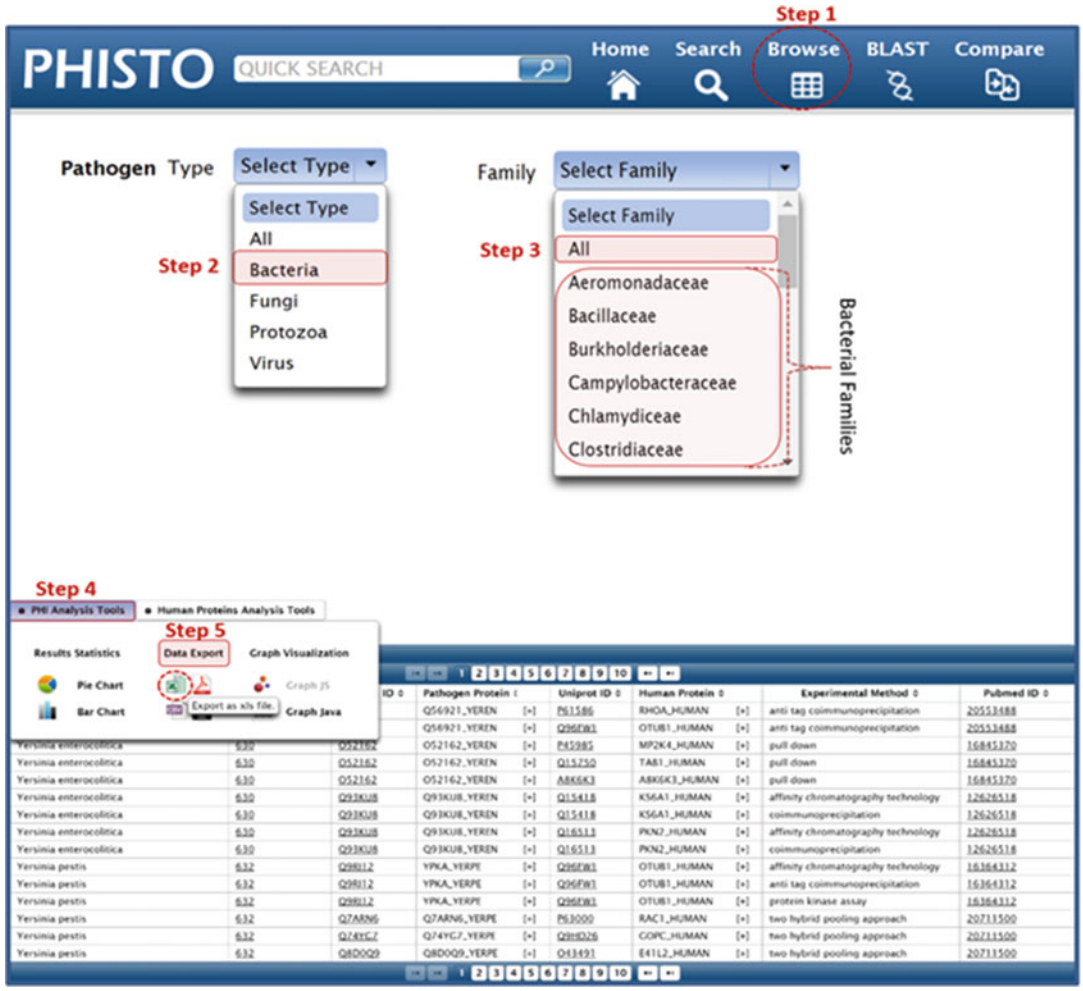

Fig. 1 Procedure to export all bacterial PHI data from PHISTO to an .xls file

4. The PHI data results are presented in an ID-based formatted table, which includes information about eight identifiers as taxonomy ID and name of pathogen, Uniprot ID and name of pathogen protein, Uniprot ID and name of human protein, experimental method and literature reference.
5. Use "Data Export" option under "PHI Analysis Tools" to download the search results. The data can be exported to different file formats as .xls, .pdf, .csv, and .xml.
6. Figure 1 presents the simple five-step procedure to export sample PHI data belonging to all bacterial pathogens in PHISTO to an .xls file.

3.2 Topological Analysis of PHI Networks

The analysis of topological properties of interacting pathogen and host proteins as PHI network components is crucial to get initial insights on infection mechanisms without thorough investigations

(*see* **Note 4**). For instance, proteins with higher number of interacting partners probably have critical roles in infection processes.

1. From a PHI dataset extracted from the databases, obtain the unique binary interactions between pathogen and host proteins (*see* **Note 5**).
2. Calculate the number of interactions involved for each protein.
3. Sort the pathogen proteins and host proteins independently, with decreasing number of interactions involved.
4. The top proteins with the highest number of interactions are the hub proteins within the PHI network. Take the top portion of the lists to obtain highly interacting proteins. Investigate these few highly interacting pathogen and host proteins one by one, in order to determine their roles within the infection processes.
5. It is also possible to determine the highly interacting proteins by visualization of PHI networks with moderate total number of interactions (Fig. 2).

3.3 Preparation of Host Protein Sets to Be Analyzed

Recent computational systems biology of PHI networks mainly focus on the properties of host proteins since critical ones may serve as drug target potential for the next-generation anti-infectives [33, 47–50]. Comparative analysis of host proteins targeted by different types of pathogens may provide results for further design of broad and pathogen-specific therapeutics. For a comparative analysis of PHI networks based on the targeted host mechanisms,

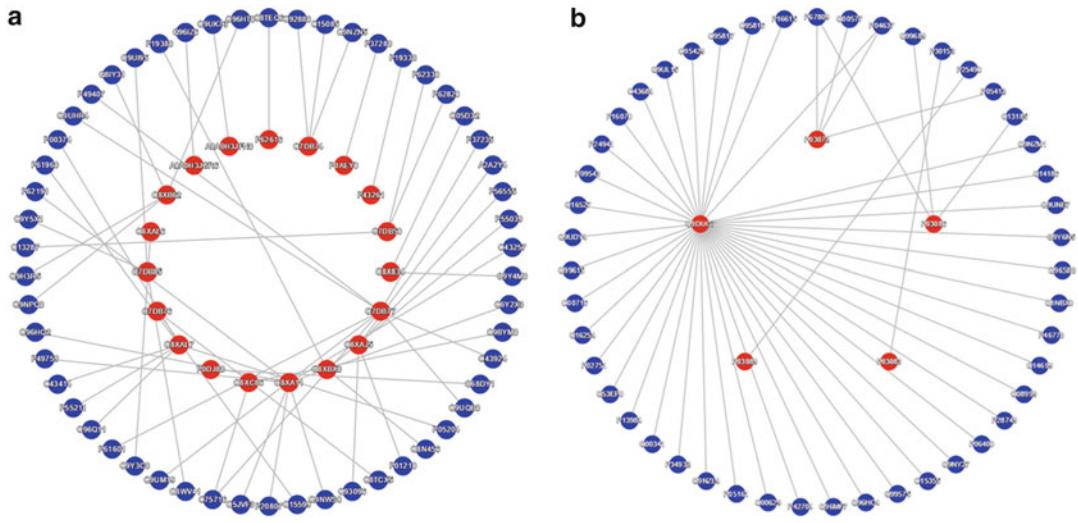

Fig. 2 Experimentally verified PHI networks. (**a**) A sample bacteria-human PHI network for bacterial pathogen *Escherichia coli* O157:H7 (**b**) A sample virus-human PHI network for JC polyomavirus. The networks were drawn in PHISTO using "Graph Visualization" tool under "PHI Analysis Tools" with "Circle Layout" option. Red nodes are pathogen proteins and blue nodes are human proteins

host protein sets should be constructed from the PHI data, according to the focus of the research. Followings are the host protein sets constructed from PHI data belonging to all bacterial and viral pathogens for an example analysis for comparison of bacterial and viral infection mechanism.

1. The host proteins targeted by all bacteria (bacteria-targeted set) and those targeted by all viruses (viruses-targeted set) to observe the general characteristics of the pathogen types.
2. The host proteins targeted by only bacteria, i.e., not targeted by any viruses (only bacteria-targeted set) and those targeted by only viruses, i.e., not targeted by any bacteria (only viruses-targeted set) to search the attack strategies specific to each pathogen type.
3. The host proteins interacting with at least a specified number (n) of pathogen families (n-bacteria-targeted set and n-viruses-targeted set). A deeper comparison between bacteria and viruses is possible with these host protein sets targeted highly by multiple pathogens.
4. The host proteins targeted by bacteria and/or viruses (pathogen-targeted set) and by both bacteria and viruses (bacteria-virus-targeted set) to observe the common infection strategies based on the commonly targeted host mechanisms.

3.4 Functional Analysis of Pathogen-Targeted Host Proteins

To characterize the molecular targets of infectious agents it would be a good idea initially to perform a functional analysis of host proteins involved in PHIs (*see* **Note 6**). Here, as examples of such analysis, we introduce Gene Ontology (GO) and pathway enrichment analyses of pathogen-targeted host protein sets in Subheading 3.3.

3.4.1 GO Enrichment Analysis of Pathogen-Targeted Host Proteins

The information regarding sequences, genes and their products has been rapidly accumulated through the advances in the experimental methods. The Gene Ontology Consortium launched an initiative in order to construct a unified vocabulary including standardized representation of this accumulating information. GO aims to characterize the components of data sets via three terms: "Biological Process", "Molecular Function", and "Cellular Component" [51, 52]. Biological processes are made of the reactions including physical or chemical transformations of substances in the cell (e.g., DNA replication initiation, translation, and cell recognition). Molecular function refers to the activity of a gene product at the molecular level (e.g., phosphotransferase activity, ATP binding and oxidoreductase activity). Cellular component is used in order to provide the localization in which the related gene product is active (e.g., cell wall, ribosome and nucleosome) [52]. Thus, GO terms can form a framework for an understanding of cellular systems.

These terms can be detected by a variety of Web servers and tools including agriGO [53], g:Profiler [54], AmiGO [55], BiNGO [56], GOEAST [57], DAVID [58], Gorilla [59], GOstat [60, 61], and Ontologizer [62]. For a protein set under investigation, GO enrichment analysis gives the significantly enriched GO terms for the components of the set. Followings are the steps for performing a GO enrichment analysis using the BiNGO app of Cytoscape [56].

1. Download Cytoscape from www.cytoscape.org and then install it.
2. Install BiNGO app via App Manager of Cytoscape.
3. Start BiNGO under Apps menu in order to manage BiNGO settings (Fig. 3) and then start the GO enrichment analysis.
4. Enter the name of your host protein set into the box "Cluster name". This name will also be the name of your output file.
5. Select the option "Paste Genes from Text" and then paste a host protein set (e.g., a list of Uniprot IDs for the host proteins) to be analyzed.
6. Select Hypergeometric or Binomial test for calculation of *p*-values (*see* **Note 7**).
7. Use Benjamini–Hochberg False Discovery Rate (FDR) or Bonferroni Family-Wise Error Rate (FWER) correction method (*see* **Note 8**).
8. Set the significance level (*p*-value) to the default value (i.e., 0.05) or any other desired value. Then GO terms whose *p*-values smaller than the significance level will be included in the results as enriched ones for the protein set.
9. Set the desired type of ontology file under "Select ontology file". You may select one of GOSlim options. GOSlim is a tailored cut-down (slim) versions of all GO terms that provides a well-summarized view of the ontology content without the details [63–65]. In other words, GO slims consist of high-level terms reflecting each of the three ontologies by far the best without overlapping in paths in the GO hierarchy; considering proteins can be associated with more than one GO term and they can be assigned to these all terms [66, 67]. They can be generated in line with their requirements by users and they might be species-specific as opposed to generic GO slims or model organism-specific (e.g., GOSlim_Plants and GOSlim_Yeast) [65]. Moreover, to detect all versions of GO terms other options (i.e., GO_Cellular_Component, GO_Molecular_Function, GO_ Biological_Process or GO_full comprising all of them) can be selected.

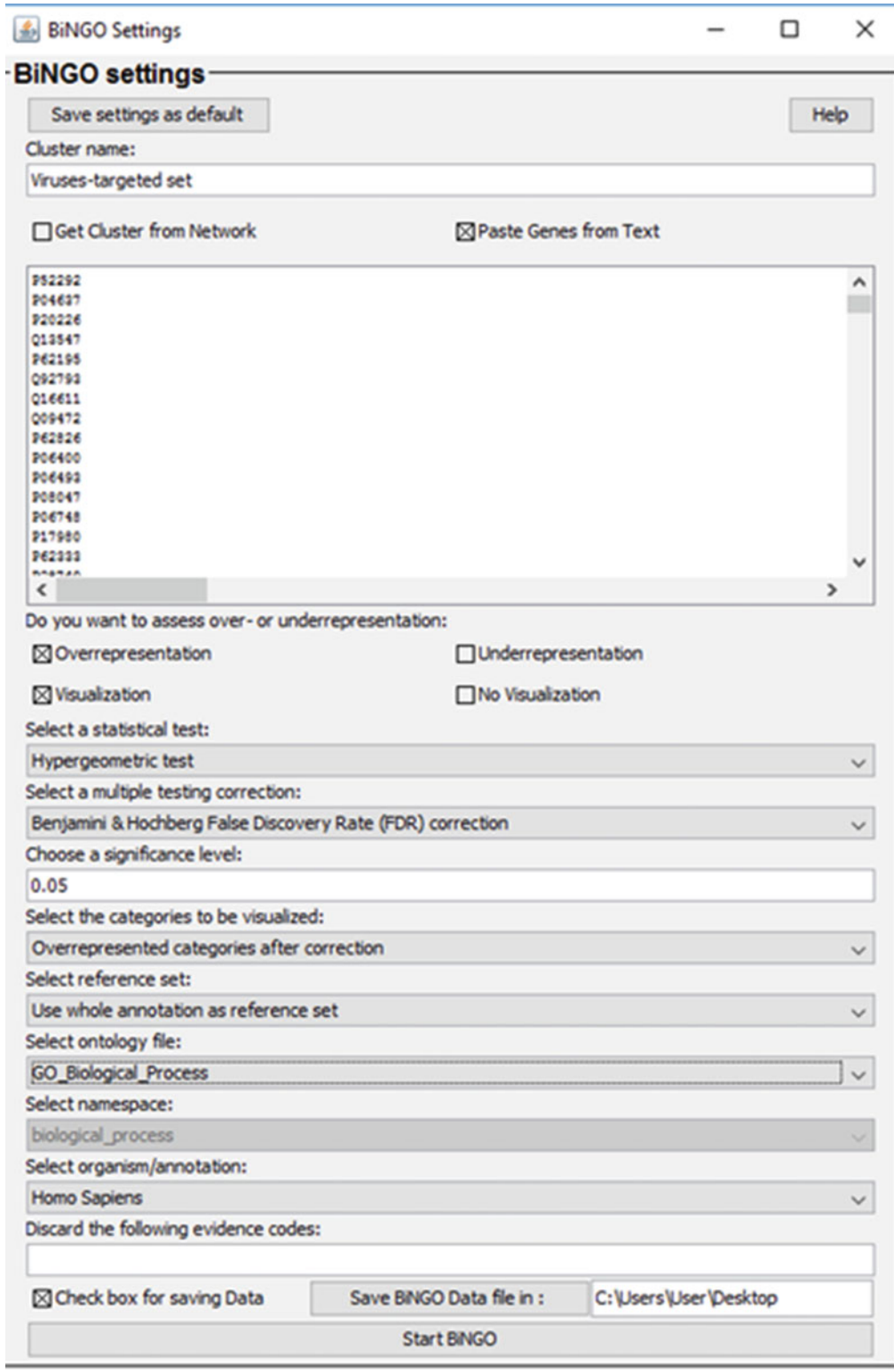

Fig. 3 Screenshot for user interface of BiNGO

10. Select the host organism under "Select organism/annotation" menu.
11. Check box for saving data and specifying a location via "Save BiNGO Data file in" button.
12. To start the enrichment analysis, use the "Start BiNGO" button.
13. You can obtain the enriched GO terms you specify in **step 9** from the output file saved in the location you specify in **step 11**.

3.4.2 Pathway Enrichment Analysis of Pathogen-Targeted Host Proteins

Pathway-based analyses are usually performed to understand complex diseases by investigating defective parts in the unhealthy systems [68]. In the case of infectious diseases, pathway enrichment analysis of pathogen-targeted host proteins is a powerful tool in order to unravel the disease mechanisms and to determine potential host-oriented drug targets by detection of the affected host pathways. Some of the corresponding analysis tools are g:Profiler [54], DAVID [58], FunSpec [69], KOBAS [70], PANTHER [71], sigPathway [72], and WebGestalt [73]. Here, we focus on how to use KOBAS online software in order to determine the enriched pathways for pathogen-targeted host protein sets.

1. Access the KOBAS 2.0 Web page at http://kobas.cbi.pku.edu.cn
2. There are three options on the top toolbar: Annotate, Identify and Annotate + Identify. "Annotate" is for annotating your input proteins to pathways and diseases. "Identify" is for identifying enriched pathways and diseases for the protein set you enter. "Annotate + Identify" is a shortcut which combines "Annotate" and "Identify". For a pathway enrichment analysis of human protein sets, use "Annotate + Identify" option.
3. Select the type of your dataset via "input type" option. Using "UniProtKB AC" is recommended since most of the PHI databases store Uniprot IDs of interacting pathogen and host proteins.
4. Using "species" button, select the species of interest among a wide range of organisms which your host protein list will be mapped to.
5. Enter the input (e.g., a protein/nucleotide sequence in FASTA format, BLAST output or a list of IDs) proper to the input type you select in **step 3**. You can directly paste the input into the text field or upload the relevant file by clicking "Choose File" button.
6. Select your desired databases from the alternatives for pathway databases (KEGG PATHWAY, Reactome, BioCyc, PANTHER) and diseases databases (OMIM, KEGG DISEASE, NHGRI GWAS Catalog).

7. Select one of the four statistical methods supported by the KOBAS 2.0 in the identification of statistically significant pathways and diseases.
8. To reduce the ratio of the Type-1 errors as a result of the multiple hypothesis tests, select one of the alternative FDR correction methods.
9. Set the *p*-value cutoff to any specified value to obtain only statistically significant results with *p*-values smaller than the cutoff.
10. Press 'Run' to start search for the enriched pathways for the input host protein set.

4 Notes

1. Databases may not be maintained regularly. You should check the final data update date before you extract data from a database. It is important, because currently pathogen–host PPI data are producing at higher rate than ever. If you extract your data from an outdated database, you may lose the recently produced experimental data.
2. To obtain the most comprehensive data as much as possible, you should check the intersection of the alternative databases. If the interaction is small, it would be better to use multiple data sources together.
3. Even though databases are invaluable resources of major knowledge regarding pathogens, hosts, and their interactions, they cannot store all available experimental PHI data. There is a considerable amount of PHI data embedded in the literature. Some rare efforts have been performed to develop text mining techniques to obtain hidden PHIs from the literature [4, 11]. If you want to obtain experimentally found PHI data as much as possible for a specific pathogen–host system, you should use text mining techniques to retrieve data directly from articles in addition to the online databases.
4. Topological network properties of the pathogen-targeted host proteins within the intranetwork of host PPIs are also important to unravel the characteristics of the targeted host mechanisms. Therefore, analysis of the topological network properties of the targeted host proteins within the host's own PPI network may be performed in addition to the topological analysis of interspecies PHI networks.
5. Some PHI data may be presented in PHISTO more than once, if it is obtained in more than one publication and/or using more than one experimental method.

6. The results drawn from this type of an analysis of PHI networks should be interpreted with caution since the data for lots of pathogens are still scarce.
7. Binomial test takes less time; however, it can be only preferred for very large datasets such as in the case of the number of proteins in the given set being several thousand.
8. Both correction methods are useful to remove the false positives. However, Bonferroni FWER correction is stricter and can cause an increase in the number of false negatives. Therefore, care should be taken when using this approach.

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Chapter 11

Phage Therapy: Various Perspectives on How to Improve the Art

Stephen T. Abedon

Abstract

Use of phages as antibacterial agents has a long and, even, storied history. During that time much has been learned but, to a degree, also forgotten. As a consequence, today we experience a largely preclinical development of a field which already has been subject to substantial clinical practice. This development, as well, is now occurring within a much more rigorously regulated environment than previously had been the case. The consequence is not only a need to reinvent standards of practice but to do so within a more explicitly pharmacological context. Of particular concern is that the application of phages to bacterial infections does not always result in control of the latter, necessitating ongoing thought on how to refine treatment protocols. Here I consider a number of issues relevant to such refinement, focusing on areas which, in my opinion, phage therapy researchers—perhaps especially those new to the field—might struggle with. In order of presentation, I consider how best to describe phage therapy within publications toward achieving a more coherent literature, the importance of Poisson distributions along with killing titers toward understanding phage dosing, the associated importance of establishing sufficient phage numbers in situ to achieve adequate bacteria killing, various problems with the use of multiplicity of infection (MOI) as a description of phage dosing, how to anticipate the basic kinetics of phage–bacteria absorptive interactions, how to distinguish passive from active treatments, and basic approaches toward addressing disappointing efficacy outcomes.

Key words Bacteriophage therapy, Biocontrol, Biocontrol using phages, Biological control, Killing titer, Multiplicity of infection, Phage biocontrol, Phage therapy, Phage therapy pharmacology, Poisson distribution

1 Introduction

In the 1980s funding for phage research in the USA to a large extent dried up. The result was a great upheaval which saw many phage labs switching to other, more easily funded systems [1]. At approximately the same time, and presumably not coincidentally, these more easily funded, mostly more complex systems also became more amenable to the kind of deep molecular inquiry which previously had been possible only with relatively simple model systems, such as phages [2]. Indeed, Kropinski and Clokie

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[1] trace what possibly is a substantial decline in numbers of phage publications that appears to have occurred over the course of the mid-1980s. Young [3] in fact refers to the 1940s through the early 1980s as "the golden age of phage biology."

In the decades leading up to this point, roughly the 1960s onward, there also had been relatively little ongoing ecological tradition within these same laboratories, e.g., witness the emphasis placed on especially the molecular in the 1994 "Epilogue" written by Bruce Alberts on "the T4 paradigm" [4]. Coincidentally, however, this relative dearth of ecological thinking began to change relatively soon after funding for more traditional phage biology had declined. Increased attention to phage ecology as it can affect global biogeochemical cycling in particular began in the late 1980s, especially with observation of substantially more virus particles in seawater, largely phages, than previously had been thought to be the case [5]. Earlier but less influential consideration of phage ecology is well documented as well in the 1987 monograph by Goyal et al. [6].

As phage therapy resurged in the mid-to-late 1990s [7], it was not phage ecology which served as its intellectual foundation but instead more traditional aspects of phage molecular biology, molecular genetics, and biochemistry. A number of issues relevant to the practice of phage therapy, however, were not the primary interest of those researchers who persisted in these more traditional phage studies. Simple, less explicitly molecular phage–bacterial interactions in particular were less of a concern, even though these same phenomena, e.g., phage adsorption rates or host range, serve as much of the basis of using phages as antibacterial agents. Thus, phage therapy in our more modern era grew out of a phage biology tradition which itself was in decline, but which also had already moved well beyond much of the appreciation of those aspects of phage biology, both ecological and less molecular in their emphasis, which could have been particularly useful to phage therapy preclinical redevelopment. These include in particular a phage's ability simply to infect, kill, and lyse target bacteria, in situ, as well as release new phages, which in turn represent the basics of phage ecological study.

A result of these various tendencies is that there are a number of aspects of phage therapy which could benefit not just from deeper thinking but also from thinking which already to a fairly substantial extent has been undertaken. Here I consider a number of such issues. My general goal is to provide an overview of what can be thought of as basic but often misunderstood or insufficiently appreciated as well as *quantitative* underpinnings of phage therapy. I have previously published a general outline for phage therapists on how especially phage therapy experimentation might be more rationally approached [8]. My goal in this current publication is to discuss a number of basic, related issues of the pharmacology of phage–bacterial interactions which could be helpful toward phage therapy preclinical development.

2 Methods

2.1 What's in a Word? Phage "Therapy" Versus Phage-Mediated "Biocontrol"

Though having little to do with the issues discussed in the Introduction, nevertheless a good place to start our discussion is with the question of just what, as a concept, is "phage therapy" along with what is not "phage therapy." One reason that this is relevant may simply be for the sake of not confusing people. Another reason, however, is to perhaps come to appreciate why the phrase "phage therapy" is not always as prominently placed within seeming phage therapy publications, i.e., as may be expected or desired. In fact, in a Google Scholar search it becomes very obvious that historically the phrase "phage therapy" was not necessarily being used by everyone, in publications, at the same time that the actual technique in fact was originally being developed [7]. In more modern times, however, the important distinction which needs to be made is between therapy, on the one hand, and biological control or biocontrol using phages on the other.

One distinction, which I favor [9], is to limit the use of "therapy," as in phage therapy, to circumstances where treatment in fact is therapeutic. The concept of therapy tends to be medical, or veterinary, involving the treatment especially of individuals toward improvement in health. Thus, as applied to humans or animals either to treat or to prevent disease, then phage *therapy* is an appropriate descriptor. For the sake of finding studies via online searches, the term "phage therapy" thus should always be prominently used versus, e.g., "bacteriophage therapy," "therapy using phages," "phage therapeutics," or other derivations of the phrase. In other words, if a researcher cannot type "phage therapy" into PubMed or Google Scholar and find a phage therapy publication, then an author should have less expectation of that study being happened upon. Note, though, that I now also try to use "bacteriophage therapy" as a keyword in my publications.

When phages instead are used to treat environments, such as water supplies to remove problem bacteria, then "biocontrol" as a descriptor is more appropriate. Unfortunately, an equivalent key word or phrase to "phage therapy" does not exist for biocontrol using phages, and "biological control" also often is used instead of "biocontrol," thereby complicating attempts to be fully aware of this literature. For the moment the impetus is on individual authors to do what they can, in terms of key words—using both "biocontrol" and "biological control," "phage" and "bacteriophage" (the latter versus solely, e.g., "bacterial virus"), etc.—to make sure that their publications are not immediately "lost" to this literature in terms of online searches. It would be helpful, however, were prominent authors involved in these studies to publish as a dedicated, open-access commentary a consensus phrase which could be employed as a keyword in such publications, e.g., "phage

biocontrol." The latter I suggest would be preferable to "biocontrol using phages" since, though this alternative is more grammatically correct, there will always be conflicts among individuals as well as journals in terms of preference for "phage" versus "phages" as a plural [10], which can easily stymie a keyword search.

2.2 Understanding Poisson Distributions, and Killing Titers

Shifting gears, let us consider the basic mathematics of either phage therapy or phage-mediated biocontrol of bacteria [11]. In doing so, let us make three basic assumptions. First, there is some density of phages within a given environment which exists independent, so far as this discussion is concerned, of questions of how those phages ended up in that environment (that is, we are ignoring issues of increases in phage densities which are a consequence of external dosing versus as due to in situ phage reproduction). Second, all of the phages we are describing have demonstrably adsorbed to bacterial hosts, that is, rather than still existing as free phages. Third, all targeted bacteria are phage adsorbable and die given the adsorption of a single phage, that is, they serve as bactericidal targets of the therapeutic phages employed. What we are interested in is how many of the phage-infectable bacteria within an environment will die given the adsorption of a certain number of phages to a certain number of those bacteria. Without an ability to address this simple issue, then no rational basis for phage therapy dosing can exist, or biocontrol dosing (for simplicity, I use the phrase "phage therapy" here on out, unless otherwise indicated, to imply either).

One additional assumption needs to be made and that is that free phages will adsorb with some approximation of what is known as a Poisson distribution, e.g., [11–16]. Poisson distributions can be used to determine what fraction of bacteria may have been adsorbed by no phages as well as what fraction have been adsorbed by some specific number of phages, e.g., a total of one phage, or a total of two phages, etc. These fractions are related in that together they should add up to one. That is, the total number of bacteria which have been adsorbed by zero phages plus the total number that have been adsorbed by only a single phage plus the total number that have been adsorbed by a total of two or more phages all together should add up to the total number of bacteria which our phage in question is capable of adsorbing within an environment. Particularly for the zero-adsorbed case, which turns out to be the most relevant to phage therapy, the calculation itself is simple: The fraction of unadsorbed bacteria among phage-adsorbable bacteria should equal e^{-M}, where M is the ratio of *adsorbed* phages to target bacteria. This "M" stands for "Multiplicity of infection," but it is important to keep in mind, as I reiterate in a subsequent section, that "infection" is not found by accident in the phrase, multiplicity of *infection*. M thus is the ratio of actually *infecting* phages to targeted bacteria.

Phage therapy, like any medical procedure, cannot be precisely controlled and therefore M in practice often will not be known. Nevertheless, it is crucial for phage therapists to understand that simply achieving a "high" ratio of phages to bacteria, even if all of those phages succeed in adsorbing, still will not in and of itself necessarily eradicate a bacterial population. For example, if the ratio is 5 adsorbed phages to one ($M = 5$), then the rate of bacterial survival will be e^{-5} or roughly 7×10^{-3}. As 7 is close to 10, this then is nearly 10^{-2} surviving bacteria, or 1%. A 99% reduction in bacterial load may or may not be sufficient to cure a bacterial infection, but nonetheless is inevitable if "only" five phages are adsorbing for every bacterium targeted. Reduce that number further and killing becomes even less effective, e.g., and classically, a ratio of 1 adsorbed phage to every targeted bacterium will yield 37% bacterial survival.

If you are employing phages which either cannot or do not replicate, then *at best* your killing effectiveness will be defined by that initial Poisson distribution. At worst phage killing effectiveness will be defined also by a phage's ability to reach, adsorb, infect, and then kill targeted bacteria. Thus, if you are adding five phages for every bacterium targeted but observing only a 90% reduction in bacterial density, then you will have learned that your phages are not reaching, etc., your targeted bacteria effectively, nor necessarily replicating effectively in situ. In short, when treating bacteria with phages, *if not also estimating how many bacteria will be expected to die using assumptions of a Poisson distribution*—particularly given highly controlled experimental conditions (versus treatment, e.g., of model animals or in the clinic)—then there can exist no rational basis for predicting how effective added phages should be as antibacterial agents.

Based on assumptions of Poisson distributions, one also can estimate phage numbers even when employing phages which are not able to replicate. These calculations require knowledge of the fraction of bacteria which have survived phage attack. Specifically, this involves the calculation of a killing titer [14, 17]. A phage stock's killing titer is equal to the opposite of the product, (number of starting bacteria) $\times$ ln (fraction of surviving bacteria), where "ln" refers to the natural logarithm. Thus, for example, if 50% of bacteria survive (0.5) and you started with 10^8 bacteria/mL, then your titer of successfully adsorbing and bacteria-killing phages would be estimated as approximately 7×10^7/mL, i.e., as $-\ln(0.5) = 0.7$.

2.3 It Takes a Large Number of Phages to Eradicate a Bacterial Population

As noted, phage densities in the vicinity of target bacteria can result from phage addition to bacteria-containing environments or from phage replication within those environments. Some combination of these processes, as well as mechanisms which result in phage losses, will define what density of phages will be present, that is, the phage titer in situ. Collectively these issues are equivalent to the pharmacokinetics of a drug [18, 19]. In any case, a phage's attained

in situ titer is a key variable determining the extent of phage impact on target bacteria. Two other variables are the phage adsorption rate, that is, how readily phages adsorb to target bacteria that are found in their vicinity, and the likelihood that phages will in fact kill targeted bacteria once those bacteria have been adsorbed. With these various issues in mind, we thus can ask: How many phages does it take to kill off a population of bacteria?

To answer this question, it is important to define what degree of phage-sensitive bacterial survival is acceptable in the course of killing off a population of bacteria [17]. That is, since phages adsorb with a Poisson distribution, we will always expect some degree of fractional bacterial survival following phage adsorption. If that fraction multiplied by the original size of the bacterial population is <1, then at least ideally we can expect no survival of phage-sensitive bacteria following phage adsorption. Alternatively, Kasman et al. [20] define reasonable bacterial survival as approximately 10^{-4}, that is, 10,000-fold reductions. That number, 10^{-4}, is equal to roughly e^{-10} (actually e^{-9}) and this in turn implies a ratio of *adsorbed* phages to bacteria of nearly 10, or more if greater killing is desired. What phage densities are required to achieve this ratio of adsorbed phages to bacteria, or indeed even larger ratios if greater levels of bacteria killing are desired?

Unfortunately, a key problem in answering these questions is that it takes time for phages to adsorb to bacteria. In addition, generally in making these calculations it is not reasonable for phage therapists to assume that phages will be allowed infinite lengths of time to adsorb bacteria. Therefore, one has to come up with some reasonable length of time over which some desired degree of phage adsorption should have occurred. Here, for the sake of simplicity, I will assume 100 min. It is possible that this number is too long for some applications, or unreasonably short for others, but some number has to be chosen as a basis for making calculations, and here it will be 100.

Another assumption must be made, and that is the magnitude of the phage adsorption rate constant. I tend to use Stent's [13] value of 2.5×10^{-9} mL/min, or equivalently with units of mL^{-1} min^{-1} as both representations of these units are used. If you use hours instead of minutes as your time unit, however, then it is crucial that this change be taken into account in your calculation. In any case, your phage may adsorb faster than this, or it may adsorb more slowly, or adsorb at different rates in situ versus in vitro, which also can affect calculations. A last assumption is that phage densities will remain constant over time. The latter is simplifying but still reasonable if bacterial densities are relatively low, or if phages are constantly being added to the environment either through dosing or instead as a consequence of in situ phage replication; the more complicated scenario where phage numbers

decline over time due to bacterial adsorption is handled elsewhere [17]. So, given $t = 100$ min and Stent's adsorption rate constant ($k = 2.5 \times 10^{-9}$ mL/min), then what phage density (P) is required to result in 10 phage adsorptions per target bacterium? The answer to that specific question is $P = 10/tk = 4 \times 10^{7}$ phage particles/mL.

Phages may not adsorb in situ to all bacteria as quickly as assumed, you may want phages to adsorb over shorter periods, you might prefer higher levels of bacterial eradication, and phages may decline in density over time for reasons other than bacterial adsorption or, indeed, *because* of bacterial adsorption. I therefore tend to round this density, P, up to 10^{8} phage particles/mL [19, 21]. Thus, to be moderately confident that bacteria are being killed off at a reasonably high rate during phage therapy or biocontrol of bacteria, you should be aiming to achieve a phage density of 10^{8} phage particles/mL. This goal is complicated, however, since as noted it can result either from explicit phage dosing or instead from in situ phage replication. Furthermore, these phage numbers may need to be sustained over long periods if bacteria become only transiently available to phage adsorption, e.g., such as during phage treatment of bacterial biofilms [22].

Alternatively, an animal's entire body need not possess titers of 10^{8} phage particles/mL for phage therapy to be successful. Certainly, however, phage titers of this magnitude must be achieved in the immediate surroundings of the bacteria being targeted for phage therapy to be reasonably likely to succeed. Alternatively, if 10^{8} or higher phage particles/mL are demonstrably achieved in the immediate vicinity of target bacteria but desired levels of efficacy are not observed, then a careful exploration of the reasons for this lower-than-desired efficacy could include, e.g., considerations of unexpected incompatibilities between phages and bacteria, poor phage adsorption conditions in situ, and/or bacteria presence within somewhat virion-impenetrable biofilms. Unfortunately, toward addressing these issues, determining just what phage densities have been achieved during phage therapy, particularly within the immediate vicinity of target bacteria, is not always easy.

2.4 Multiplicity of Infection (MOI) Is a Poor Means of Describing Phage Therapy Dosing

Above we saw the usefulness of multiplicity of infection, MOI, for determining the likelihood of bacterial survival following phage adsorption, that is, as M. The greater the MOI then the fewer bacteria which should survive, which for phage therapy or biocontrol is a good thing. Despite the obviousness of the utility of MOI, the usefulness of MOI to phage therapy nevertheless often is hugely exaggerated in publications. I will not go so far as to say that MOI should never be used in phage therapy or other studies, but MOI nevertheless should *not* be used without at least some attempt at justification, if only to place limits on its current abuse. What is the problem? There are at least three answers to that question [16]:

(1) The term is typically used incorrectly, (2) it is often used misleadingly, and (3) too often MOI is used, as well, in a manner that prevents readers from easily replicating phage dosing. Thus:

1. Multiplicity of infection means multiplicity of *infection*. It does not mean multiplicity of *addition*, though a myth exists that it in fact does mean multiplicity of *addition*, that is, addition of phages to bacteria-containing environments. In reality, properly used, multiplicity of infection is usually better stated, for phages, as multiplicity of *adsorption*. This, in discussion of the Poisson distribution, above, is explicitly how M is defined. Simply adding 10 phages to a culture for every bacterium present is, by contrast, absolutely not an MOI of 10. Why the lack of equivalence? It takes time for phages to adsorb to bacteria—in many cases extremely longer periods of time (next paragraph)—and therefore it can take long periods of time for multiplicities of addition to become multiplicities of adsorption.
2. If there are not many bacteria present within an environment, e.g., if fewer than roughly 10^6 bacteria per mL are present, then the amount of time required for multiplicity of *adsorption* to come to approximate multiplicity of *addition* can be quite long. Naïvely, however, one imagines that when an MOI of 10 for example is stated within a publication, then bacteria will be killed off 10,000-fold (above). That degree of killing, however, may take days, weeks, months, or even years depending on the bacterial density and given MOI defined in terms of addition rather than adsorption [23]. MOI, as often used, thus tends to be misleading because it can substantially overstate the actual ratio of *adsorbed* phages to target bacteria, and the lower the density of bacteria which are targeted within an environment, then the greater the overstatement.
3. When phage dosing is reported solely in terms of multiplicity of infection then it can be difficult or even impossible for readers to work out how many phages have actually been added. It should be obvious that a lack of explicit indication of how many phages have been added during phage therapy should represent a substantial burden to impose upon readers, that is, when phage numbers are obscured by employing MOI as the sole dosing parameter. By way of illustration, imagine a drug trial in which drug densities used were not explicitly stated.

A related issue is that it is not always obvious what researchers consider to be densities of target bacteria at the point of dosing with phages. As a consequence, it is sometimes impossible to calculate the numbers of phages actually added as based upon reported MOIs. Again, substitute "drug" for "phage" and then imagine the publication of a "drug therapy" study where the readers were unable to calculate how much drug was added during patient

dosing. It therefore is my firm opinion that no phage therapy study should be published which does not describe either the titer (and volume) of phages applied per dose—including, explicitly, the titer of every phage type applied as within a cocktail—or at least the total number of phages applied per unit body mass, per dose.

2.5 The Rate that Bacteria Are Adsorbed Is a Function of Phage Density

The rate at which newly infected bacteria are formed within a culture is, ideally, equal to NPk, where N is the density of uninfected bacteria, P is phage density (specifically, the titer of free virions), and k is the phage adsorption rate constant. From this calculation it is obvious that by having more bacteria (greater N) then there will be a faster absolute rate of conversion of uninfected bacteria to infected bacteria. That conclusion in fact is true. It is not the *absolute* rate that bacteria are killed, however, that is particularly relevant to phage therapy so much as the relative rate. For example, killing 10,000 bacteria per minute is fairly impressive if an environment contains only 100,000 bacteria, but is fairly abysmal if the environment instead contains one billion bacteria. In other words, in increasing bacterial numbers while keeping all other factors constant, i.e., P and k, then bacteria will indeed be killed off at a faster rate, but so too more bacteria will be present to kill off.

In standard medical microbiology terms this issue is obvious: It is always preferable to be treating smaller bacterial populations with antibacterial agents versus larger bacterial populations. With phages, because of what appears to be a misinterpretation of the above calculations, as well as a second issue not yet discussed, the obviousness of this point unfortunately seems to have been obscured. The second issue is considered in the following section while the first issue I elaborate on in this section. Specifically, only by increasing P, or instead by increasing k, as per the formula presented at the start of the previous paragraph, will the relative rate of bacteria killing be increased.

Holding bacterial densities constant, the result of adding more phages to an environment is an increase in *both* multiplicity of addition and multiplicity of adsorption. Indeed, unless bacteria are so prevalent within an environment that they substantially reduce the number of phages present, i.e., as due to phage adsorption to those bacteria, then it really does not matter how many bacteria are present when determining especially multiplicity of *adsorption*. That value instead is equal simply to Pkt, where t is the duration over which bacteria are exposed to phages, where again we are simplifying calculations by assuming that phage densities are held constant [24]. If $P = 10^8$ phages/mL, $k = 2.5 \times 10^{-9}$ mL/min, and $t = 100$ min, then the resulting multiplicity of *adsorption* will be 25. For $P = 10^7$, 10^6, or 10^5, the equivalent numbers are 2.5, 0.25, and 0.25. Changing bacterial density, however, has no impact on multiplicity of adsorption as so calculated since the variable, N, is not found in the calculation.

2.6 Passive Versus Active Treatment

The concepts of passive versus active treatment during phage therapy were introduced by Payne et al. [25] with additional consideration by Payne and Jansen [26, 27]. I have also commented on these ideas rather extensively [17, 18, 28]. Passive treatment can be described also as inundation therapy or passive therapy, and so too active treatment as active therapy. A variation on these concepts is "mixed passive/active therapy" [27], which I have suggested could serve as a reasonable default approach to and/or consequence of phage therapy [28]. In addition I have introduced the related concept of active penetration [18]. What do all of these terms mean?

2.6.1 Passive Treatment

Use of standard antimicrobial substances constitutes passive treatment as defined by Payne et al. [25]. As we point out elsewhere [18], the use of "passive" here is semantically confusing since it seems to imply that the actor, e.g., the clinician, is not actively doing anything, whereas in fact with passive treatment the actor not only *is* actively doing something, by dosing with the antimicrobial, but the clinician in fact may be more actively participating in treatment than would be the case for so-called active treatment. Instead, what passive means here, at least by my interpretation, is that the agent (the antimicrobial) is not showing "active replication" [25]. Passive treatment by default thus occurs when employing phages for treatment which are not able to replicate, whether this inability to replicate is or is not intentional on the part of the therapist, or when phage-like "tailocins" [29] are employed instead, i.e., phage tail-like, high molecular weight bacteriocins.

With passive treatment, antibacterial success is entirely dependent on how many phages are explicitly applied to target bacteria as well as the ability of those phages to reach all target bacteria. To be successful, all targeted bacteria must be adsorbed by an average of approximately 10 phage virions (above), or more. To accomplish this degree of adsorption, as noted, one should be striving toward attaining roughly 10^8 phages/mL in the vicinity of target bacteria, with those phages present over periods that are as long as required for these phages to successfully penetrate to and then adsorb to targeted bacteria.

2.6.2 Active Treatment

With active treatment, phages by necessity are actively replicating. This can be confusing because with active treatment the actor, e.g., the clinician, is "passively" allowing phages to increase their dose. The hallmark of active treatment is that fewer phages are applied to bacteria than are required to eliminate bacteria. Typically this would be seen as a multiplicity of *addition* of <1, though, as we've discussed, in reality it means a multiplicity of *adsorption* of added phages that is somewhat <10. In addition, the requirements for successful active treatment are much greater than those for passive treatment [19, 28], as phages must not only be able to adsorb and then kill bacteria but also must be able to (1) lyse

infected bacteria, (2) in so doing release sufficient numbers of virion progeny, and (3) as a population reach inundative phage densities (e.g., 10^8 phages/mL). The latter is possible only if densities of target bacteria are suitably high to support sufficient in situ increases in phage titers, e.g., ~10^6 bacteria/mL or greater. This requirement for higher bacteria numbers to support active treatment is the second issue alluded to in the previous section (2.5) that may have resulted in phage therapists occasionally perceiving that an initial presence of more target bacteria might be preferable to fewer during phage therapy.

Another thing to keep in mind with active treatment is that its use does not mean that phages should *not* be applied in multiple doses, since not all bacteria may be reachable by phages at all times and higher doses attained through in situ phage replication may not persist over sufficient time frames, or be present in optimal locations to penetrate to all target bacteria. Active treatment instead only means that, for treatments to be successful, phages are required to replicate, that is, to increase their numbers at least locally over that amount supplied by dosing alone.

When looking at phage treatment protocols, as I have done particularly when exploring phage treatment of biofilms [15], what I look for in particular is whether indicated degrees of bacteria killing align with possible ratios of added phages to bacteria. If as many phages are added as there are bacteria present (i.e., multiplicity of *addition* of 1), and only about half of the bacteria are killed, then not only was active treatment attempted, but it was not successful: A multiplicity of addition in this case would be far <10 and total bacteria killing in this example approximates what one would expect based on a Poisson distribution given a multiplicity of adsorption of 1, suggesting a lack of substantial increase in phage numbers in situ as a consequence of phage replication. On the other hand, if as many phages were added as there are bacteria, and viable bacteria were reduced in number by orders of magnitude, then that almost certainly is a consequence of active treatment.

If one is merely determining whether phages have increased in number following their addition, then that is not strictly an indication of active treatment since instead it could be mixed passive-active treatment (next paragraph). What matters instead, for the sake of concluding that active treatment has occurred, is whether bacteria are being killed off to a degree which is greater than expected as based on the number of added phages which reach target bacteria. If you do not add enough phages to achieve the degree of killing that you actually observe, then either active treatment has taken place or some other mechanism besides phage infection is resulting in bacteria killing. In any case, note that to a first approximation figuring out whether you are getting more killing than you would expect through purely passive treatment requires an understanding of Poisson distributions (above).

2.6.3 Mixed Passive/Active Treatment

Mixed passive/active therapy is a long phrase that describes what in my opinion is phage therapy as it often takes place. Specifically, if you are dosing as though passive treatment is intended (e.g., 10^8 or more phages/mL, final in situ concentration) and are employing replication competent phages, then you may be achieving mixed passive/active therapy. All this really means is that while the number of phages added may be sufficient to achieve adequate killing of target bacteria, at the same time these phages are increasing their in situ numbers via replication, thereby providing a "margin of efficacy" in terms of how much antibacterial substance is present.

2.6.4 Inundation Is not Lysis from Without

Inundation, whether achieved through standard dosing or, instead, due to in situ phage replication, in any case should be defined as phage presence at densities sufficient to achieve a multiplicity of adsorption of 10 or greater. Contrast "inundation therapy" which by definition, as equivalent to passive treatment, requires for success an achievement of a multiplicity of adsorption of 10, or greater, through standard dosing alone. Regardless of how inundation is achieved, however, inundation should not be assumed to be synonymous with lysis from without [30]. This is despite the semantic similarity, as both concepts could be construed as meaning that cells have been overwhelmed by relatively large numbers of phages in a manner that is not necessarily also associated with successful phage infection and replication. Specifically, and as alluded to above under the heading of Poisson distribution, inundation statistically requires overwhelming bacteria with phages, resulting in reduction of the zero-adsorption bacterial population to an approximation of zero even without—for strictly passive treatment—in situ production of new phages. Lysis from without, by contrast, is a physical or, more accurate, enzymatic overwhelming of a bacterial population in which lysis is achieved without associated phage infection. Lysis from without, in particular, should be assumed to be a somewhat rare and thereby a relatively unlikely phenomenon [30].

2.6.5 Active Penetration

Active penetration is "active" because, as with active treatment, it involves active phage infection of bacteria, that is, rather than solely bactericidal effects by phages [18]. The term was invented to describe the hypothesized requirement by phages to at least lyse biofilm bacteria in order to penetrate more deeply into bacterial biofilms. What lysis achieves is a combination of (1) removing an adsorbable target potentially able to protect underlying bacteria, (2) allowing physiological improvements in the same underlying bacteria which then may then be able to support more effective phage infections, (3) releasing progeny phage virions which may then be able to more directly reach those underlying bacteria, and (4) potentially also releasing extracellular polymeric substance

(EPS) depolymerases. We thus can speculate that lytic and productive phage infections as "active" may be more effective against bacterial biofilms than phage infections which are only bactericidal (as, at least, *less* "active").

2.7 Delivering more Phages

It can be important in experimentation to not be unduly satisfied with merely statistically significant results, e.g., [31]. This point is perhaps here most relevant in terms of the survival of bacteria following phage application. The goal generally is not solely to reduce bacterial numbers but instead to reduce bacterial numbers to below some medically and/or biologically relevant level. It is important, therefore, to have in mind what that level might be *prior* to initiating experiments. Of further issue is not just what fraction of bacteria are killed as a consequence of phage application but also to what level they are reduced. For example, to 10 bacteria per gram or mL? Lower? Higher? The point is that it is not good enough to just show reductions. The reductions, as experimental endpoints, need to be, for example, medically meaningful as well.

Given that perspective, it then becomes natural to ask the question: If degrees of bacteria killing are not sufficient, then how might levels of bacteria killing be improved? Starting with the assumption that methods of phage application conform, in model experimental systems, to how application would occur in the real world, then the biggest variables are qualitative aspects of phage formulated products, quantitative aspects of individual phage delivery, and how often or indeed over what time spans phages are being applied.

Confounding aspects can be abundant. Are applied numbers of phages adequate? Can improvement be achieved by packaging virions such as into liposomes [32], e.g., toward survival through the stomach [33]? Alternatively, if excessive reliance has been placed on active treatment, then it may be reasonable to try using higher phage numbers per dose. Lastly, it is not at all unusual for clinical therapy to involve multiple phage doses, e.g., [34, 35]. Therefore it can be relevant to attempt multiple dosing during preclinical development, perhaps over relatively long time frames, depending on the characteristics of the experimental system. Remember, above all, that the goal is not merely demonstration of an ability to reduce numbers of target bacteria but instead an ability to reduce numbers to clinically meaningful low levels. In a well-controlled experimental system, in other words, the expectation is not that one will be able to approximate real-world success, i.e., as may be observed in the clinic using phage therapy, but instead that one will be able to perhaps substantially exceed that success.

3 Conclusion

Phage therapy is unusual among relatively novel medical techniques in that clinical development appears to have outstripped preclinical development [34, 36]. Presented here have been a number of issues which may have had the effect of blunting especially the preclinical development of phage therapy. My philosophy in presenting these issues is not necessarily to dramatically change phage therapy practice but instead to make phage therapy practitioners, as well as reviewers and more casual readers, at least aware of the existence and possible impact of various somewhat established but nevertheless potentially mistaken perspectives.

Based upon clinical results, phage therapy appears to represent a promising alternative approach to antibiotics toward combatting pathogenic bacteria as well as, in the guise of biocontrol, combatting simply nuisance bacteria. It is important, therefore, that preclinical phage therapy development achieve similar success, and to do so within a pharmacologically rigorous context. Successful consideration of the various issues presented here, along with those addressed in [8] as well as [37] and the appendix of [38], should serve to aid in both endeavors: improvement not only in experiment success but in experimental rigor as well.

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Part II

Microbe-Microbe Interaction Section

Chapter 12

Application of RNA-seq and Bioimaging Methods to Study Microbe–Microbe Interactions and Their Effects on Biofilm Formation and Gene Expression

Cristina Isabel Amador, Claus Sternberg, and Lars Jelsbak

Abstract

Complex interactions between pathogenic bacteria, the microbiota, and the host can modify pathogen physiology and behavior. We describe two different experimental approaches to study microbe–microbe interactions in in vitro systems containing surface-associated microbial populations. One method is the application of RNA sequencing (RNA-seq) to determine the transcriptional changes in pathogenic bacteria in response to microbial interspecies interactions. The other method combines flow cell devices for bacterial cultivation and growth with high-resolution bioimaging to analyze the microscale structural organization of interacting microbial populations within mixed-species biofilms.

Key words Microbe–microbe interactions, Bacterial physiology, Interspecies interactions, RNA sequencing, Bacterial pathogens, Biofilms

1 Introduction

It is becoming increasingly clear that interactions between pathogenic bacteria and the commensal microbiota or other microbial species present at the infection site can influence disease phenotype or the clinical outcome of the infection. Research is now starting to unravel the complex interactions between the microbiota, the host and pathogenic bacteria, and several studies have shown that interactions between bacterial pathogens and other microbial species can result in altered pathogen behaviors, and function to either limit pathogen colonization [1–3] or potentiate pathogen expansion, virulence, or antibiotic resistance [4–6]. These and related studies illustrate the need for both molecular characterization of interspecies interactions mediated by bacterial metabolites and gene products, as well as determination of how these interactions modify pathogen physiology and behavior. Overall, the results emerging from this exciting new research area points toward

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potential novel strategies for control of infectious diseases, which rely on manipulations of the commensal microbiome to increase its resistance against bacterial pathogens.

It is obviously challenging to identify and characterize microbial interspecies interactions and their effects within infected hosts, but interdisciplinary approaches that combines classical microbiological in vitro cultivation techniques with advancing omics technologies such as transcriptomics [7], metabolomics [6, 8], and development of realistic and controllable in vitro model systems [9] now makes it possible to begin to systematically investigate in which way microbe–microbe interactions change the physiology of pathogenic bacteria.

In this chapter, we describe two different experimental approaches to study microbe–microbe interactions in vitro. Each of the methods interrogates different aspects of how microbial interactions change pathogen physiology. In one approach, application of RNA sequencing technology (RNA-seq) is used to obtain molecular level insight into the functional consequences of microbe-microbe interactions in terms of effects on the transcriptome. The other approach uses high-resolution bio-imaging to visualize and analyze the spatial and physical organization of interacting populations within surface-attached mixed-species biofilms. The protocols described focus on *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but can easily be extended to other bacterial species or strains with minor modifications depending on the specific characteristics of particular species, or the specific questions addressed.

We have recently used agar plate model systems to study how microbial interspecies interactions modulate metabolite production, physiology, and behavior in the opportunistic pathogen *Pseudomonas aeruginosa* [6]. Although agar based models do not realistically mimic the natural habitat of the pathogen, such models enable systematic in-depth analysis of the mechanisms, metabolites, and genes that underlie the interaction. In the first sections of the chapter we will describe how to determine the transcriptional response in interacting microbial colonies on agar plates. In this approach, total RNA is harvested directly from the surface grown colonies and the relative amount of all mRNA determined by RNA-seq. Protocols for bacterial cultivation on agar plates, RNA purification, RNA-seq, and data analysis will be described.

Growing and analyzing microbial biofilm communities under hydrodynamic conditions in flow cell devices allow direct microscopic investigation of how microbe-microbe interactions modulate the microscale structural organization and activities of microbial biofilm communities [9]. In this approach, microbial cells are labeled with fluorescent biomarkers, cultivated in flow cell devices, and biofilm formation process analyzed by confocal laser scanning microscopy. The latter sections of the chapter will

describe protocols for flow cell assembly, device inoculation, experimental conditions, confocal laser scanning microscopy, and image analysis. In addition, we describe protocols for introducing fluorescent biomarker genes (e.g., *gfp*) into bacterial strains by chromosomal engineering.

2 Materials

2.1 General Material and Equipment

1. Luria–Bertani rich medium (LB): tryptone 10 g/L, granulated yeast extract 5 g/L, NaCl 4 g/L, pH 7.4. Sterilize by autoclaving 15 min at 121 °C.
2. LB agar: LB medium with 20 g/L bacteriology grade agar.
3. 0.9% NaCl solution: 9 g/L NaCl.
4. Sterile scalpel.
5. Glass tubes.
6. 10-μL sterile disposable inoculation loops.
7. 10 mM dNTPs.
8. Ultrapure agarose.
9. 50-bp DNA ladder.
10. Taq polymerase and 10× Taq buffer.
11. Milli-Q water.
12. 37 °C oven to dry LB plates.
13. 37 °C incubator.
14. Spectrophotometer and cuvettes.
15. Thermocycler and PCR tubes.
16. Vortex mixer.

2.2 Coculture on Agar Plates

1. Inoculum of strains to be coinoculated.
2. Tweezers.
3. 0.45 μm HA filter, previously cropped with 1 × 2.5-cm size.
4. 2-mL nuclease-free microcentrifuge tubes.

2.3 RNA Isolation from Spot Cultures (See Note 1)

1. Disposable, nuclease-free pipette tips with filter.
2. Nuclease-free 1.5-mL microcentrifuge tubes.
3. Nuclease-free water (not DEPC-treated water).
4. RNase-ZAP to remove ribonuclease (RNase) contamination from glass and plastic (*see* **Note 1**).
5. RNeasy® Mini Kit.
6. RNase-Free DNase Set.
7. β-mercaptoethanol.

8. <106 μm acid-washed glass beads.
9. Tubes and screw-on caps for homogenizer.
10. Homogenizer/bead-beater instrument.
11. 100% and 70% Ethanol.
12. 3 M sodium acetate (NaOAc) solution, pH 5.2.
13. 5 mg/mL Glycogen.
14. Phenol–chloroform–isoamyl alcohol 25:24:1.
15. 2-mL Phase-Lock Gel tubes.
16. Low-volume spectrophotometer.
17. Turbo DNase-free Kit.
18. RNase OUT (Recombinant Ribonuclease Inhibitor).
19. Primers for high expression genes: L28 F 5′ TAACCGG-TAAGGGTCCGGTT and L28 R 5′ GAATTT TTCGCCGCGGGCA (*P. aeruginosa*); 16S F 5′ AGCC-GACCTGAGAGGGTGA and 16S R 5′ TCTGGA CCGTGTCTCAGTTCC (*S. aureus*).
20. RNA6000 Nano Kit.

2.4 rRNA Depletion and Library Preparation

1. Ribo-Zero® rRNA Removal Kit (Bacteria).
2. Magnetic Core Kit.
3. ScriptSeq™ v2 RNA-Seq Library Preparation Kit.
4. ScriptSeq Index PCR Primers.
5. AgenCourt RNAClean XP Kit.
6. Magnetic rack or stand for 1.5 mL tubes.
7. 0.2 or 0.5 mL RNase-free tubes.
8. Water bath, heating block, or other temperature control device for 1.5-mL tubes.
9. Ice-cold 100%, 80% 70% ethanol, for precipitation and washing.
10. RNA6000 Pico Kit.
11. High Sensitivity DNA Kit.

2.5 Analysis of RNA-Sequencing Data

1. Computer and CLC Genomics Workbench software.

2.6 Fluorescently Tagging of the Strains to Be Studied (See Note 2)

1. Recipient strain to be tagged (e.g., *Pseudomonas aeruginosa*).
2. *E. coli* with transposase-harboring plasmid (e.g., pUX-BF13).
3. *E. coli* with helper plasmid for mobilization (e.g., pRK600).
4. *E. coli* with Tn7 delivery plasmid (e.g., pBKminiTn7-Gm *gfp2* or pHA51).

5. Pseudomonas Isolation Agar (PIA) for selecting *Pseudomonas aeruginosa* conjugants: peptone 20 g, magnesium chloride 1.4 g, potassium sulfate 10 g, Irgasan 25 mg, agar 13.6 g/L. For PIA plates, add 45 g of PIA powder in 1 L of distilled water and autoclave for 15 min at 121 °C. As carbon source, add 30 mL of glycerol 80% (V/V) per liter and the appropriate antibiotic/s.
6. Antibiotic stocks: 50 mg/mL Gentamicin, 100 mg/mL Ampicillin and 6 mg/mL Chloramphenicol stocks solubilized in 10 mL milli-Q water (or 70% ethanol for chloramphenicol) and filtered through a syringe filter. Preferably, prepare small aliquots to avoid frequent thawing-freezing cycles.
7. 0.2 μm GTTP polycarbonate membrane filters.
8. Primers to check Tn7 integration: Tn7-GlmS 5′AATCTGGCC AAGTCGGTGAC and Tn7R109 5′ CAGCATAACTG-GACTG
ATTTCAG.

2.7 Assembling and Sterilizing the Flow-Cell System (See Note 3)

1. Flow cells.
2. Bubble traps.
3. 5-mL syringe, with 12.5 mm diameter and caps.
4. Marprene tubing, 3 mm outer diameter, 1 mm inner diameter (*see* **Note 3**).
5. Silicone tubing, 3 mm outer diameter, 1 mm inner diameter.
6. Silicone tubing, 4 mm outer diameter, 2 mm inner diameter.
7. Silicone tubing, 7 mm outer diameter, 5 mm inner diameter.
8. Multichannel peristaltic pump.
9. Medium bottles.
10. Waste container.
11. Clear polypropylene plastic connectors and T-connectors, 1/8 in. (3.175 mm) and 1/16 in. (1.588 mm). Reduction connectors 1/8–1/16 in.
12. Scalpel.
13. Silicone glue (Super Silicone Sealant Clear, *see* **Note 3**).
14. 0.5% (w/v) sodium hypochlorite solution.
15. 70% and 96% (v/v) ethanol.
16. 50 × 24-mm glass coverslips or other appropriate material for substratum.
17. Rolling cart for flow systems and pumps (optional).
18. Sterile distilled water.

2.8 Inoculation of the Flow Cells System

1. Inoculum, e.g., fresh overnight culture of the microorganisms under study.
2. Fe-EDTA-AB (FAB) medium: 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM Fe-EDTA, 0.15 mM $(NH_4)SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, and 0.5 mM NaCl [10]).
3. Syringes with needles (27 G × 1.2″ (0.4 × 12 mm), 0.5 mL).
4. Clamps.

2.9 Equipment for Confocal Laser Scanning Microscopy of Flow-Cell-Grown Biofilms

1. CLSM microscope.
2. Computer software:

   Imaris (Bitplane; http://www.bitplane.com).

   COMSTAT version 2 (DTU-Systems Biology, Technical University of Denmark, http://www.comstat.dk).

   Java runtime environment (needed for Comstat v. 2, http://www.java.com).

3 Methods

3.1 RNA-seq of Coculture Spots

3.1.1 Coculturing in Agar Plates

1. Plate out the two strains (*S. aureus* and *P. aeruginosa*) on LB agar plates from −80 °C glycerol stocks and incubate them for 2 days at 37 °C.
2. Set starting cultures of both strains in triplicate in 20 mL LB in 100-mL flasks from independent colonies and incubate at 37 °C and 200 rpm for 20 h.
3. Crop 0.45-μm filters with 1 × 2.5 cm dimensions and set them for autoclaving for the following day.
4. Measure optical density of the six cultures with a spectrophotometer by diluting the overnight cultures 1:10 in LB–0.9% NaCl.
5. Calculate the volume needed for OD = 1 in 1 mL final volume and transfer those culture volumes to 1.5-mL centrifuge tubes. Spin for 2 min at 12470 × *g* at room temperature (RT) in 1.5-mL centrifuge tubes.
6. Discard supernatant and suspend the pellet in 1 mL 0.9% NaCl using vortex mixer. Spin again for 2 min at 13,000 rpm and RT.
7. Discard supernatant and resuspend the cell pellet in 1 mL 0.9% NaCl. These are the OD-1 cultures to be used for the coculture spotting.
8. Dry one LB plate per biological replicate for 20 min in a 37 °C oven (*see* **Note 4**).
9. Once the plates are dry, use sterile tweezers to place five sterile, cropped filters sufficiently separated from each other on the agar surface (*see* Fig. 1). Use one plate for each condition and biological replicate (*see* **Note 5**).

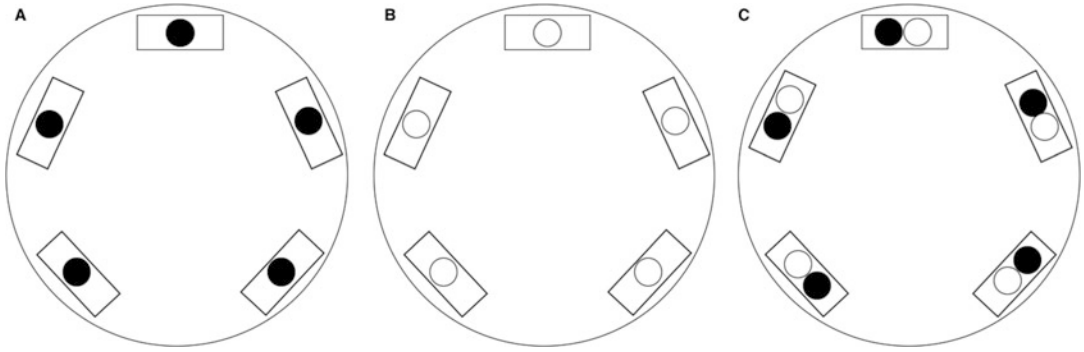

Fig. 1 Cospot inoculation on agar plates. LB-agar plates were dry and five individual 1 × 2.5-cm HA filters were placed on top. To get colonies for RNA isolation, 10-μL of OD 1 cultures from *P. aeruginosa* (**a**), *S. aureus* JE2 (**b**), or both (**c**) were spotted on top of the filters

10. Spot 10-μL spots of *S. aureus*, DK2 (mono-culture) or both (co-culture) on top of the filters. Let dry for at least 10 min at room temperature (*see* Fig. 1).
11. Incubate the plates upside down for 24 h.

3.1.2 Colony Harvesting

1. Label 2-mL tubes with the different strains/conditions and biological replicates (i.e., DK2 monoculture BR#1). Cool them down at −80 °C for 30 min.
2. Take one plate at a time and start removing the filters containing the outgrown colonies. For that, use sterile tweezers and place the filters individually in 2-mL tubes. Freeze immediately at −80 °C.
3. For the coculture spots, take a brand new sterile scalpel and cut the filter into two at the interaction area. Then, place them separately onto 2-mL tubes and freeze immediately at −80 °C (*see* **Note 6**).

3.1.3 RNA Isolation from Harvested Colonies

Multiple choices for bacterial RNA isolation are commercially available. There are in-column based isolation kits but also reagents based on RNA protection followed by precipitation. The method which works better for our samples comprises a first step of cell mechanical disruption followed by purification of the lysate using the RNeasy® Mini Kit. We used a supplementary protocol from the manufacturer to use this kit with bacteria by adding some modifications (*see* **Note 7**).

1. Take out from −80 °C one sample per condition and strain and place them on ice. Add 500 μL RLT buffer (RNeasy® Mini kit) containing β-mercaptoethanol (10 μL β-ME/mL RLT) per tube and resuspend the biomass in the filter with vortex until fully suspended.

2. Transfer the suspended bacteria to homogenizer tubes with screw-on cap and 300 mg of <106 μm acid-washed glass beads.
3. Disrupt the cells by using the homogenizer instrument with the following settings: 2 cycles of 40 s at maximum power followed by 2-min stop on ice.
4. Spin the homogenizer tubes containing the disrupted cells for 10 min at 13,000 rpm and 4 °C in a microcentrifuge to pellet the cell debris and beads.
5. Transfer the supernatant (around 350 μL) to a new 1–5-mL tube and add 250 μL 100% EtOH. Mix 10 times by inverting the tube.
6. Load lysate onto RNeasy column and follow the manufacturer's instructions, performing also the optional step of in-column DNase treatment (*see* **Note 6**).
7. Elute RNA with 50 μL RNase-free after and freeze samples at −80 °C.
8. Quantify RNA to check RNA concentration and absorbance ratios.
9. Apply a DNase treatment to the RNA samples for removal of any residual DNA contamination. For that, use the Turbo DNA-free Kit (Ambion): follow the manufacturer's recommendations to prevent RNA degradation.

   Incubate for 30 min at 37 °C (*see* **Note 8**).
10. Add 10 μL DNase inactivation reagent from the kit to the reactions and incubate for 2 min at RT. Then spin for 2 min at 13,000 rpm to pellet DNase and residues.
11. Transfer the supernatant very carefully (around 100 μL) to a new 1.5 mL tube.
12. Make a 3-μL aliquot for the Subheading 3.1.4 and freeze the rest at −80 °C.

3.1.4 Assessment of DNA Contamination

When the RNA is going to be used for sensitive downstream applications, such as RNA-Seq or qRT-PCR, it is very important to make sure that there is not residual DNA in the RNA samples. For that purpose, we apply conventional PCR using the RNA samples as a template, but with DNA-binding primers. These primers target genes highly represented in the cell, such as those encoding ribosomal proteins or ribosomal RNA. In this case, we use primers hybridizing in the gene coding for the ribosomal protein L28 (*P. aeruginosa*) or for 16S rRNA (*S. aureus*).

1. Prepare a PCR mix as follows: 25 μL 10× Taq buffer, 25 μL 16S F/L28 F, 25 μL 16S R/L28 R, 5 μL, 10 mM dNTPs, 2 μL Taq 5 U/μL, 130 μL RNase-free water, 220 μL total volume

2. Aliquot 22 μL of the different mixtures in PCR tubes and add 3 μL of RNA template (500 ng). As a positive control use 50 ng genomic DNA of *Pseudomonas aeruginosa* DK2 or *S. aureus* and as negative controls just RNase-free water.
3. Use the following PCR program to run the reactions: 98 °C × 2 min; 25 cycles (98 °C × 30 s, 60 °C × 30 s, 72 °C × 30 s); 72 °C × 5 min.
4. Run the PCR products in a 2% agarose gel using a 50-bp DNA ladder, bands of 50/75 bp are expected if DNA contamination is present (*see* **Note 8**).

3.1.5 Assessment of RNA Quality and rRNA Depletion

The RNA quality of the samples is crucial for the results obtained afterward from RNA-seq. In order to increase the coverage of mRNA during sequencing, it is important to remove as much rRNA as possible, so that the mRNA portion is enriched. Assessment of RNA quality can be performed with chip-based electrophoresis using the RNA 6000 Nano Kit. The assay determines the quality of the RNA by quantifying the 23S and 16S rRNA and assigning a value from 1 to 10 or RIN (RNA Integrity Number). If the samples have RIN > 9, continue immediately with the rRNA depletion step detailed below. There are different kits commercially available for rRNA removal with different performance depending on the target organism or input RNA. Thus, it is recommendable to evaluate in advance which one is more suitable for the species of interest (*see* **Note 9**).

1. Follow the instructions in the kit manual for preparing the magnetic beads and keep them at room temperature until ready to use with the RNA solution (*see* **Note 10**).
2. For each sample, use 0.5 μg total RNA and add the following reagents in a 0.2 or 0.5 mL RNase-free microcentrifuge tube. Combine in the order given: *x* μL RNase-Free Water, 4 μL Ribo-Zero rRNA Reaction Buffer, *y* μL RNA sample, 8 μL Ribo-Zero Removal Solution, 40 μL total volume.
3. Fully mix by pipetting 10–15 times. Incubate at 68 °C for 10 min.
4. Remove the tubes from heat and centrifuge briefly to collect any condensation.
5. Incubate the tubes at room temperature for 5 min.
6. For each sample add the 40 μL probe-hybridized RNA sample to the 1.5 mL tube containing 65 μL washed magnetic beads, prepared in **step 1**. This order of addition is critical and can impact rRNA removal efficiency if done incorrectly.
7. Without changing the pipette tip, immediately mix the contents of the tube by pipetting 10–15 times. Set the tubes aside at room temperature.

8. Vortex for 10 s and incubate at room temperature for 5 min.
9. Incubate the tubes at 50 °C for 5 min.
10. Remove the tubes from heat and immediately place them on a magnetic stand for at least 1 min until the solution appears clear.
11. Carefully remove the supernatant (85–90 μL) containing the rRNA-depleted sample, and transfer to an appropriately sized RNase-free microcentrifuge tube.
12. Place the supernatant (rRNA-depleted sample) on ice and proceed to **step 14** or, alternatively, keep the supernatant at −20 °C overnight or at −65 °C to −80 °C for long-term storage.
13. For purifying the rRNA-depleted samples, use the AMPure RNAClean XP Kit, following manufacturer's instructions for "Single Tube Format."
14. Use 11 μL of RNase-free water to elute the depleted RNA.
15. Place on ice the purified rRNA-depleted RNA to continue with Subheading 3.1.6, or keep at −20 °C overnight or −65 °C to −80 °C for long-term storage.

3.1.6 Library Preparation

It is recommendable but not necessary to assess the quality of depleted RNA before proceeding to library preparation. Typically, <8% of the amount of input RNA is recovered (e.g., 1 μg total RNA input will yield <80 ng of RNA depleted of rRNA). It is recommended to use a chip-based capillary electrophoresis to quantify the yield of mRNA after rRNA removal with RNA6000 PicoChip and load 1 μL.

For the library preparation the ScriptSeqTM v2 RNA-Seq Library Preparation Kit was used. Library preparation comprises RNA fragmentation, synthesis of cDNA, terminal tagging of cDNA, amplification and checking library quality.

1. RNA fragmentation. In a 0.2-mL PCR tube, assemble the following reaction mixture: 9 μL treated RNA, 1 μL RNA Fragmentation Solution, 2 μL cDNA Synthesis Primer, total 12 μL. Incubate for 5 min at 85 °C in a thermocycler and then place on ice to stop the fragmentation reaction.
2. On ice, prepare the cDNA Synthesis Master Mix: For each reaction, combine: 3.0 μL cDNA Synthesis PreMix, 0.5 μL 100 mM DTT, 0.5 μL StarScript Reverse Transcriptase. Add the 4 μL of cDNA Synthesis Master Mix to each reaction from **step 1** on ice. Mix by pipetting and incubate at 25 °C for 5 min followed by 42 °C for 20 min.

3. Cool reaction to 37 °C and pause the thermocycler. Then add 1 μL of Finishing Solution to each reaction and mix by pipetting. Incubate at 37 °C for 10 min.
4. Incubate each reaction at 95 °C for 3 min. Then, cool the reactions to 25 °C and pause the thermocycler.

   During the 95 °C incubation, prepare the Terminal Tagging Master Mix as follows: 7.5 μL Terminal Tagging Premix, 0.5 μL DNA Polymerase, 8.0 μL total volume. Mix thoroughly by pipetting.
5. Add 8.0 μL of Terminal Tagging Master Mix to each reaction. Mix by pipetting and incubate reaction at 25 °C for 15 min.
6. Incubate each reaction at 95 °C for 3 min. Then, cool the reactions to 4 °C on ice or in the thermocycler.
7. Purify the cDNA with a method of convenience (*see* **Note 11**). Transfer the 22.5 μL of the ditagged cDNA from each tube to a new 0.2-mL PCR tube, place it on ice and proceed to library amplification or at −20 °C for longer-term storage.

3.1.7 Library Amplification and Sequencing

1. Prepare PCR mixtures for library amplification following the manufacturer's instructions and using a different barcoding primer for each sample library (*see* **Note 12**).
2. Place the tubes in a thermocycler and run the following program: 95 °C for 1 min; 15 cycles (95 °C for 30 s, 55 °C for 30 s, 68 °C for 3 min); final extension of 68 °C for 7 min.
3. Purify the amplified libraries similarly to **step 7** in Subheading 3.1.6 but eluting the purified amplified libraries in 20 μL RNase-free water.
4. The quality and quantity of the DNA libraries can be subsequently assessed on a chip-based electrophoresis using the High Sensitivity DNA kit and a fluorometric assay, respectively.
5. The libraries are multiplexed into one pool with 4 nM of each library (in equimolar concentrations) and loaded in one lane of a flow cells for cluster formation.
6. Finally, the libraries are sequenced (100 bp paired-end reads) on an Illumina HiSeq platform according to the manufacturer's recommendations.

3.2 Analyis of RNA-Seq Data Using the CLC Genomics Workbench (See Note 13)

Detailed protocols and instructional videos on performing RNA-seq analysis using CLC Genomics Workbench 7.5.1 can be obtained from the CLC Web site (http://www.clcbio.com/products/clc-genomics-workbench). However, we have chosen the RNA-Seq Legacy Tool, which unifies the read mapping to the chosen reference genome and calculation of expression values.

3.2.1 Quality Check of the Reads

1. Import de-multiplexed fastq files into CLC Genomics Workbench using the function "Import—Illumina" function. If importing Paired reads, tick the Paired Reads box and include both _1.fastq and _2.fastq files. Select paired-end distance 100–350 and Quality scores "Illumina Pipeline 1.8 and later."
2. Perform a quality check of the imported read files: NGS Core Tools→Create Sequencing QC Report. Use the "batch" option and choose the folder containing all samples. Select destination folder and finish.
3. Check the following parameters:

   % GC should be as expected based on the bacterial species.

   Quality distribution: PHRED-score ~65.

   Nucleotide contribution is similar throughout the read.

   Check presence of enriched 5mers, such as adaptor sequences in the read.

3.2.2 Read Mapping and Expression Value Calculation

1. Download the reference genomes of the most closely related strain/species as a GenBank file and import into CLC Genomics Workbench using the "Standard Import" function.
2. Remove the rRNA and tRNA genes from both genomes.
3. In the "Tools" menu, select "Legacy Tools" and then "RNA-Seq Legacy." A dialog menu will open and under the tab "Select sequencing reads," enable the batch function. More than one reads file can be uploaded at once. Select the folder containing the reads files from the different biological replicates for each sample/condition (*see* **Note 13**).
4. In the next tab, "Set reference" click the option to use reference with annotations and select the tRNA and rRNA curated genomes as reference genomes, which will be used to map the reads with the Legacy Tool.
5. Use the following settings in the tab "Read mapping settings": use annotations for gene and transcript identification = Yes; additional upstream bases = 0; additional downstream bases = 0; maximum number of mismatches allowed (applies to short reads) = 2; unspecific match limit = 10; use colorspace encoding = No; strand = Forward; minimum paired distance = 100; maximum paired distance = 350;
6. In organism type, select the option "PROKARYOTE."
7. In the tab "results handling" select the following settings: expression value = RPKM. Also, enable the option to save into separate folders.

3.2.3 Pairwise Analysis of Monoculture and Coculture Samples

In the Transcriptomic Analysis tools, select the function "Set up Experiment," which allows us to perform comparative analysis when there are groups of samples, as it is the case in this example.

A dialog menu will open and then we select the following settings:

1. Select the RNA-Seq Legacy newly generated files where the RPKM values have been calculated. In this case, we select all samples belonging to one strain (i.e., *S. aureus* or PADK2).
2. In the next tab, "Define experiment type," select the following settings: two group comparison and unpaired.
3. Assign names to the two groups generated: i.e., monoculture and coculture.
4. Assign groups to the selected samples and click finish.
5. Add an annotation table to the data with at least the following information:

   Feature ID: locus number/gene name

   Gene symbol: locus number

   Gene description, with information about the putative function of the gene.
6. Select the "Gene symbol" column to merge annotations with data, and enable the option "Removed leading zeros before matching."

3.2.4 Statistical Analysis of the Pairwise Comparison Experiment

1. Select "Transcriptomics Analysis→Statistical Analysis→On Proportion
2. Select the experiment to be analyzed.
3. Select the Baggerley's test to compare proportions, the reference group to be used, original expression values and calculate FDR corrected *p*-value.
4. Create a subexperiment by using the following filtering parameters:

   Baggerley's test-Weighted proportions fold change: abs value >2

   Baggerley's test-FDR *p*-value <0.05
5. Save it with a different file name and export it as an Excel file.

3.3 Flow-Cell Biofilms to Study Microbial Interactions

Caldwell and colleagues developed a flow cell constructed of Plexiglas and mounted with a microscope slide similar to the flow cell described in this section [11]. The use of these flow cells, combined with confocal laser scanning microscopy (CLSM), allows examination of biofilms in a near-natural state as well as real-time monitoring of the 3D biofilm structure as it develops. However, CLSM requires fluorescence emission from the bacteria subjected to examination.

Strains can be stained with fluorescent dyes right before visualization, or can be engineered to express a fluorescent protein (e.g., Gfp) from either a plasmid or from a chromosomal insertion. Even though prestaining of the strains can eliminate the need of genetic manipulation, it would require injection of the dye prior to microscopy, with may potentially cause biofilm disruption if not performed carefully. Expression of a fluorescent protein from a high-copy number plasmid would in turn require the addition of antibiotic to the culture media to prevent plasmid loss. In addition, antibiotic selection to plasmid loss may in many cases not work when cells are growing as biofilms due to the increased inherent tolerance against antibiotics. Fluorescent protein encoding genes inserted into the host chromosome can circumvent these limitations. In this section, we provide a method to fluorescently tag the strains by chromosomal insertions of Tn*7* transposons containing fluorescent protein encoding genes.

Additionally, the protocol comprises assembly and sterilization of the flow cell system, inoculation of the flow cells, running of the system, confocal laser scanning microscopy, disassembly, and cleaning of the system. Image analysis software such as COMSTAT can be used for calculation of a variety of parameters from three-dimensional CLSM images.

3.3.1 Fluorescently Tagging of the Strains Using the Tn7 System

This system is used to insert the sequence cloned between the transposon ends, Tn7L and Tn7R, as single copy into the chromosome. The insertion site is well-defined and located downstream of the coding region of the gene *glmS* (in most of the studied bacteria). Furthermore, the insertion only occurs in one orientation and it is stably maintained during growth.

The mini-Tn*7* tagging system used here is based on the constructs developed by Koch *and* colleagues [12, 13]. We will perform a differential tagging of two *P. aeruginosa* strains by using delivery plasmids with Gfp (pBKminiTn*7*-Gm *gfp2*) or mCherry (pAH51), both providing resistance to gentamicin and ampicillin. To mobilize the Tn*7* fluorescent cassette we chose a four-parental conjugation strategy.

1. Inoculate the four bacteria (a, b, c, and d) separately, each in 10 mL LB medium with the proper antibiotics and incubate overnight at a suitable temperature (*see* **Note 2**).
   (a) recipient bacterium.
   (b) bacterium with helper plasmid (pUX-BF13, encoding the Tn*7* transposases, grow with 100 μg/μL ampicillin).
   (c) bacterium with delivery plasmid (harboring the miniTn*7* transposon, modified with the desired insert, i.e., gene encoding a fluorescent protein).

(d) bacterium with the plasmid that can mobilize the other plasmids (e.g., pRK600, grow with 6 μg/mL chloramphenicol).

2. Wash 1 mL of each culture twice with fresh LB to eliminate any antibiotic residues.
3. Mix samples of the four cultures (for example 50 μL of each) and place them as one large spot on a nonselective nutrient agar plate, e.g., an LB-plate or filter the bacteria onto a 0.2 μm polycarbonate filter (this increases the plasmid transfer frequency and thereby also the tagging frequency) and place the filter on an LB-plate and incubate at 37 °C overnight or at another temperature, depending on which strain is tagged.
4. Transfer cells from the LB plate or the filter and suspend in 1 mL LB medium/saline buffer.
5. Dilute the cell suspension and spread on PIA selective plates with the 40 μg/mL gentamicin (*see* **Note 2**). Incubate overnight at 37 °C, depending on the strain. Remember also to plate a negative control on a selective plate containing only the recipient strain.
6. Restreak single colonies on fresh selective plates and incubate overnight at 37 °C.
7. Prepare colony lysates of candidate clones to test insertion by PCR. For that, take a small fresh single colony from the selective plate suspend in 50 μL sterile water. Boil 5 min and spin to pellet the cell debris. Transfer 40 μL of supernatant to a new tube.
8. Use that lysed supernatant as a template in a PCR mix: 2.5 μL 10× Taq buffer, 0.5 μL 10 mM dNTPs, 2.5 μL 10 μM Tn7-GlmS, 2.5 μL 10 μM Tn7-Tn7R, 5 μL Lysate, 0.2 μL Taq polymerase 5 U/μL, 11.8 μL Milli-Q water, 25 μL total volume.
9. Use the following PCR program to run the reactions: 98 °C × 2 min; 25 cycles (98 °C × 30 s, 60 °C × 30 s, 72 °C × 30 s); 72 °C × 5 min.
10. Run the PCR products in a 2% agarose gels together with a 50-bp DNA ladder. Bands of approximately 150 bp are expected if Tn7 has been integrated into the chromosome, *see* example in Fig. 2 (*see* **Note 14**).

3.3.2 Assembly of the Flow-Cell System

The flow-cell system consists of the elements shown in Fig. 3. The elements of the flow-cell system are the following: a medium bottle, lead-in silicone tubing, Marprene® pump tubing, a peristaltic pump, lead-out silicone tubing, a three-channel bubble trap, connecting silicone tubing, a three-channel flow cell, thin effluent connecting silicone tubing, wide waste silicone tubing (silicone,

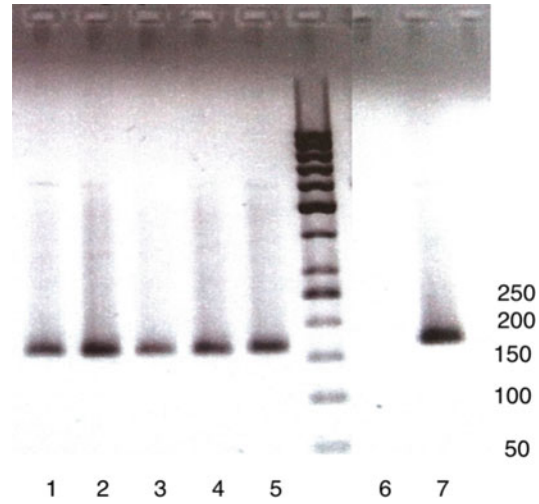

Fig. 2 Checking integration of Tn7 fluorescent cassette into the chromosome. PCR products were amplified from different colony lysates (lanes 1–5) using the primers Tn7-GlmS and Tn7-R109. We also included a negative control with a recipient strain without the Tn7 integration (lane 6) and a positive control with a strain already tagged with Gfp using the Tn7 system (lane 7). The expected band size for Tn7-integrants was approximately 150 bp

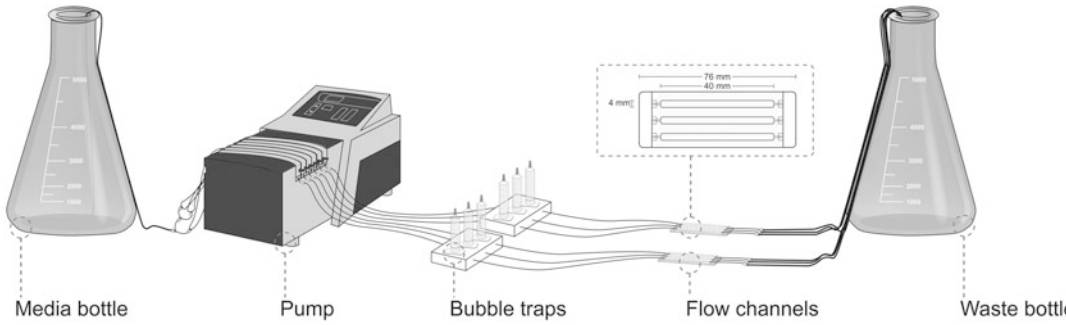

Fig. 3 Diagram of the flow-cell system setup [19]. Reproduced with permission of Springer

2 mm inner diameter), and finally a waste collection container (*see* **Note 15**).

All elements of the flow-cell system (*see* Fig. 3), except the pump and flow-cells can be autoclaved and it is recommended to do so prior to assembly.

The flow cells and bubble traps can be purchased from several sources, for instance, from DTU Bioengineering, at the Technical University of Denmark (*see* **Note 3**).

The assembly process can be divided into a couple steps: preparation of medium flask with appropriate medium, preparation of bubble traps and flow cells, and mounting of silicone tubing and connectors.

Prior to the assembly, you need to determine how many channels are needed. A 16-channel peristaltic pump can accommodate up to 16 channels, which will allow for five 3-channel flow chambers. This will help to estimate the size of the medium bottle, the medium volume to prepare and the number of flow cells you need to assemble (*see* **Note 16**).

1. To prepare media flasks, fit the bottle to contain the medium with an approximately 1-m-long silicone tube, 2 mm inner diameter. Attach a straight connector to the end of the tubing and cover it with metal foil. The connector should allow connection of the 2-mm inner diameter tubing for the medium side and 1 mm inner diameter for the pump side (i.e., a 1/8–1/16 straight reduction connector). Place the other end of the tubing inside the medium bottle and fix the tubing with autoclave tape to the neck of the flask. It is important to clamp the tubing off before autoclaving to prevent a siphoning effect. Fill the suitable medium into the container, cover the opening with aluminum foil, and autoclave. Some media must be autoclaved before addition of certain salts or carbon source to prevent precipitation during the autoclaving [10].
2. For preparing the flow cells, note they should be mounted prior the assembly of the whole system, at least the day before to allow proper sealing. Cut off the wide end of a 200-μL pipette tip and place it onto a 2-mL syringe where the piston has been removed. Use the piston to press the tip into the outlet port of the syringe. Remove the piston again and fill about 1 mL silicone glue into the syringe from the open end and push the piston to pump the silicone to the tip. Make thin threads of silicone between the flow channels and at each end of the flow cell. Make sure that the silicone makes continuous threads; gaps or bubbles might lead to leakage or cross-contaminations across the channels (*see* **Note 15**). Carefully place a 50 × 24 mm coverslip (substratum) on top of the silicone threads and gently press using a cover slide to apply even pressure to the substratum. Remove small gaps by cautiously pressing with the tip of a piston or 1-mL pipette tip, paying attention to high fragility of the coverslip glass. Let the silicone glue cure overnight at room temperature until the silicone has completely solidified and sealed the substratum to the base. Alternatively, incubate them for 3 h at 60 °C.
3. It is not essential but recommended to rule out that the inlets/outlets of the flow cells have not been blocked with silicone. For that, insert an insulin syringe with needle (inoculation), in each hole of the flow cell, until reaching the channels.
4. For assembly of the bubble traps, use a 3-channel bubble trap base and three 5-mL syringes (12.5 mm inner diameter) without piston per flow chamber. This bubble traps are supplied

with rubber rings which seals the syringes to the base to avoid leaking. Press each syringe onto the vertical columns of the base and put a stopper at the tip of the syringes. For that, we used the caps of the inoculation needles, but screw-on caps/stoppers and syringes can alternatively be used. Subsequently, the silicone tubing and connectors can be mounted.

5. Prepare the medium-feeding tubes with a fan-out connector. Start with 50 cm silicone tubing 1 mm inner diameter and attach a 1/16 in T-connector and cover the other end with aluminum foil. The T-connector can attach three pieces of tubing. Attach short pieces of silicone tubing (e.g., 10 cm) to each free end of the T-connector and if needed attach another T-connector, until there is the same number of free tube ends as channels in the system.
6. Cut one piece of Marprene® tubing per channel, with a 25-cm length depending on the dimensions of the pump. Place a 1/16 straight connector in each end of the tubing and use autoclave tape to fix the end of the tubing that will be on the inlet side. This will prevent the pump from pulling the tubing off the connector.
7. For each channel make the following pieces of silicone tubing (1 mm inner diameter):

   One piece 35 cm, 1 mm inner diameter.

   One piece 150 cm, 1 mm inner diameter.

   One piece 10 cm, 1 mm inner diameter.

   One piece 120 cm, 2 mm inner diameter.
8. Connect the ends of the fan-out tubing to the pump tubing ends with the autoclave tape. Connect the other end of the pump tubing to the 35-cm pieces. Attach the bubble traps to the other end of the 35 cm pieces, with the highest part of the central columns toward the pump. Attach the 150 cm pieces to the outlets of the bubble traps and connect the other ends to the inlets of the flow cells. On the outlets of the flow cells attach the short 1 mm inner diameter tubing and put a 1/16–1/8 reduction straight connector to the end. Finally, on the other side of the straight connector attach the 120 cm × 2 mm inner diameter waste tubing.
9. Mount the flow system onto the pump: Place the system on a rolling cart or near the microscope. Make sure that you connect one line at a time in order, so it is possible to follow the line from, e.g., position #1 on the pump to the first bubble trap position 1 and on to the first channel in the first flow cell. Likewise follow the position all the way for position 2 and so on. It is also recommended to label the channel in the pump and/or the flow cells. It will make leakage identification easier

(*see* **Note 15**). Place all waste lines into an appropriate collecting container, such as a 5-L flask and secure them to prevent them from slipping out prematurely.

3.3.3 Sterilization of the Flow-Cell System

At this point the system can be considered nonsterile, but it is highly recommended to use sterile distilled water in all steps.

1. Place all components at the same height level, e.g., on a table. Specifically, do not place the medium or waste containers on a higher or lower level than the flow cells.
2. Remove the foil from the fan-out connector and put it in a 1-L bottle of distilled water, remove the stoppers of the bubble traps and store them in a petri dish with 70% ethanol.
3. Start the pump at maximum speed (90 rpm) and fill up the system. The bubble traps will fill slowly and, when water is flowing over the tip, place back the stoppers. Then the rest of the system will fill with water. Let water run through the system until the flow cells are filled and water is flowing from the waste tubing into the waste container.

   Pay special attention to water leaks at this moment. If any leakage is observed, it must be sealed before proceeding to sterilization (*see* **Note 15**).
4. Empty the system by lifting the inlet tube out of the water bottle and allow air to be pumped into the system.
5. Fill up the system again as in **steps 2** and **3** with a solution of 0.5% sodium hypochlorite in sterile distilled water. When the bubble traps are filled, place back the stoppers.
6. If any air bubbles are observed in the flow channels, remove them by tapping the flow cells vertically on a hard surface while the pump is running at maximum speed (*see* **Note 17**). Then reduce pump speed to minimum (0.5 rpm) and leave the system to sterilize for at least 2 h, but not more than 6 h.

   From this point on the system is sterile and care should be taken when handling tubing and stoppers.
7. The hypochlorite is removed from the system by three consecutive fillings with air followed by complete filling with sterile water. Remove any possible air bubbles from the flow cells after each filling and conclude by passing 1 L of sterile water through the system at lower flow rate (0.5 rpm) (*see* **Note 18**).
8. Prior to the experiment, purge the system with air as in **step 4**. Attach the media bottle and fill the system as in **steps 2** and **3**, taking care not to contaminate the tube ends that are to be connected (*see* **Note 19**).
9. Equilibrate the system at the appropriate temperature overnight to avoid bubble formation after inoculation with low flow rate of the medium (0.5 rpm) (*see* **Note 16**).

3.3.4 Inoculation of the Flow Cells

The flow-cell system is now ready for inoculation and we will use it to study the impact of strain cocultivation on biofilm structure. We will use pure cultures (monoculture condition) as a control for biofilm architecture and compare against mixed cultures with two bacterial species/clone types (coculture condition).

1. Grow bacteria to be inoculated overnight and dilute appropriately. In our case, for *Pseudomonas aeruginosa* prepare 1:1000 dilutions of the overnight cultures in 2 mL saline buffer (0.9% NaCl) using 1:10 serial dilutions. To prepare the coculture inoculum, mix 200 μL of a 1:100 dilution of each strain and 1.8 mL saline buffer, so that each strain is diluted 1:1000. The dilution depends on the bacterial strains used and must be determined empirically (*see* **Note 20**).
2. Stop the pump, clamp off the tubing right before the flow cells to avoid contamination of the upstream system due to backflow from the flow cells.
3. Fill a 0.5-mL syringe with needle (e.g., insulin syringe) with 250 μL of the diluted inoculum. Make sure that there are not air bubbles in the syringe, otherwise they may be introduced into the flow cells.
4. Wipe the tubing before the inlet with 70% ethanol. Inject the inoculum very slowly by perforating the tube with the needle until reaching the inlet of the flow channel. Again, be careful not to inject air bubbles in the process.
5. Seal the injection hole by adding a thin layer of silicone.
6. Place the flow cells downward so the substratum is facing the table and leave in this position for 1 h with the flow stopped. This will allow the bacteria to make initial adhesion to the glass substratum.
7. Turn the flow cells over, with the substratum facing up, remove the clamps and resume flow at a low flow rate (1.75 rpm, *see* **Note 16**).

3.3.5 Running of the Flow-Cell System

The system can run for several days or weeks without interruption. After inoculation, the system does not require any maintenance but periodical leak check is highly recommended. We also have to ensure that enough medium is supplied at all times until the end of the experiment, especially if running the system over the weekend or periods without supervision. Medium shortage might lead to biofilm dispersal/destruction.

Ensure also that biological waste is not flowing over the waste container and it is disposed according to the correct regulations, e.g., by autoclaving the waste containers before disposal.

3.3.6 Confocal Laser Scanning Microscopy of the Flow-Cell Biofilms

The method of choice for microscopic inspection of flow-cell-grown biofilms is to use a confocal laser scanning microscope (CLSM). It is recommended to try first the setup explained below with an empty flow cell before mounting a real experiment.

1. Place the flow cell system close to the confocal microscope.
2. Carefully move one flow cell to the microscope object table while observing the medium and waste lines. If you followed the instructions for tubing length, the access to the microscope should not be a problem. Firmly attach the flow cell in the specimen holder. Since the flow cell still is connected to the system it is occasionally necessary to use adhesive tape to assist firmly mounting the sample on to the microscope.
3. Examine the sample. Choose the proper objective depending on the microscope used (e.g., 40× oil or 63× oil immersion objectives) and slowly get closer to the sample until it touches the coverslip. To focus the biofilm cells, slowly move the objective away from the flow chamber. This way is possible to focus while avoiding to break the coverslip, which would lead to medium leakage and bubble formation.
4. The procedure for confocal imaging will vary with the microscope to be used, but it will usually require to select an *x*–*y* plane and create a z-stack with the thickness of the biofilms. Then, scanning of the samples will need excitation with suitable lasers, which will vary depending on the fluorescent proteins used.

An example of a mixed biofilm consisting of two distinct *P. aeruginosa* clones can be observed in Fig. 4, where the *P. aeruginosa* clone-type DK1 (green), colocalizes with the DK2 clone-type (red) and grows on top of it when cocultured.

3.3.7 Disassembly and Cleaning of the Flow-Cell System

After completion of the experiments the system must be cleaned and the components prepared for reuse.

1. Empty remaining liquid from the still assembled system by filling with air.
2. Refill once with water, using the same protocol as for preparing the system and make sure that any visible colonization is removed by massaging the tubing while the pump is still running.
3. Finalize by emptying the system one last time and detach the flow cells. All other components can be left assembled for next use. Collect all waste and dispose of following regulations.
4. The flow chambers are disassembled using a scalpel to remove the substratum. To facilitate that, soak the flow cells in 96% ethanol at least for half an hour. If the substratum is made of fragile material such as glass, it will inevitably break in this process.

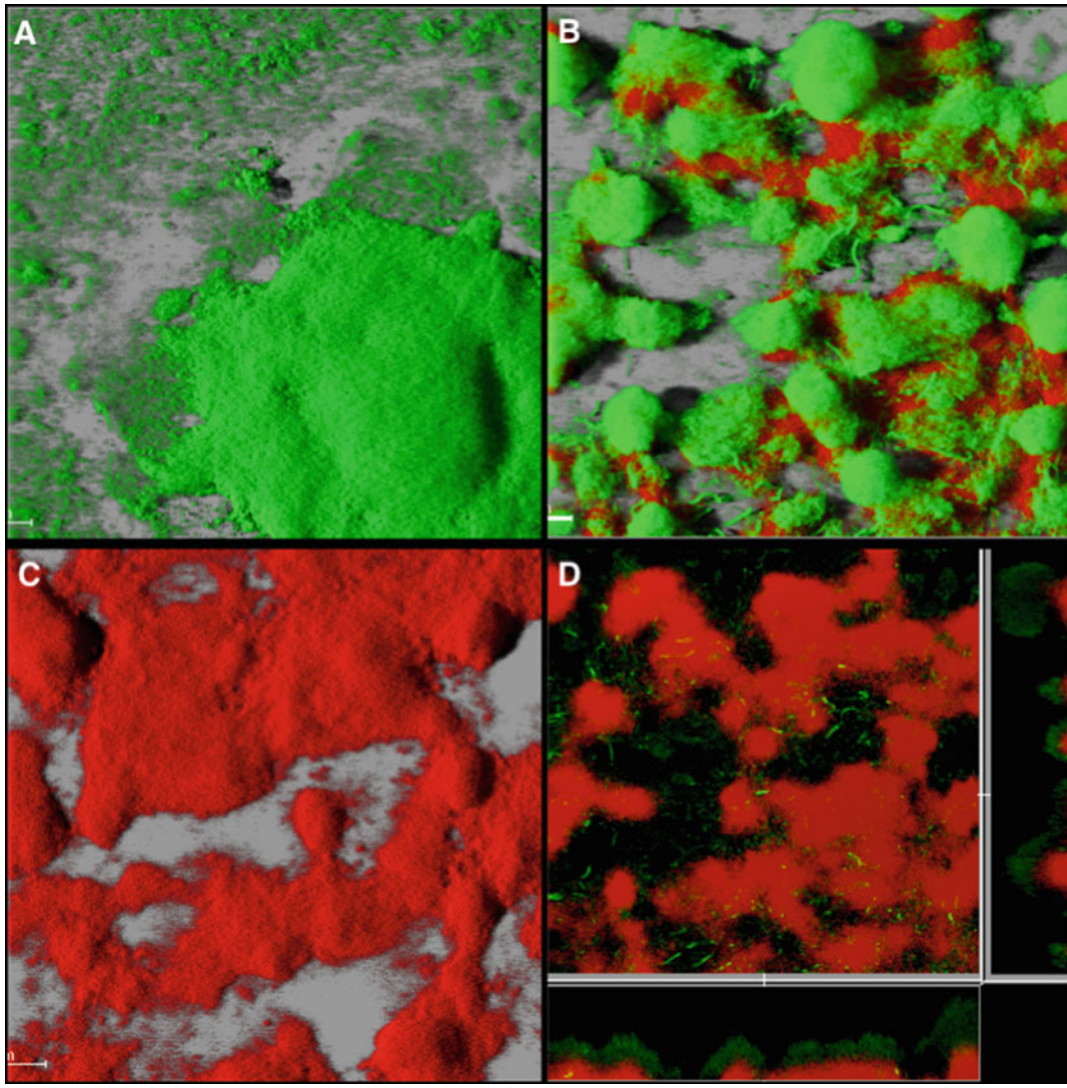

Fig. 4 Architecture of monospecies vs. mixed-species biofilms in *P. aeruginosa*. Two distinct *P. aeruginosa* clone types, namely DK1 (Gfp-tagged) and DK2 (mRfp-tagged), were grown either individually (**a**, **c**) or in a mixed culture (**b**, **d**) for 8 days in a flow-cell system with FAB medium. (**a**), (**b**), and (**c**) are 3D reconstructions of CLSM stacks while (**d**) is a cross section of it, showing a layer in the *Z*-axis. As it can be seen in panels (**b**) and (**d**), DK1 colocalizes with DK2 microcolonies and grows on top of them when in a mixed-species biofilm

5. Scrape off the glass using a scalpel. Small pieces of glass can jump off in this step and it is recommended to wear protection goggles and rubber gloves.
6. The rest of components of the flow-cell system can remain assembled. They can be wrapped in aluminum foil, autoclaved, stored, and reused for subsequent experiments.

3.3.8 Image Analysis

CLSM Images from biofilm experiments can be used, in principle, in two different ways: as qualitatively descriptive images or for quantitative measurements. If the biofilm structure of our conditions varies substantially from one condition to the other, image analysis may not be needed.

For qualitative analysis an appropriate software package is needed and there are several packages available. One popular commercial package is the Imaris® software suite (www.bitplane.ch) which can create three dimensional visualizations as well as animations. This program is also very useful if time series experiments have been recorded. Simple measurements can be performed in this suite, depending on which add-on modules have been purchased. ImageJ is a freeware alternative [https://imagej.nih.gov/ij/] which can be supplemented by user-written plug-ins to perform several graphical visualizations of CLSM images and extensive qualitative measurements. ImageJ does require more from the user to get full benefit of its capabilities than do commercial dedicated packages.

For quantitative analysis several programs have been developed, most prominently the ISA3D [14], Comstat 2 [15] (http://www.comstat.dk), and Daime [16] (http://rsb.info.nih.gov/ij). All these programs can extract basic parameters from CLSM image stacks, such as biomass, roughness, and average thickness. The output of quantitative analysis programs is values, rather than images and provides a way to directly evaluate both reproducibility of experiments and compare different biofilms which qualitatively seem similar but may have characteristic differences.

4 Notes

1. When working with RNA, special care must be taken to create a RNase-free environment, given that ribonucleases are ubiquitous, very stable, and difficult to inactivate. They can be frequently found on skin, dust particles or laboratory glassware for instance. Special care must be taken throughout the whole workflow: from purification to sample handling. Always wear gloves and use sterile conditions when possible. Use disposable RNase-free plasticware, sterile solutions, and, preferably, chemicals with RNA grade. Allocating a RNA working space with reserved equipment, pipettes and other material is also highly recommended. Use RNase decontamination solution, such as RNase-ZAP or 0.2 N NaOH to decontaminate work surfaces and equipment prior to RNA purification/manipulation.
2. Plasmid pUX-BF13 provides the transposase genes (*tnsABCDE*) needed for insertion of the mini-Tn*7* transposon [17]. This plasmid also encodes resistance to ampicillin, and it

contains the R6K origin. The plasmid can only be maintained in bacterial strains expressing the *pir*-protein (replication factor). The plasmid pUX-BF13 and the Tn7 delivery plasmids also carry the *mob* genes which allow their mobilization in the presence of a helper plasmid, such as the plasmid pRK600 [18]. Plasmid pRK600 enables the mobilization of both the Tn7 delivery and Tn helper plasmids into the recipient strain and harbors resistance to Chloramphenicol. The Tn7 delivery plasmids will vary based on the specific needs. For example, intrinsic antibiotic resistance of the recipient strain, number of strains to be tagged, etc. In this case we have used two Tn7 delivery plasmids encoding different fluorescent proteins, both harboring gentamicin and ampicillin resistance genes and the *mob* genes: pBKminiTn7-Gm *gfp2* [12] has the *gfp* gene fused to a *lac*-derivative promoter, providing constitutive expression of the Gfp protein, while pHA51 carries the *mcherry* gene fused to the *trc* promoter (kindly received from Tim Tolker Nielsen, University of Copenhagen). Both plasmids provide resistance to ampicillin and gentamicin.

3. DTU Bioengineering offer flow cells and bubble traps on a nonprofit basis, further information can be found in http://comstat.dk/reswiki/doku.php?id=biofilm_setup. To glue the glass substratum to the flow cells it is important not to use silicone glue intended for sanitation applications as this contains antimicrobial compounds, we recommend using the one listed in the materials section. For the tubing that runs through the pump it is important to use a particularly durable material, e.g., Marprene®. Otherwise, the tubing will wear out and break during the mechanical stress on the tubing.
4. The dry status of the plates is highly important to get proper culture spots. If the plates are not dry enough the drops will not acquire a perfect round shape and may probably also be bigger. On the contrary, plates dried in excess will favor the emergence of bubbles between the agar and the petri dish. Find the optimal drying time for your 37 °C oven.
5. For this protocol we are using *S. aureus* and *Pseudomonas aeruginosa* DK2 in monoculture and coculture. For the coculture spots we just need one plate per biological replicate, while for the monoculture spots we will use three LB plates for *S. aureus* and another three for PADK2 (one per biological replicate). Other variations can be included, such as using more biological or technical replicates for each condition.
6. Different RNA protecting reagents are available and can be added to bacterial cultures prior to pelleting samples or stop solution based on 95% ethanol and 5% phenol. While the purpose of these reagents is to preserve RNA integrity,

improved quality RNA samples can be obtained by simply working swiftly to freeze the bacterial pellet as quickly as possible.

7. The RNeasy® Mini Handbook provides protocols for purification of total RNA from animal cells, animal tissues, and yeast. Nonetheless, the manufacturer has additional protocols to use the kit with bacteria, such as the supplementary protocol "Purification of total RNA from bacteria using the RNeasy® Mini Kit." This protocol suggests the use of guanidine-thiocyanate-containing lysis buffer, but alone this is not sufficient for lysis of gram-positive bacteria. However, a combination of mechanical disruption with the RNeasy® Mini Kit resulted in intact and high quality RNA with our samples. According to the manufacturer, DNase digestion is not required since RNeasy technology efficiently eliminates most of the DNA without DNase treatment. Nonetheless, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA such as quantitative RT-PCR analysis but also RNA-sequencing assays. Commonly used DNase buffers are not compatible with on-column DNase digestion using this technology and may influence the binding of RNA to the RNeasy membrane, reducing RNA yield and integrity. However, the RNase-Free DNase Set provides efficient on-column digestion of DNA during RNA purification and it is compatible with the RNeasy technology and the DNase is efficiently removed in subsequent washing steps.
8. If DNA contamination is suspected to be higher, perform two subsequent DNase treatments, do not extend incubation time, this may lead to RNA degradation. If any positive bands are observed after PCR amplification using the purified RNA samples as templates, perform an additional DNase treatment and repeat the PCR reactions and electrophoresis until no trace of DNA is observed.
9. Different commercially available kits for bacterial rRNA-depletion have been developed. Many of them use capture oligonucleotides targeting specific regions of the 16S and 23S rRNAs, while others degrade processed 5′-phosphorylated RNA molecules such as rRNAs by utilizing a 5′-monophosphate-dependent exonuclease. An alternative kit enriches for non-rRNA in NGS libraries during cDNA synthesis. The diverse available kits can display different performance depending on the target organism or input total RNA. Thus, it is recommendable to evaluate in advance which one is more suitable for the species of interest and conditions.
10. The Ribo-Zero® rRNA Removal Kit manual indicates not to freeze the magnetic beads or place them on ice. This might

decrease kit performance. When not in use, store the magnetic beads at 2–8 °C. The manufacturer recommends at least 1 μg total RNA as input material, however, 0.5 μg worked better for our conditions and species. The total RNA sample volume allowed for 1–2.5 μg is 28 μL and 26 μL for 2.5–5 μg and 8–10 μL Removal Solution, respectively.

11. Different methods can be performed for DNA purification, such as DNA precipitation, in-column purification or magnetic-bead binding. Nonetheless, we utilized the latter one given that it provides superior DNA quality with no salt carryover and does not require centrifugation or filtration.
12. If preparing libraries for 12 different samples and performing sequencing in the same lane, each library needs to be prepared using a different index or barcode so that the different libraries can be pooled and sequenced together. Therefore, if having 12 samples as our case, the number of barcodes needed is 12.
13. In this study we had two strains, two different conditions and three biological replicates per condition, making a total of 12 samples. We used *Staphylococcus aureus* subsp. aureus USA300 FPR3757 genome (NC_007793) as a reference for the *S. aureus* samples and an in-house genome assembly of *Pseudomonas aeruginosa* PADK2 genome for DK2 samples. In order to run the RNA-seq Legacy tool, we loaded all biological replicate samples belonging to the same condition, i.e., *S. aureus* monoculture with the different biological replicates BR1, BR2, and BR3 and likewise for the other conditions.
14. Besides PCR check of Tn7 integration, inspection of the candidate clones in an epifluorescence microscope is recommended, using a suitable filter to excite the fluorescent tag used (e.g., blue light for Gfp). If no fluorescence is observed, the transposon integration should be double checked and/or start over with the tagging procedure.
15. The system is highly sensitive to leakage and ideally this should be detected while filling up the system with water. The common sources of leakage are: insufficient sealing by the silicone, loose connection between tubing and connectors/inlets, blockage of inlets/outlets of bubble traps or flow cells or breaks in the glass coverslip (substratum). If a leak is detected in the tubing-connector, stop the flow, disconnect the tubing from the connectors, tightly reconnect tubing and connectors and resume the flow. When leakage is observed in inlets/outlets of a bubble trap or flow cell, disconnect the tubing and insert an inoculation needle until reaching the flow channel. Repeat for each hole in the flow chamber, reconnect and check again for leaks. In case of leaks in the sealing or by cracked glass,

we advise to replace the whole flow cell by a spare one, given that external sealing with silicone glue could interfere when visualizing with CLSM. However, if leakage is detected after the system has been inoculated, use silicone glue to seal the coverslip. In this case it is important to remove water or leaked medium as much as possible as this could prevent the silicone from working properly.

16. Using the Watson-Marlow 205S pump at 1.75 rpm with the system described in the methods section, medium consumption is approximately 0.0038 L/h and channel. However, if using tubing with different inner diameter than 1 mm and/or length or a different pump, a previous estimation of the flow may be necessary.
17. Air bubbles can interfere in the sterilization process but also alter or destroy the biofilm structure, so care must be taken to prevent them from entering the flow cells. The system contains bubble traps to catch air in the medium supply. Moreover, it is recommended not to cool the medium after autoclaving, but to place it immediately at the correct temperature for the experiment. If the medium is colder than the temperature of the flow cell and tubing, air bubbles tend to emerge throughout the system when it is subsequently placed at the desired temperature for the experiment. Furthermore, running of the flow cell system at high temperatures (e.g., 37 °C) gives rise to bubble formation. In this case it may help to run the flow chambers in a vertical position.
18. Any trace of hypochlorite may inhibit bacterial growth after inoculation, so special attention must be taken at the filling-emptying cycles with water and the final wash overnight. Emptying the waste bottle after the washing steps is also necessary, in case any hypochlorite remaining.
19. Always wear gloves when removing the bubble-trap stoppers and place them in a 70% ethanol solution. Apply the same principle to the fan-out and medium bottle tubing and immerse it in 70% ethanol before connecting them. This will prevent the media from getting contaminating upon tubing connection.
20. Biofilms are intrinsically heterogeneous and the asymmetric nutrient supply along the flow cell accentuates this heterogeneity. Thus, technical replicates should be inoculated in parallel in different flow channels. Furthermore, independent flow-cell biofilms with biological replicates should be performed. We advise to include at least two technical/biological replicates per condition (monoculture or coculture) and to run at least two independent biofilm systems as replicates.

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Chapter 13

Serial Dilution-Based Growth Curves and Growth Curve Synchronization for High-Resolution Time Series of Bacterial Biofilm Growth

Fernando Govantes

Abstract

The ability to form stable surface-attached communities called biofilms is of paramount importance to both beneficial and harmful interactions between microbes and microbial, plant or animal partners. Assessment of biofilm formation ability is often performed by growing the organisms in microtiter plate wells and staining the well-attached material, a method whose use for time-course analysis is limited by its destructive nature. Here we combine a serial dilution-based biofilm growth curve method with online monitoring of planktonic growth and a serially diluted growth curve synchronization algorithm to reconstruct the time-course of planktonic and biofilm growth. As demonstrated here with the rhizosphere bacterium *Pseudomonas putida*, the method allows accurate determination of the growth rate and doubling time, a robust depiction of the biofilm formation and dispersal dynamics and assessment of the biofilm development defects in mutant strains.

Key words Biofilm development, Bacterial growth, Bacterial physiology, Bacteria–host interactions, Computing approaches

1 Introduction

Most bacterial cells are found in nature as part of structured polymeric matrix-encased sessile communities, named biofilms [1]. Biofilms offer numerous advantages to the organisms involved, including increased resistance to multiple stress conditions. Mature biofilm communities are heterogeneous due to the generation of microniches in which organisms are in different physiological states. Biofilm growth promotes positive interactions between microorganisms, such as syntrophism, and horizontal genetic transfer and, because of their ubiquity and the metabolic efficiency achieved through metabolic interactions, biofilms have a major

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ecological impact [2]. Biofilms are also responsible for numerous infectious processes that, due to their low susceptibility to antimicrobials, are difficult to treat [3] and, in any case, the ability to form surface-attached communities on the hosts' surfaces has been shown to be an important determinant of microbial positive and negative interactions with plant and animal partners [4–8].

Evaluation of biofilm growth on microtiter plate wells [9] has become a staple of the analysis of biofilm-related phenotypes. One caveat of this approach is that, due to the destructive nature of the staining procedure, direct monitoring of biofilm evolution on microtiter plates over time is not possible. For this reason, time-course studies of biofilm growth require inoculation and independent processing of replicate plates for each time point [9]. This approach is time-consuming and inaccurate, as considerable plate-to-plate variability often arises due to small changes in the incubation conditions or the washing and staining procedures [10].

According to the exponential cell growth model, under an ample set of conditions, serial dilution of an inoculum produces growth curves shifted in time to reflect the differences in initial cell density. By using an algorithm to compensate for this time shift, van Ditmarsch and Xavier [11] devised the growth curve synchronization method. This general approach that combines the results of end-point measurements of a given parameter obtained from serially diluted cultures with highly accurate time-resolved growth and/or fluorescence data obtained from online monitoring of the same dilution series to reconstruct an accurate time-resolved quantitative measurements of the chosen parameter. This method was applied successfully to determine the correlation of the time-course of rhamnolipid (a virulence factor in *Pseudomonas aeruginosa*) secretion and induction of rhamnolipid synthesis gene transcription with the entry into stationary phase and quorum sensing induction [11]. In a similar vein, we devised the serial dilution-based growth curve method, an alternative approach to time-course measurements of planktonic and biofilm growth in which strains are serially diluted in the wells of a microtiter dish and incubated for a defined period of time prior to planktonic and biofilm growth assessment. The growth measurements were then plotted vs. the initial OD_{600} of each dilution in the series which acts as a surrogate of the time scale [12], as the dilution series recapitulates the time-course of both planktonic and biofilm growth. We have used this method extensively for the characterization of biofilm formation- and dispersal-defective mutants in the plant growth-promoting rhizosphere bacterium *Pseudomonas putida* [12–15].

Here we propose a combination of the serial dilution-based growth curve and growth curve synchronization methods described above to produce accurate and reproducible reconstructed planktonic and biofilm growth and dispersal curves plotted on a time scale. We believe that this method will have broad

application in the characterization of biofilm development defects that may be relevant to the interaction of microbes with other microbial, plant and animal partners.

2 Materials

2.1 Bacterial Growth

1. Bacterial strains: *Pseudomonas putida* KT2442 and derivatives.
2. Luria–Bertani (LB) broth (For 1 L): 10 g tryptone, 5 g yeast extract, 5 g NaCl. Sterilize by autoclaving 15 min at 121 °C. Add 20 g agar for solid medium.
3. Microtiter polystyrene flat bottom plates.
4. Microtiter plate reader/incubator: Spark® multimode plate reader (Tecan) equipped with a PC running the SparkControl™ software.
5. 25 mL sterile glass tubes with caps.
6. 12 mL sterile polycarbonate tubes
7. Sterile wooden applicator sticks.
8. Sterile 10 cm Ø Petri dishes.
9. Multichannel pipette (100–300 μL) and sterile tips
10. 30 °C shaking incubator.
11. Spectrophotometer and cuvettes.
12. Vortex mixer.
13. Desktop shaker.

2.2 Biofilm Growth Quantification

1. Crystal violet 0.1% (w/v) solution in distilled water.
2. Ethanol 96%.
3. Large (>2 L) containers with tap water.
4. Stacks of filter paper.

2.3 Software

1. SparkControl™ Spark® reader control software (Tecan).
2. Microsoft Excel®.
3. GraphPadPrism®.

3 Methods

3.1 Serial Dilution-Based Growth Curves

This procedure is essentially as described in López-Sánchez et al. [12], with some modifications. An overnight inoculum is serially diluted and eight samples from each dilution are allocated into the wells of a microtiter plate column. Columns 1 and 12 are reserved for blanks and filled with sterile growth medium. In this version of the protocol, growth is performed in a microtiter plate reader/

incubator and monitored online. At the end of the experiment, the biomass of the surface-attached populations is measured by staining with crystal violet, extracting with ethanol and measuring absorbance of the ethanolic crystal violet solution [9].

3.1.1 Setting Up the Dilution Series

1. Streak out the desired *P. putida* strain on an LB agar plate with antibiotics (when required) from a −80 °C glycerol stock and incubate overnight at 30 °C.
2. Using a wooden applicator stick, set up an inoculum from an isolated colony in 3 mL of LB and incubate overnight at 30 °C with 180 rpm shaking.
3. Measure optical density at 600 nm (OD_{600}) with a spectrophotometer after diluting 1:10 in LB.
4. Dilute the culture to an OD_{600} of 0.025 in 10 mL LB in a 12-mL sterile polycarbonate tube.
5. Prepare nine additional 12-mL sterile polycarbonate tubes containing 6 mL LB each.
6. Perform a fourfold dilution by adding 2 mL of the diluted culture to the first tube prepared in **step 5**.
7. Vortex and perform serial fourfold dilution steps by repeating **step 6** with each of the additional tubes containing LB (*see* **Note 1**).
8. Vortex again and transfer the contents of each of the tubes to a sterile container (e.g., a sterile petri dish) suitable for multichannel pipetting (*see* **Note 1**).
9. Transfer 150 μL from each dilution to all eight wells in columns 2–11 of a flat-bottomed 96-well polystyrene microtiter plate (*see* **Note 2**). Transfer 150 μL of sterile LB to all eight wells in columns 1 and 12. These columns will be used as blanks (*see* **Note 3**).

3.1.2 Online Monitoring of Planktonic Growth

1. Transfer the inoculated microtiter plate with its lid to the tray of a microtiter plate reader equipped with temperature and shaking control modules (*see* **Note 4**).
2. Using the appropriate software, select a 14-h incubation cycle at 30 °C with 150 rpm shaking, and an OD_{600} read in 15 min intervals (*see* **Note 5**).
3. At the end of the incubation period, remove the plate and export the data to an Excel® file (Planktonic data file).

3.1.3 Serial Dilution-Based Biofilm Growth Curves

1. Remove the supernatant and planktonic cells by inverting and gently tapping the plate on a stack of filter paper (*see* **Note 6**).
2. Wash remaining planktonic cells by sequentially submerging the plate in three containers with tap water.

3. Remove excess water by inverting and gently tapping the plate on a stack of filter paper.
4. Using a multichannel pipette, dispense 200 μL of 0.1% (w/v) crystal violet solution in distilled water per well. Incubate 15 min at room temperature.
5. Remove the crystal violet solution by inverting and gently tapping the plate on a stack of filter paper.
6. Wash the excess crystal violet in the wells by sequentially submerging the plate in three containers with tap water.
7. Remove excess water by inverting and gently tapping the plate on a stack of filter paper.
8. Using a multichannel pipette, dispense 200 μL of 96% ethanol per well. Incubate in a desktop shaker at 600 rpm for 15 min at room temperature to make the ethanolic crystal violet solution homogeneous.
9. Transfer the microtiter plate with the lid removed to the tray of a microtiter plate reader.
10. Perform an A_{620} reading of the whole plate. Save the data as an Excel® file (Biofilm data file).
11. To obtain serial dilution-based planktonic and biofilm growth curves [12], plot planktonic growth (the final OD_{600} reading of the time series) and biofilm biomass (A_{620} from the crystal violet-stained wells) against the initial OD_{600} for each dilution (Fig. 1).

3.2 Growth Curve Synchronization

Here we adapt the growth curve synchronization method developed by van Ditmarsch and Xavier [11]. This method is based on the premise that the serial dilution series results in a reproducible set of time-shifted growth curves (Fig. 1a). By using a correction factor for the time-shift between dilutions, a single combined growth curve can be reconstructed from the complete set (Fig. 1b). End-point measurements of any parameter (in our case, quantification of biofilm formation) performed on the serially diluted samples can then be assigned to time points on the time-shifted growth curve (Fig. 2c). Calculations are performed on an Excel calculation template (Time-shifted growth curves template.xlsx), available as Electronic Supplementary Material [16], which is adapted from that developed by van Ditmarsch and Xavier [11] for this application (*see* **Note 7**).

3.2.1 Processing the Planktonic Growth Data

1. Copy columns A-M from the planktonic data file, containing the experiment settings and the absorbance readings, to columns A-M of the Result sheet in the calculation template.
2. The Sorted planktonic data sheet automatically sorts the data in columns, representing the time course of the absorbance

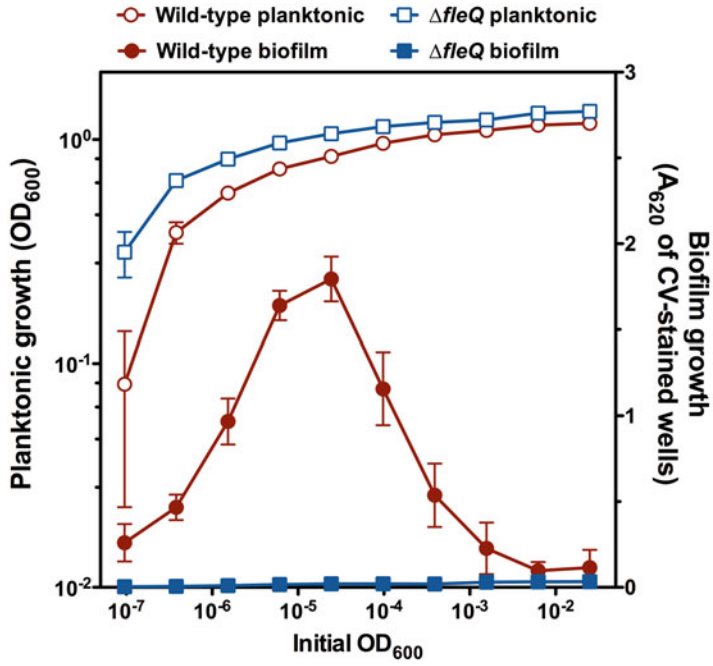

Fig. 1 Serial dilution-based growth curves of wild-type and Δ*fleQ Pseudomonas putida* strains. Planktonic (open symbols) and biofilm (closed symbols) growth values from serial dilutions of the wild-type KT2442 (circles) and Δ*fleQ* mutant MRB52 (squares) strains incubated for 14 h as described in the text are plotted against the OD_{600} values at time 0 of the dilution series. Values and error bars represent the medians and median absolute deviations of the eight replicates

readings at each well. Columns are grouped in color-coded blocks representing the eight replicates of each dilution or blank.

3. The Calculated OD sheet automatically subtracts the blank values in Column 1 from the absorbance data of each dilution. Next, it rejects all corrected readings with OD values below 0.005 (*see* **Note 8**). Finally, it calculates the average, median, standard deviation, and median absolute deviation for the eight replicates of each dilution (*see* **Note 9**).

3.2.2 Processing the Biofilm Growth Data

1. Copy columns A-M from the biofilm data file, containing the experiment settings and the absorbance readings, to columns A-M of the Biofilm sheet in the calculation template.
2. The Biofilm data sheet automatically subtracts the blank values in Column 1 from the absorbance values of each dilution, then calculates the average, median, standard deviation and median absolute deviation for the eight replicates of each dilution (*see* **Note 9**).

3.2.3 Synchronization of the Growth Curves

1. Insert the initial OD_{600} and dilution factor values used at the corresponding cells of the Plotting data sheet (*see* **Note 10**).

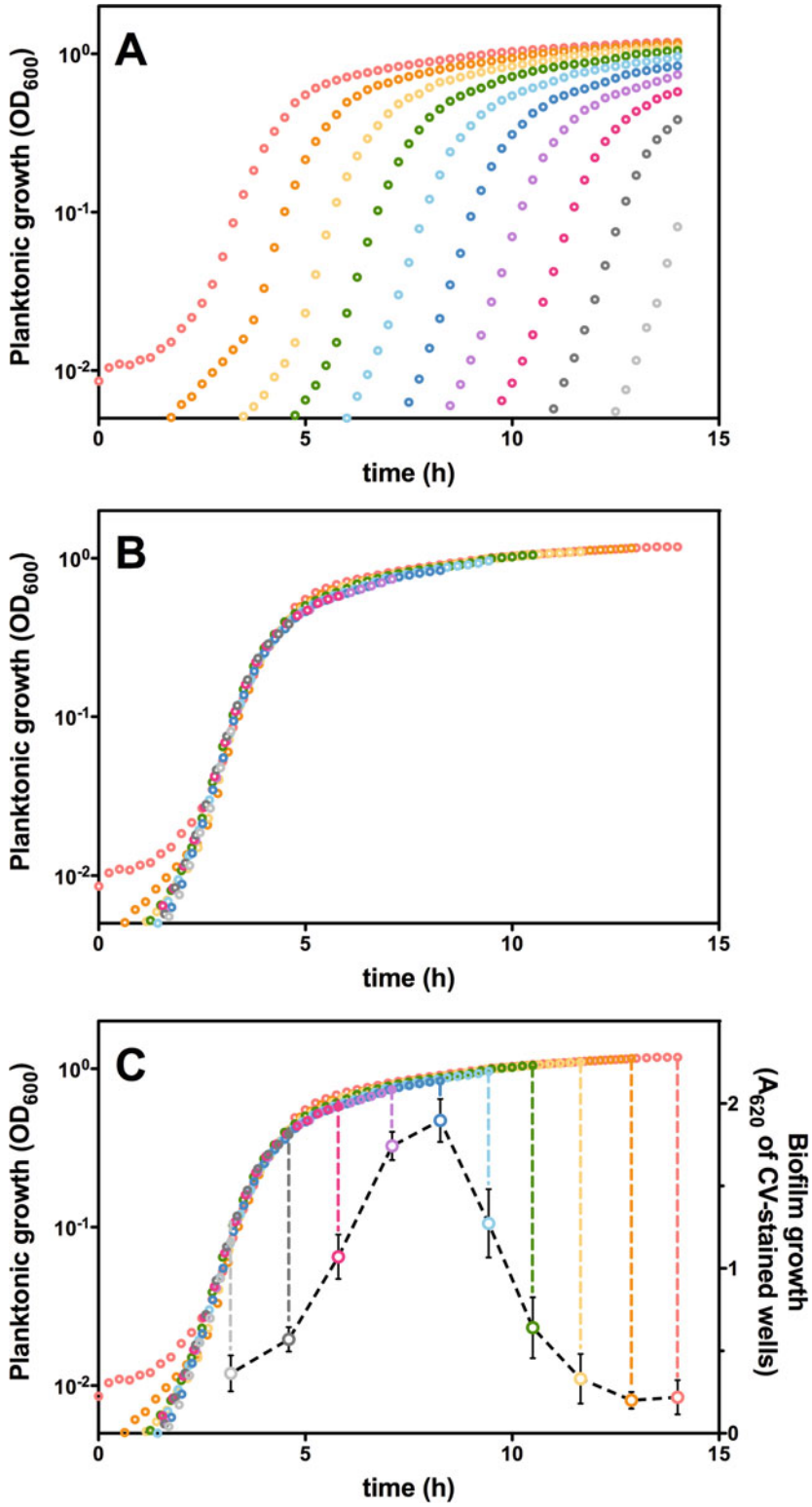

Fig. 2 Depiction of the growth curve synchronization method. (**a**) Time-shifted planktonic growth curves obtained from serially diluted wild-type *P. putida* KT2442 after 14-h incubation with OD_{600} measurements

2. Growth curves are synchronized by subtracting a time-shift coefficient (*tau*) to each of the time values. The *tau* values are chosen to minimize the difference between the data points of each time-shifted curve and those in the curve of the least diluted sample (i.e., Column 2 or Dilution 1), which is used as the reference. To perform this task, *tau* values are inserted at the corresponding cells of the Plotting data sheet, and these are used to modify the time scales for the corresponding planktonic growth curves at the Calculated OD data sheet. The magnitude of the errors between each curve and the reference is computed at the "Alignment error calculations block" of the OD alignment error data sheet and copied to the "OD align error" table at the Plotting data sheet. There are two methods to assign the *tau* values: (1) *tau* values are assigned using the Goal SeekExcel® function, using the minimal value of the error (i.e., assigning a value of 0) as the goal; (2) *tau* values can also be assigned manually by trial and error, and tested repeatedly until the lowest possible value of the alignment error is obtained (*see* **Note 11**). The Plotting data sheet also provides visual feedback for the alignment in the form of not time-shifted (in which the original time values are used) and time-shifted (in which the original time values are replaced by the time-shifted values) plots of the different growth curves (*see* **Note 12**). Finally, the Plotting data sheet displays a plot of *tau* versus the natural logarithm of the dilution factor of each curve relative to the reference curve (ln X_2/X_1). The slope of the linear fit to this plot is used to automatically calculate the growth rate and doubling time, and the R-squared value of the fit is used a quality control of the reliability of the *tau* values chosen (*see* **Note 13**).

3.2.4 Plotting the Planktonic and Biofilm Growth Curves in a Common Time Scale

1. In order to generate a single plot in which continuously measured planktonic growth and end-point-measured biofilm growth are shown in a shared time scale, the time-shifted values of the last time point for each dilution (i.e., the one recorded immediately before the plate was processed for biofilm measurement) is automatically paired with the biofilm measurements of each dilution at the Biofilm data sheet. The biofilm

Fig. 2 (continued) performed every 15 min. (**b**) Synchronized planktonic growth curves obtained after application of the time-shift correction factor (*tau*) as described in the text to the data plotted in **A**. (**c**). Synchronized planktonic and biofilm growth curves obtained after application of the time-shift correction factor (*tau*) as described in the text to the data plotted in (**a**). and the biofilm quantification data obtained after 14 h incubation as described in the text. Values and error bars (only for the biofilm data) represent the medians and median absolute deviations of the eight replicates

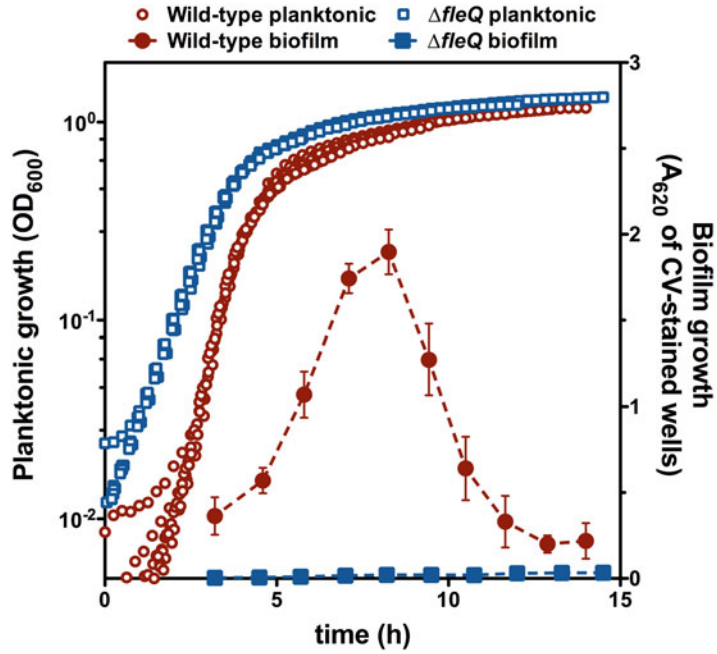

Fig. 3 Serial dilution-based synchronized growth curves of wild-type and Δ*fleQ* *Pseudomonas putida* strains. Synchronized planktonic growth curves (open symbols) and biofilm (closed symbols) obtained after application of the time-shift correction factor (*tau*) as described in the text growth from serial dilutions of the wild-type KT2442 (circles) and Δ*fleQ* mutant MRB52 (squares) strains incubated for 14 h as described in the text are plotted against incubation time. Values and error bars represent the medians and median absolute deviations of the eight replicates.

biomass curve is then plotted along with the time-shifted growth curves at the Plotting data sheet (Fig. 3) (*see* **Note 14**).

4 Notes

1. It is important that the cell suspensions are vortexed vigorously in this step. As *P. putida* attaches quickly to most materials, there is a risk that a significant fraction of the population remains bound to the inner surface of the tube and is not diluted.
2. We routinely use eight technical replicates and ten dilutions, and therefore each plate is used for a single strain and growth condition. However, we have on occasion used only four replicates, which allows having two strains or conditions in the same plate with satisfactory results.
3. Even though some microtiter plate readers minimize evaporation at the edges of the plates, we prefer not to use columns

1 and 12 with experimental samples, and we save them for the experimental blanks.

4. We use the Tecan Spark® reader, but of course other readers are acceptable as long as they provide temperature and shaking control. However, the Excel template is written to match the output format of the Spark®, so users of other readers will likely have to modify the template to fit their own output format.
5. Other intervals and durations are possible, but they may require minor editing of the Excel template.
6. The plate should be processed promptly after removal from the reader to prevent further growth, as the biofilm measurements are linked to the OD_{600} readings obtained at the final time point.
7. Even though the original authors developed the curve synchronization algorithm in MATLAB, they also suggest the Excel-based method shown here, which in my opinion yields excellent results and requires little prior training.
8. Rejection of readings below 0.005, which are highly noisy as they are near the detection threshold of the reader, results in higher quality alignment of the curves during the synchronization procedure. This threshold may be increased or decreased as required by modifying the threshold value in the corresponding data sheet.
9. Median and median absolute deviation are used for plotting, as they provide more robust central value by minimizing the effect of occasional outliers in the series. However, average and standard deviation are also automatically calculated for other possible uses.
10. These values will be used later during the synchronization procedure.
11. In our experience, Goal seek does not always work as intended. On the other hand, excellent synchronization is obtained by manually assigning *tau* values (in hours) with a single decimal place and then increasing or decreasing the values in 0.1 h intervals.
12. Individual curves of the time-shifted plot shift up and down the X-axis as *tau* values change. Good visual fitting to the reference curve is normally consistent with a low alignment error value.
13. We routinely obtain R-squared values above 0.995, indicating excellent fit of the data to the fitted line.
14. We routinely export the data from Excel® to Graphpad Prism® to obtain highly customized publication-quality plots.

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Chapter 14

Detection of Bacterial Quorum Sensing Molecules

Elke Stein and Adam Schikora

Abstract

Bacterial cells use the quorum sensing system to communicate with each other. The gram-negative species very often use *N*-acyl homoserine lactones for this purpose. One of the easiest ways to detect these molecules is the use of particular reporter strains, which possess different kinds of reporter genes under the control of AHL-responsive promoters. Here we present some of the possibilities available today, even for not specialized researchers.

Key words Acyl homoserine lactones, Quorum sensing, GFP, Image processing, HPLC-MS

1 Introduction

Detection and quantification of bacterial quorum sensing molecules gained a lot of attention in the last years. It is very likely to be in focus also in the coming years, since quorum quenching might become important strategy to lower the virulence of pathogenic bacteria. Obviously medical aspects are the driving force in this topic, many features of bacterial pathogens' virulence are controlled by the quorum sensing system [1–4] and the increasing number of new multiresistant strains requires alternatives to the conventional antibiotic-based treatments. However, also other aspects are of great interest, for example the impact of bacterial populations on the performance of crop plants, which can be either positive as in the case of induced resistance, or negative as in the case of diverse phytopathogenic bacteria.

Quorum sensing was described for the first time in *Vibrio fischeri* that lives in symbiosis with squid and produces bioluminescent blue–green light at high cell densities [5–7]. The quorum sensing system of *Vibrio fischeri* is based on *N*-acyl homoserine lactones (AHLs) and until now is the best characterized bacterial communication system [8]. Besides the AHL molecules, bacteria require the AHL-synthase (LuxI), the receptor (LuxR), and signal response regulator protein(s), which regulate the so-called

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QS-regulon [2]. The variation in quorum sensing systems within the bacterial populations depends on the chemical structure of the communication molecules. For example, AHLs can vary in the length of the acyl chain (4–18-carbons) and in the substitutions at the carbon chain. In addition to AHLs, 2-alkyl-4-quinolones, long-chain fatty acids, fatty acid methyl esters, and furanones (autoinducer-2) can be used in gram-negative bacteria [9]. Moreover, quorum sensing and the subsequent gene expression are not restricted to gram-negative bacteria. Also signaling compounds from gram-positive bacteria, which include cyclic peptides, autoinducer-2, or butyrolacetone, have been reported to function as population density-dependent activators of their QS-regulon [10].

Given the diversity of possible systems, we will focus this chapter on the detection and quantification of AHLs as the best characterized quorum sensing system today.

2 Materials

2.1 Bacterial Strains

Reporter bacteria should be grown on LB medium with specific antibiotics.

1. *Pseudomonas putida* (F117 pKR C12 GFP): 20 mg/L Gm, 50 mg/L Kan [11].
2. *Serratia liquefaciens* (MG44 pBAH9 GFP) 100 mg/L Amp, 10 mg/L Tet, 50 mg/L Kan [12].
3. *Escherichia coli* (MT102 GFP pJBA89) 100 mg/L Amp [13].
4. *E. coli* strain (Top10 pSB403) 10 mg/L Tet [14] expressing luxR + luxI::luxCDABE from *Vibrio fischeri*, detecting a range of AHLs from C6-HSL to oxo-C14-HSL.
5. *S. meliloti*.

2.2 Bacterial Media

2.2.1 Media for Reporter Strains

1. LB medium (for 1 L): 24 g LB Luria Miller. Autoclave at 120 °C for 20 min.
2. LB agar (for 200 mL): 8 g LB Luria Miller and 3 g agar-agar. Autoclave at 120 °C for 20 min.
3. Add the appropriate antibiotics when the medium is cooled down to approximate 40 °C and pour each 50 ml portion into one square and sterile petri dish.

2.2.2 Media for Ensifer meliloti (Sinorhizobium meliloti):

1. TY Broth (for 1 L): 5 g tryptone, 3 g yeast extract. Autoclave at 120 °C for 20 min, thereafter add sterile 10 mL of 1 M $CaCl_2$. The final concentration of $CaCl_2$ should be 10 mM.
2. TY agar (for 500 mL): 2.5 g tryptone and 1.5 g yeast extract. Add 1.5 g agar per 100 mL. Autoclave at 120 °C for 20 min,

Table 1
AHL molecules used as reference for detection

Molecule	Length	MW (g/mol)
N-Hexanoyl-DL-homoserine lactone	C6	199.25
N-(−3-Oxooctanoyl)-L-homoserine lactone	C8	241.48
N-(−3-Oxodecanoyl)-L-homoserine lactone	C10	269.34
N-(−3-Oxododecanoyl)-L-homoserine lactone	C12	297.39
N-(−3-Oxotetradecanoyl)-L-homoserine lactone	C14	325.44

thereafter add sterile and prewarmed 1 mL of 1 M $CaCl_2$. The final concentration of $CaCL_2$ should be 10 mM. At the same time add the appropriate antibiotics. Pour each portion into 4–5 petri dishes.

2.3 Chemicals and Laboratory Equipment

1. Antibiotics: prepare all antibiotics as 1000× stock solutions: 250 mg/mL streptomycin sulfate, 10 mg/mL tetracycline, 100 mg/mL ampicillin, 50 mg/mL kanamycin, 20 mg/mL gentamycin. Sterilize the stock solutions using a 0.2 μm filter.
2. Reference molecules: the reference solutions of *N*-acyl homoserine lactones (used as positive controls during the experiments) have to be prepared as 60 mM stock solutions in acetone (Table 1). For the concentration standards use: 60, 600 nM, and 1.2, 6 and 60 μM stocks solved in acetone.
3. Chloroform.
4. Refrigerated speed-vac.
5. Epifluorescence microscope (GFP filter).
6. 96-well plates.
7. Luminiscence microplate reader.

3 Methods

All steps should be performed under sterile conditions using clean bench. Handling with acetone should be performed with appropriate caution. Growth and manipulation of transgenic organisms requires an S1 level laboratory. Here, we use the example of *Ensifer meliloti* (*Sinorhizobium meliloti*).

3.1 Preparation of the Bacterial Cultures

1. Prepare the bacterial preculture by inoculation of 5 mL TY broth the day before the scheduled experiment. Cultivate upon shaking (150 rpm) at 23 °C overnight.
2. On the experiment day use 10 μL of this culture to start the experimental culture in TY medium.

3. Grow the culture until the expected density ($OD_{600\ nm}$). A minimum of 1 mL of culture is required for the following AHL extraction.

3.2 AHL Extraction from the Bacterial Culture

1. Use 1 mL of the culture designed for AHL extraction and pipet it into a new 2 mL Eppendorf tube and add 400 μL chloroform.
2. Shake this mix vigorously using a horizontal shaker for 15 min or as an alternative vortex the mix 3–5 times for 15 s at full speed.
3. Afterwards centrifuge the culture–chloroform mix for 10 min at 20.000 × *g* in a tabletop centrifuge at 4 °C.
4. Handle the resulting separated phases (Fig. 1) with care since they will easily collapse after accidental shaking.
5. Pipet out the upper (hydrophilic) phase and discard it (*see* **Note 1**).
6. The remaining chloroform-solved phase should be desiccated as quickly as possible; the best is to use a refrigerated speed-vac instrument (*see* **Note 2**).
7. Solve the remaining, AHL-containing pellet in 40 μL acetone (*see* **Note 3**).
8. If necessary acetone-solved AHLs might be stored at −20 °C for a short period (*see* **Note 4** and **Note 5**).

3.3 Preparation of GFP-Reporter Bacteria

1. The day before the scheduled experiment, inoculate 100 μL of already prepared preculture onto petri dish with the appropriate combination of medium and antibiotics.

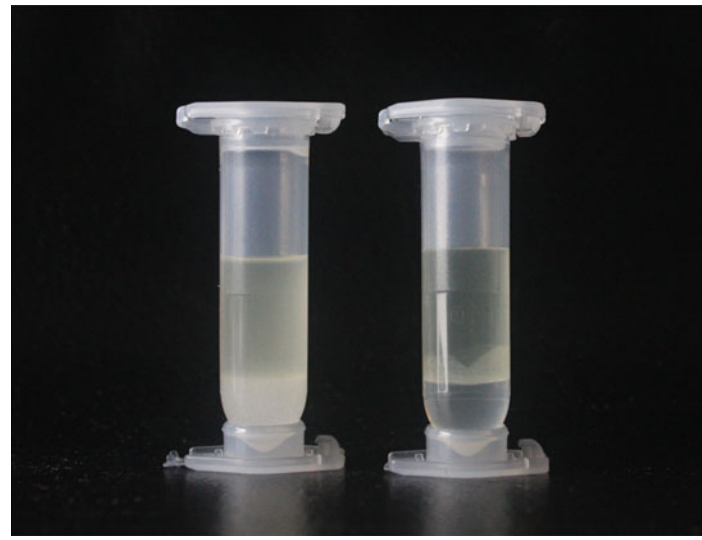

Fig. 1 Phase separation. Phase separation after shaking of the mix between bacterial culture and chloroform (left tube) and the additional centrifugation (right tube)

2. Spread the bacteria equally on the plate using sterile glass beads.
3. Incubate at 22–25 °C overnight.
4. The resulting bacterial lawn should evenly cover the entire plate.

3.4 Detection with GFP-Marker Strains

1. Design a grid on the lower surface of each plate with reporter bacteria, for example 5 × 5 squares.
2. Drop directly on the bacterial lawn 10 µL (better to drop 2 × 5 µL) of the AHL standards solved in acetone. Pure acetone serves as control.
3. Proceed equally with the extracted AHL samples.
4. Bacterial lawn should be photographed after 4–6 h using a fluorescence microscope and a GFP filter: excitation 480/40 nm and emission 510–550 nm.
5. Resulting images (Figs. 2 and 3) can be taken for intensity calculation or used as raw data [15, 16].

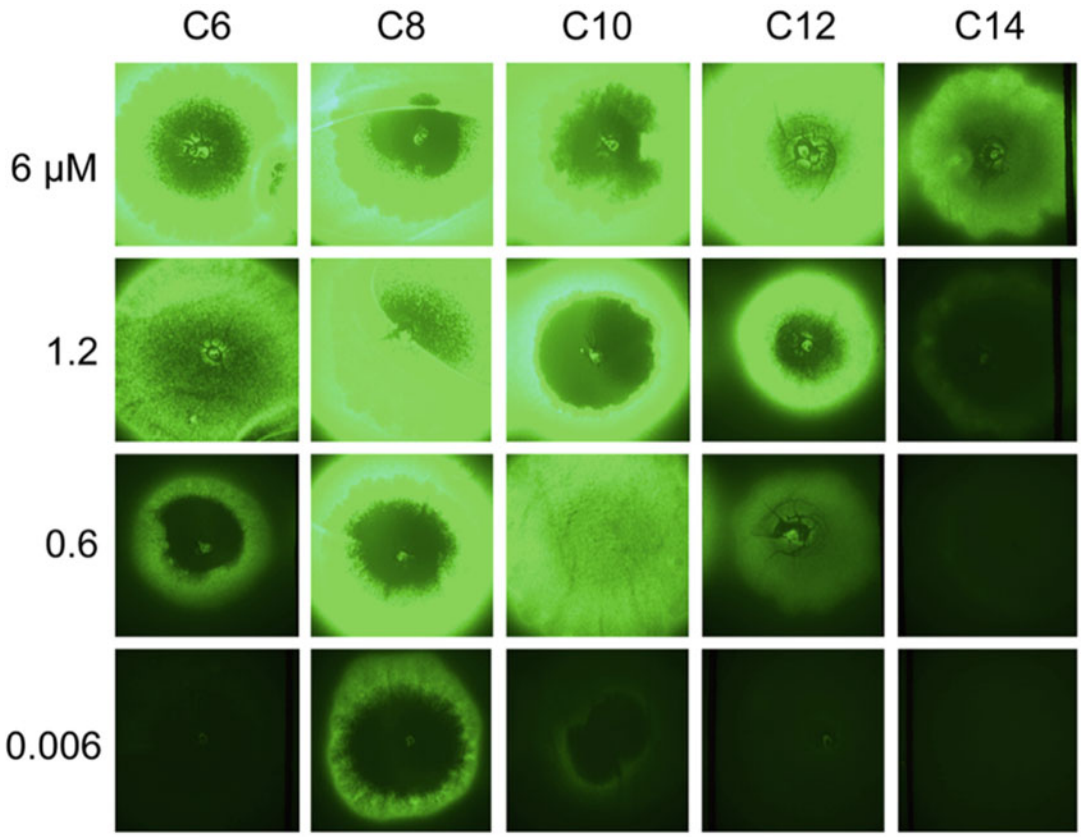

Fig. 2 Detection of AHL molecules using GFP-based system. Images resulting from AHL detection using the *Escherichia coli* GFP reporter strain and an AHL standard series containing molecules with different acyl chain lengths and different concentrations of the molecules

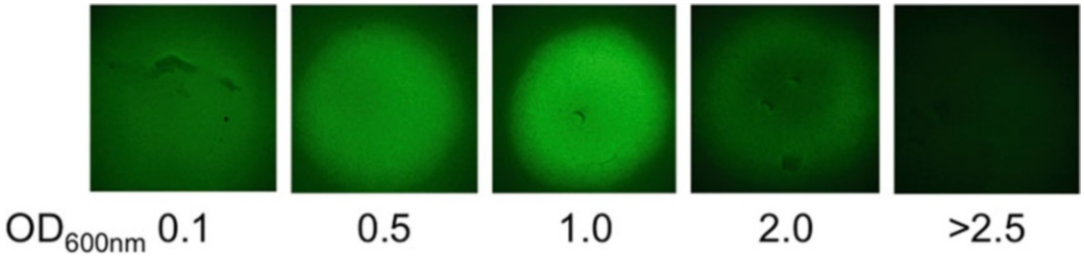

Fig. 3 AHL Production during bacterial growth. Images resulting from AHL detection using the *Escherichia coli* GFP reporter strain and AHL extracted from *Ensifer meliloti* (*Sinorhizobium meliloti*) culture at different stages of the growth

3.5 Preparation of LUX-Reporter Bacteria

1. Prepare a preculture of the *E. coli luxCDABE* reporter strain in 5 mL LB medium.
2. Incubate overnight while shaking (150 rpm) at 37 °C.
3. On the scheduled experiment day inoculate 25 mL of LB medium and grow under constant shaking until the culture reaches a $OD_{600\ nm} = 0.8$.
4. For an entire 96-well plate usually used during this experiment for detection 10 mL of reporter strain culture are required.

3.6 Measuring the AHL-Induced Luminescence with Escherichia coli luxCDABE

1. Prepare the 96-well multiplates and a pipetting scheme, calculate at least triplicates as technical repetitions in addition to the biological repetitions of the experiment.
2. In each well add 100 μL of reporter bacteria culture at $OD_{600\ nm} = 0.8$.
3. Add 10 μL of the corresponding samples and AHL standards to the bacterial culture according to the pipetting scheme (triplicates) prepared beforehand.
4. Use 10 μL of acetone as a negative control.
5. In addition, use a blank with bacteria without additive.
6. The luminescence measurement is carried out after 0, 2, 4, 6, 8, and 24 h after adding the standards or the extracted samples in a microplate reader using luminescence as settings and integration time = 4000 ms/well (Figs. 4 and 5) (*see* **Note 6**).
7. The measured luminescence can be easily calculated using Excel or a similar software.

4 Notes

1. Be very careful and leave only the clean hydrophobic phase in the tube.
2. If no speed-vac available, samples can be evaporated under the clean bench. This takes usually few hours. However, such

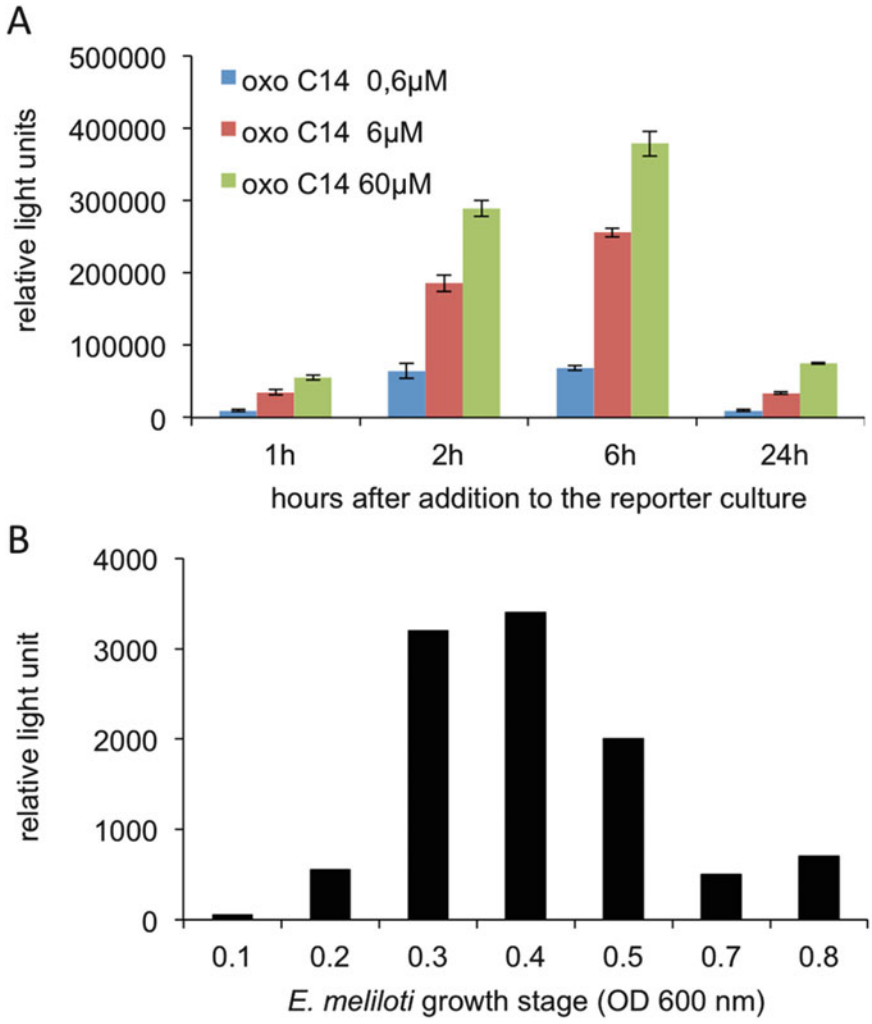

Fig. 4 Detection of AHL molecules using LUX-based system and photon counting**.** Exemplary results obtained after detection of AHLs using the *Escherichia coli luxCDABE* reporter strain and the standard C14 AHL solutions (**A**) at different time points after incubation start, and (**B**) samples extracted from *Ensifer meliloti* culture at different growth stages. Detected 6 h after incubation start

procedure is not recommended for further quantification or qualitative analysis of the samples. It might be useful though for general question addressing the presence or absence of AHLs.

3. Caution: acetone evaporates very quickly, so immediately close the reaction tubes and place them on ice.
4. The best results are obtained when the AHLs are immediately detected. Short-term storage at −20 °C is possible but not recommended.

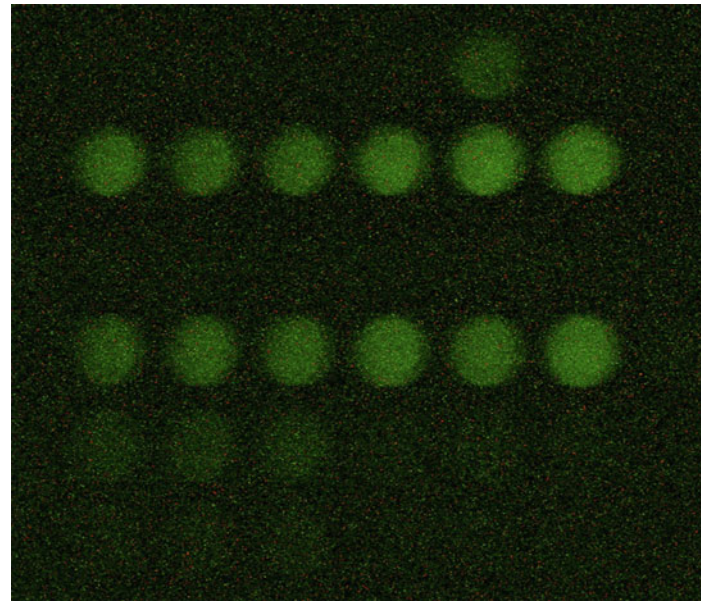

Fig. 5 Detection of AHL molecules using LUX-based system and CCD camera. Example of luminescence emitted by the *Escherichia coli luxCDABE* reporter strain after incubation with AHL, detected using a CCD camera

5. The described preparation of AHL extracts can be used for further analysis using HPLC-MS or even HPLC-MS/MS. For this kind of analysis we recommend resolving the extracted AHLs directly in the solvent system used for the HPLC and include a two-step verification procedure. For this purpose the HPLC fractions should be verified for AHL presence prior to the MS and the following MS/MS analysis. For a detailed description of such a procedure please refer to [16].
6. Instead of a plate-reader, simple visualization setup for bioluminescence (e.g., for western blots or ethidium bromide-stained gels) can be used (Fig. 5). In this case, software Image J (or a similar one) can be used to quantify the intensity of the light emitted by particular wells.

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Part III

Plant-Pathogen Interaction Section

Chapter 15

Generating Chromosome-Located Transcriptional Fusions to Fluorescent Proteins for Single-Cell Gene Expression Analysis in *Pseudomonas syringae*

José S. Rufián, Diego López-Márquez, Nieves López-Pagán, Murray Grant, Javier Ruiz-Albert, and Carmen R. Beuzón

Abstract

The last decade has seen significant effort directed toward the role of phenotypic heterogeneity in bacterial adaptation. Phenotypic heterogeneity usually refers to phenotypic diversity that takes place through nongenetic means, independently of environmental induced variation. Recent findings are changing how microbiologists analyze bacterial behavior, with a shift from traditional assays averaging large populations to single-cell analysis focusing on bacterial individual behavior. Fluorescence-based methods are often used to analyze single-cell gene expression by flow cytometry, fluorescence microscopy and/or microfluidics. Moreover, fluorescence reporters can also be used to establish where and when are the genes of interest expressed. In this chapter, we use the model bacterial plant pathogen *Pseudomonas syringae* to illustrate a method to generate chromosome-located transcriptional gene fusions to fluorescent reporter genes, without affecting the function of the gene of interest.

Key words Phenotypic heterogeneity, Gene expression, Fluorescent reporter genes, Single-cell methods, Fluorescence microscopy, Nongenetic variation, Allelic Exchange

1 Introduction

Bacterial pathogens deploy a multitude of virulence factors to colonize plants and cause disease. Many animal and plant bacterial pathogens relay on type III secretion systems (T3SS) to deliver effector proteins inside the host cell and thus modify cellular processes in order to allow bacterial survival, proliferation and spread [1]. *Pseudomonas syringae* is one of the most studied bacterial plant pathogens [2] that is both a model pathosystem and an increasing economically important pathogen in agriculture, with recent resurgence of old diseases and emergence of new ones [3, 4]. *P. syringae* is a foliar pathogen and its life history is linked to the water cycle, often reaching the leaf surface via rainfall [5]. *P. syringae* enters the

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leaf through natural openings (stomata, hydathodes) [6] or wounds, to reach the intercellular space of the leaf parenchyma, the apoplast, where it replicates. Once within the apoplast, *P. syringae* uses its T3SS to deliver effector proteins into the plant cell cytosol to suppress plant defenses, allowing bacterial colonization [7–9]. Where and when are these factors expressed during the interaction with the host is therefore of relevance for the understanding of the host-pathogen dynamics. Furthermore, recent work from our laboratory has shown that *P. syringae* T3SS genes display phenotypic heterogeneity in their expression, including *hrpL*, the gene encoding the main transcriptional activator of the system [10]. Phenotypic heterogeneity refers to phenotypic variation arising within a population living in the same microenvironment through nongenetic mechanisms [11]. Apoplastic populations of *P. syringae* pv. phaseolicola display phenotypically heterogeneous activation of the T3SS genes leading to cell-to-cell differences, which are likewise observed in the homogeneous environment of nutrient-limited culture medium, and are relevant for virulence. This finding is consistent with reports of many virulence genes displaying cell-to-cell expression differences in animal pathogens, such as *Salmonella enterica* [12–15], *Vibrio cholerae* [16], or *Yersinia pseudotuberculosis* [17]. These reports have raised the interest in using single-cell analytic methods to study bacterial behavior. Fluorescence-reporters are often used to analyze single-cell gene expression since they allow the application of techniques such as flow cytometry, fluorescence microscopy and/or microfluidics. In this chapter, we describe a method to generate chromosome-located transcriptional gene fusions to fluorescent reporter genes without affecting the function of the gene of interest, using the model bacterial plant pathogen *P. syringae*.

2 Materials

2.1 Bacterial Growth

1. Bacterial strains: *Pseudomonas syringae* pv. tomato DC3000 [18], *Escherichia coli* DH5α [19].
2. Lennox Broth (LB) [20], modification of Luria–Bertani [21] with NaCl concentration halved (For 1 L): 10 g tryptone, 5 g yeast extract, and 5 g NaCl and add to 800 mL of $_{d}H_2O$. Fill up to 1 L with $_{d}H_2O$ using a measuring cylinder. Add 10 g of bacteriological agar when necessary. Autoclave at 121 °C for 20 min. Cool down to a temperature about 50 °C and add the appropriate antibiotic. Pour about 20 mL of LB agar per 10 cm petri dish.
3. Antibiotic stock solutions: Ampicillin (Amp; 100 mg/mL), Kanamycin (Km; 50 mg/mL). LB was supplemented with either ampicillin (100 μg/mL for *E. coli* DH5α, 500 μg/mL

for *P. syringae* strains) or kanamycin (50 μg/mL for *E. coli* DH5α, 15 μg/mL for *P. syringae* strains).

4. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) stock solution (40 mg/mL in dimethylformamide).
5. TB Buffer: 10 mM HEPES pH 6.7, 15 mM $CaCl_2$, 55 mM $MnCl_2$, 250 mM KCl.
6. 25% glycerol

2.2 Plasmids Constructs

1. Plasmids (Table 1).
2. Total DNA extracted from *P. syringae* using JetFlex DNA Purification Kit (Genomed, Germany).
3. High-Fidelity polymerase system (e.g., Q5 High-Fidelity DNA Polymerase, NEB, UK).
4. dNTP mix: 10 mM each.
5. Specific primers (Table 2).
6. Gel Band Purification Kit.
7. RedSafe.
8. Vector for cloning PCR product (e.g., pGEM-T).
9. Restriction enzymes.
10. T4 DNA ligase and buffer.
11. NanoDrop spectrophotometer or similar.
12. 10 mM $MgCl_2$.
13. Agarose.
14. *E. coli* DH5α competent cells.

2.3 P. syringae Transformation

1. Sucrose stock solution, 300 mM.
2. Refrigerated microcentrifuge.
3. Electroporation cuvettes (2 mm).
4. Electroporator.

2.4 Southern Blot Analysis

1. One Kb Plus ladder.
2. HCl 0.25 N.
3. Denaturing solution: 1.5 M NaCl, 0.5 M NaOH.
4. Neutralizing solution: 3 M NaCl, 0.5 M Tris–HCl pH 7.
5. 20× SSC: 3 M NaCl, 300 mM Na-citrate.
6. Nylon membrane.
7. Blocking reagent.
8. DIG Labeling Mix.
   Anti-Digoxigenin antibody.
9. (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2 115 0-(50-chloro) tricyclo [3.3.1.1]decan}-4-yl)phenyl phosphate) CSPD

Table 1
Plasmids used or generated in this work

Plasmid	Description	Antibiotic resistance	Reference
pGEM-T	Cloning vector	Amp	Promega
pGT-*hrpL*-AB	pGemT containing the A + B fragment of *hrpL* with an *EcoRI* site	Amp	This work
pGT-YFP	pGemT derivative carrying the promoterless ORF of *eyfp* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp	This work
pGT-Turquoise2	pGemT derivative carrying the promoterless ORF of *turquoise2* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-mPlum	pGemT derivative carrying the promoterless ORF of *mplum* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-mOrange2	pGemT derivative carrying the promoterless ORF of *morange2* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-mCherry	pGemT derivative carrying the promoterless ORF of *mcherry* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-GFP$^+$	pGemT derivative carrying the promoterless ORF of *gfp* + and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pFLP2	Contains a flippase gene	Amp	[25]
pKD4	pANTS derivative containing an FRT-flanked kanamycin resistance gene	Amp, km	[26]
miniTn7(Gm) PA1/04/ 03–eyfp-a	Contains the eYFP ORF	Gm, cm	[27]
pmTurquoise2	Contains the Turquoise2 ORF	Km	[28]
pBAD-mPlum	Contains the mPlum ORF	Amp	Michael Davidson and Roger Tsien (unpublished)
pBAD-mOrange2	Contains the mOrange2 ORF	Amp	Michael Davidson and Roger Tsien (unpublished)
pBAD-mCherry	Contains the mCherry ORF	Amp	Michael Davidson and Roger Tsien (unpublished)
pZEP07	Contains the GFP+ ORF	Cm	[29]

Table 2
Primers used in this work

Name	Sequence	Restriction site[a]
HrpLA1	attcgccaaatgacggcc	NA
HrpLA2	aatgatcgagGAATTCatcgccattcaggcgaacg	*EcoRI*
HrpLB1	gaatggcgatGAATTCctcgatcatttttttctggaaccaac	*EcoRI*
HrpLB2	tcagaattgtcgagaaaggctg	NA
Prot fluor F	aaGAATTCggagatatacatatggtgagcaagggcg	*EcoRI*
Prot fluor-km R	ccagcctacacttacttgtacagctcgtcc	NA
GFP+ F	aaGAATTCggagatatacatatgagcaaaggagaagaac	*EcoRI*
GFP+ km R	ccagcctacacttatttgtagagctcatccatgc	NA
Km-Prot fluor F	ctgtacaagtaagtgtaggctggagctgc	NA
P2	tcaGAATTCcatatgaatatcctccttag	*EcoRI*
P1	tcaGAATTCgtgtaggctgga	*EcoRI*

[a] *NA* Not applicable. RS in capital letters

3 Methods

To illustrate the method, adapted from one previously developed by our laboratory to generate knockout strains in *P. syringae* [22], we will use the generation of an *hrpL* transcriptional fusion to the fluorophore-encoding gene *mturquoise2* in the model strain *Pseudomonas syringae* pv. tomato DC3000 as an example. Using the same method and primers (Table 2), and just by changing the vectors (Table 1), we also generate fusions to reporter genes encoding alternative fluorophores, namely mOrange2, mPlum, GFP+ and eYFP. The method requires the PCR-based generation of an allelic exchange fluorescent reporter DNA module to be recombined into a specific location within the bacterial chromosome. In our example, the allelic exchange module comprises: (1) the last 500 bps of the *hrpL* coding sequence including the STOP codon, (2) the *mturquoise2* ORF carrying its own ribosomal-binding site (RBS), (3) the *nptII* gene flanked by FRT sequences, and (*iv*) 500 bps immediately downstream the *hrpL* ORF STOP codon. Generation of this module is carried out sequentially.

3.1 Generating the hrpL:: mturquoise2 Allelic Exchange Plasmid

The outline of the steps described in this section is illustrated in Fig. 1.

3.1.1 Primer Design

The insertion point (where the reporter gene would be inserted) should be located 5–10 nucleotides after the STOP codon of the

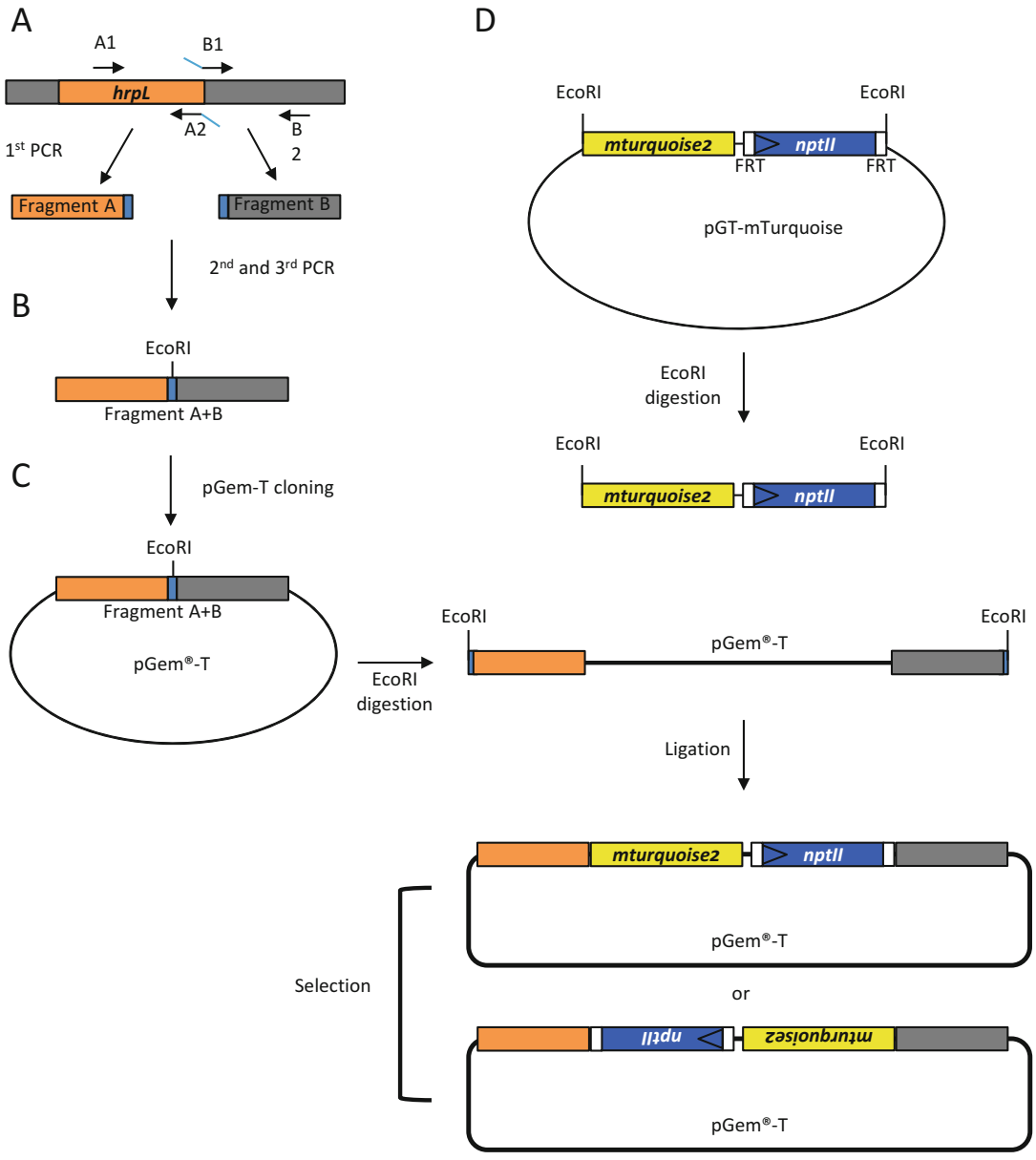

Fig. 1 Generating allelic exchange vectors for transcriptional gene fusions. (**a**) The last 0.5 Kb including the STOP codon of the target gene and the 0.5 Kb immediately downstream are amplified independently. Primers A2 and B1 share a 20 nucleotide-long homologous sequence at their 5′ ends, and incorporate a unique restriction site. (**b**) By using the resulting PCR products as both primers and template, a polymerization is carried out resulting in a joint 1 kb fragment. This is followed by amplification of the newly generated allele using primers A1 and B2. (**c**) The allele is A/T cloned into pGEM-T. (**d**) Using the same unique restriction site incorporated into primers A2 and B1, a fragment containing the *mturquoise2* ORF followed by an FRT-flanked *nptII* gene is cloned to generate the allelic exchange module, and the correct orientation is confirmed. This correctly oriented construct represents the allelic exchange vector to be transformed into *P. syringae* to obtain the double recombinants that would have incorporated the transcriptional reporter fused to the target gene of interest

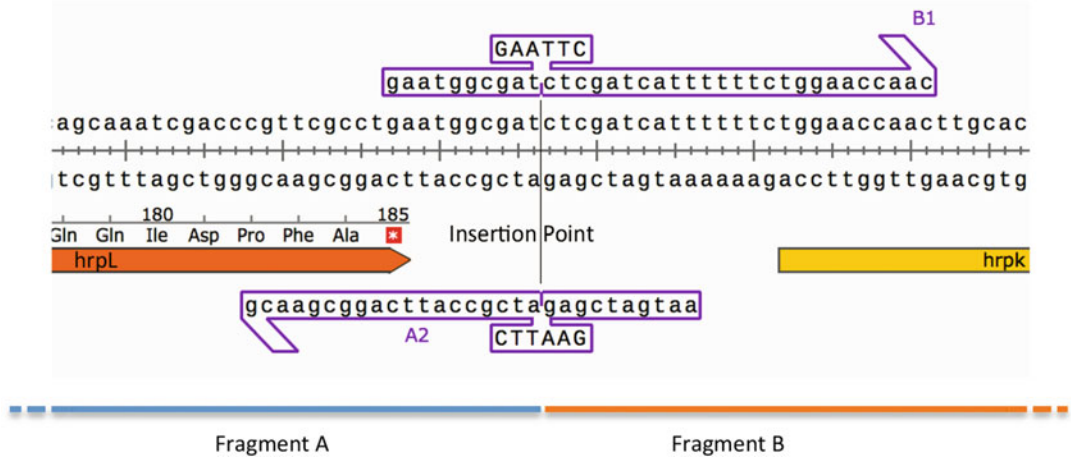

Fig. 2 Sequences and positions of primers A2 and B1 used to amplify fragments A and B to generate the allelic exchange vector used to generate chromosome located *hrpL* transcriptional fusions. Insertion point indicates the position in which, through cloning on the indicated *Eco*RI site, the promoterless reporter ORF would be inserted

gene of interest. We chose nucleotide 8 for the *hrpL:: mturquoise2* construct (Fig. 2). Fragment A in the allelic exchange module should correspond to the 500 bp (approximately) upstream of the insertion point, while fragment B should correspond to the 500 bps downstream. For each fragment, we designed a forward primer (A1 and B1, respectively) and a reverse primer (A2 and B2, respectively). Primers A1 and B2 are regular primers located approximately 500 bps upstream and downstream of the insertion point, respectively. Primers A2 (reverse primer for fragment A) and B1 (forward primer for fragment B) (Fig. 2) should include a restriction enzyme site (in this case a *Eco*RI site) upstream the insertion point, plus an additional ten nucleotide-long overlapping sequence.

3.1.2 Consecutive PCRs

Three consecutive PCR reactions are required to obtain the A + B fragment. This is achieved by following the following five steps:

1. To generate fragments A and B, a 25 μL PCR reaction with the respective primer pairs is carried out using DC3000 genomic DNA as template, 0.2 mM dNTP, 0.5 μM of each primer (Table 2) and 0.25 μL Q5 High-Fidelity DNA Polymerase (NEB, UK). The reaction is performed according to the supplier's protocol, starting with an initial 30 s at 98 °C denaturation step, followed by 35 cycles at 98 °C for 10 s, 60 °C (*see* **Note 1**) for 20 s, and 72 °C for 30 s; and finishing with a further 2 min at 72 °C.
2. Resolve the PCR samples by electrophoresis in a 1% agarose gel containing 1× RedSafe. Cut out the bands corresponding to the predicted molecular size (around 500 bp) using a blade and

purify the DNA using a Gel Band Purification Kit (e.g., Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, Spain), following the manufacturer's instructions.

3. Using equal amounts (10–20 ng) of each of the purified fragments A and B, carry out a second reaction, using 0.2 mM dNTP and 0.25 μL Q5® High-Fidelity DNA Polymerase, but without additional primers or template. Put the mix into a thermocycler and subject it to eight cycles at 98 °C for 10 s, 60 °C (*see* **Note 1**) for 20 °C and 72 °C for 45 s, with a final extension for 2 min at 72 °C.
4. Use 5 μL of the Step 3 reaction as template in a third consecutive PCR, containing 0.2 mM dNTP, 0.5 μM of each primers A1 and B2 and 0.25 μL Q5® High-Fidelity DNA Polymerase. Run the thermocycler using identical cycles and conditions as used in the first PCR—Step 1 (*see* Subheading 3.1.2).
5. Visualize the PCR sample as above by electrophoresis on a 1% agarose gel. Recover the DNA corresponding to the 1 Kb band as described.

3.1.3 pGEM®-T Cloning and Selection

1. Set up the pGEM®-T ligation reaction, using 3.3 μL (50–100 ng) of your purified DNA (*see* **Note 2**), 0.7 μL of pGEM®-T vector, 1 μL of T4 DNA ligase and 5 μL of 2× Ligase buffer, to a volume of 10 μL. Incubate overnight at 16 °C.
2. Mix 2 μL of the ligation with 20 μL of *E. coli* DH5α competent cells and transform following an appropriate protocol (*see* **Note 3**).
3. Plate the transformation onto LB agar plates supplemented with ampicillin (100 μg/mL) and X-gal (40 μg/mL). Incubate plates at 37 °C overnight.
4. Pick 2–3 white colonies and grow them overnight in LB broth supplemented with ampicillin (100 μg/mL).
5. Extract the plasmid from the bacteria using a plasmid mini-prep protocol (*see* **Note 4**). Confirm sequence of the fragment generated (Fragment A + B) by sequencing.

3.1.4 Restriction and Ligation

1. To generate the allelic exchange plasmid cut the pGEM®-T derivative obtained carrying the A + B fragment with an appropriate restriction enzyme (*see* **Note 5**). Use the same enzyme to cut the plasmid containing the *mturquoise2-nptII* construct (Table 1) (*see* **Note 6**). Mix 2 μg of each plasmid with 5 μL of 10× buffer, 1 μL of EcoRI (10 U), and $_{dd}H_2O$ up to 50 μL. Incubate the reactions at 37 °C for 1 h.
2. Analyze the sample by electrophoresis in a 1% agarose gel (*see* **Note 7**). The expected sizes in this particular example are 4 Kb for the pGEM®-T- fragment A + B digested plasmid and 2.2 Kb for the *mturquoise2-nptII* construct.

3. Purify the bands of the correct sizes as described above. Measure the DNA concentration using a NanoDrop® Spectrophotometer.
4. Set up a ligation reaction as follows: 20 ng of linearized pGEM®-T- fragment A + B, 30 ng of *mturquoise2-nptII* fragment, 1 U T4 DNA ligase, 1× T4 DNA ligase buffer, and $_{dd}H_2O$ up to 10 μL. Incubate the reaction overnight at 16 °C.
5. Mix 2 μL of the ligation with 20 μL of *E. coli* DH5α competent cells and transform following an appropriate protocol.
6. Plate the transformation in LB agar plates supplemented with kanamycin (50 μg/mL) (*see* **Note 8**).
7. Pick 2–3 colonies and grow them overnight in LB broth supplemented with kanamycin (50 μg/mL). Incubate at 37 °C overnight. Since at this stage expression of the fluorophores can be driven from a constitutive promoter from the plasmid backbone, for some fluorophores (i.e., mOrange, eYFP, and mPlum) *E. coli* transformants may appeared colored on the plate (Fig. 3).
8. Extract the plasmid from the selected clones using a plasmid mini-prep protocol (*see* **Note 4**). Check the orientation of the *mturquoise2-nptII* insert by restriction endonuclease analysis (*see* **Note 9**).

3.2 Introducing the Transcriptional Fusions into the P. syringae Chromosome

3.2.1 Preparing P. syringae Competent Cells

1. Streak from −80 °C stock the *P. syringae* strain to be transformed onto an LB plate and incubate for 2 days at 28 °C (*see* **Note 10**).
2. Scrape all biomass off of the plate and suspend it in 1 mL of chilled 300 mM sucrose. Keep the cells on ice unless otherwise stated.
3. Spin for 2 min at 12500 × *g* at 4 °C. Discard supernatant and resuspend the pellet into 1 mL of chilled 300 mM sucrose.
4. Repeat **step 3** three times.
5. Remove all the supernatant and resuspend the pellet in 100 μL of chilled 300 mM sucrose. The cells are now ready for electroporation (*see* **Note 11**).

3.2.2 Electroporation of P. syringae

1. Add a maximum of 2 μL of your purified allelic exchange plasmid to the 100 μL of competent cells. Mix by gently pipetting up and down.
2. Place the mix in a cold 2 mm electroporation cuvette and keep on ice.
3. Electroporate with a pulse of 25 kV/cm, and place the cuvette on ice immediately after. Add 1 mL of liquid LB. Mix gently.
4. Transfer the volume into a fresh Eppendorf tube and incubate for 1 h at 28 °C prior to plating on the selective medium.
5. Incubate the plates at 28 °C for 48 h.

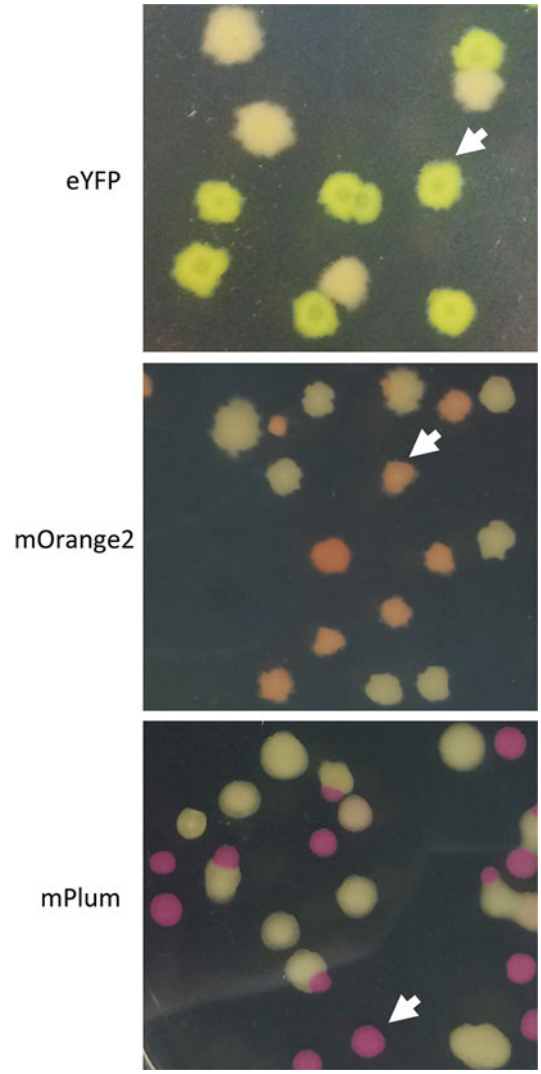

Fig. 3 Images taken from the selection plates used to get *E. coli* transformants carrying the allelic exchange vectors for: '*hrpL::eyfp* (top), *'hrpL::mOrange* (center) and *'hrpL::mPlum* (bottom). Positive transformants colonies appeared colored as indicated by arrowheads

3.2.3 Clone Selection

All kanamycin-resistant clones obtained in the previous step would have incorporated the *nptII* gene, however, some would have done so through integrating the whole plasmid (via a single recombination event in one of the homologous 500 pb regions flanking the insertion point), while others would have integrated only the fluorescent reporter module by allelic exchange (via a double recombination event in both the homologous A and B 500 bp regions flanking the insertion point). Only the latter would carry a stable transcriptional fusion of the gen of interest (*hrpL*) to the fluorescent

reporter (*mturquoise2*). These two types of recombinant clones can be differentiated on the basis of their resistance to ampicillin, since only the first type which has the pGEM®-T backbone would be resistant. To check this:

Replicate up to 50 colonies obtained following the transformation, in three different LB agar plates supplemented with: (1) 15 μg/mL kanamycin, (2) 500 μg/mL ampicillin, and (3) a not supplemented (without antibiotics) control plate (*see* **Note 11**). Incubate the plates overnight at 28 °C.

Check growth on the plates: double recombinant clones carrying the desired chromosomal fusion would grow in LB + kanamycin, would not grow in LB + ampicillin, and would grow on the control, not supplemented plate (without antibiotics) (*see* **Note 12**). Pick several clones showing the correct antibiotic resistance profile from the control plate and inoculate them into liquid LB medium supplemented with 15 μg/mL kanamycin. Incubate overnight at 28 °C with shaking. Store these clones at −80 °C in 25% glycerol. The correct chromosome insertion must be confirmed by Southern blot analysis (*see* Subheading 3.4).

3.3 Removing the Kanamycin Resistance Gene

3.3.1 *P. syringae* Transformation and Selection of the Clones

1. Prepare electrocompetent cells of the *P. syringae* strain to be used, as indicated in Subheading 3.2.1.
2. Add up to 2 μL of a pFLP2 vector preparation and transform as indicated in Subheading 3.2.2.
3. Plate the cells onto LB agar plates supplemented with ampicillin at 500 μg/mL. Incubate the plates at 28 °C for 48 h.
4. Pick up to five colonies from the selection plate and inoculate them into liquid LB medium without any antibiotic. Incubate overnight at 28 °C with aeration. Make serial dilutions into 10 mM $MgCl_2$ using the saturated cultures (*see* **Note 13**). Incubate at 28 °C for 48 h.
5. Replicate up to 5 colonies from each plate in different LB agar supplemented with: (1) kanamycin 15 μg/mL, (2) ampicillin (500 μg/mL) and (3) not supplemented (without antibiotic). Incubate at 28 °C for 48 h.
6. Check bacterial growth on the plates. The desired clones are those that do not grow in either kanamycin or ampicillin plates, but grow in the control LB plates without selection.
7. Pick one colony from each clone from the LB nonsupplemented control plate and use to inoculate LB liquid medium. Incubate overnight at 28 °C with aeration. Store the clones at −80 °C in 25% glycerol. The correct deletion of the antibiotic resistance gene must be confirmed by Southern blot analysis (*see* Subheading 3.4).

3.4 Confirming Allelic Exchange and/or Removal of the Kanamycin Resistance Gene by Southern Blot Analysis

1. Inoculate 5 mL of LB liquid medium with the strain to be tested. Incubate overnight at 28 °C with aeration. Harvest the cells by centrifugation at 12,000 × *g* for 5 min and extract genomic DNA using the Jet Flex extraction kit or equivalent, following the instructions of the manufacturer. Measure DNA concentration.
2. Digest 2 μg of DNA with the appropriate enzyme to ensure transformants carry a single insertion on the correct position and/or have lost the *nptII* gene (*see* **Note 7**).
3. Load the restrictions into a 0.7% agarose gel and separate by electrophoresis for 1–2 hours at 100 V (*see* **Note 14**).
4. Depurinate DNA by submerging the gel into 0.25 N HCl for 15 min at room temperature with gentle shaking.
5. Wash the gel by submerging it into $_{d}H_2O$. Repeat three times.
6. Submerge the gel in denaturing solution and incubate for 30 min at room temperature with gentle shaking.
7. Remove denaturing solution. Add neutralizing solution and incubate for 30 min at room temperature with gentle shaking.
8. Transfer DNA onto a nylon membrane (*see* **Note 15**), and cross-link it by exposing the DNA-bound side of the membrane to UV light (0.120 J).
9. Carry out prehybridization and hybridization stages at 65 °C, and a digoxigenin-labeled DNA fragment containing FRT-*nptII*-FRT as probe (*see* **Note 16**).
10. Develop the membrane. In our example, membrane was developed using anti-digoxigenin antibody and CSPD (Roche, Germany), following instructions of the manufacturers.

3.5 Analysis of the Strain

Once the strains carrying the chromosome-located transcriptional fusions are confirmed by Southern Blot analysis (Fig. 4a), they are ready for experimental work. Since our purpose was to use the generated strain to carry out confocal microscopy, we tested expression of the transcriptional fusion in bacteria extracted from the plant apoplast (*see* **Note 17**), where in planta activation of *hrpL* expression can be analyzed [10]. Confocal microscopy (*see* **Note 18**) on *Arabidopsis* apoplast-extracted bacteria carrying the *hrpL::mturquoise2* transcriptional fusion showed an activation of gene expression. In this particular case, this activation was characterized by a strong phenotypic heterogeneity, as previously reported for the same gene in the closely related pathogen *P. syringae* pv. *phaseolicola* 1448A in bean leaf apoplasts [10] (Fig. 4b).

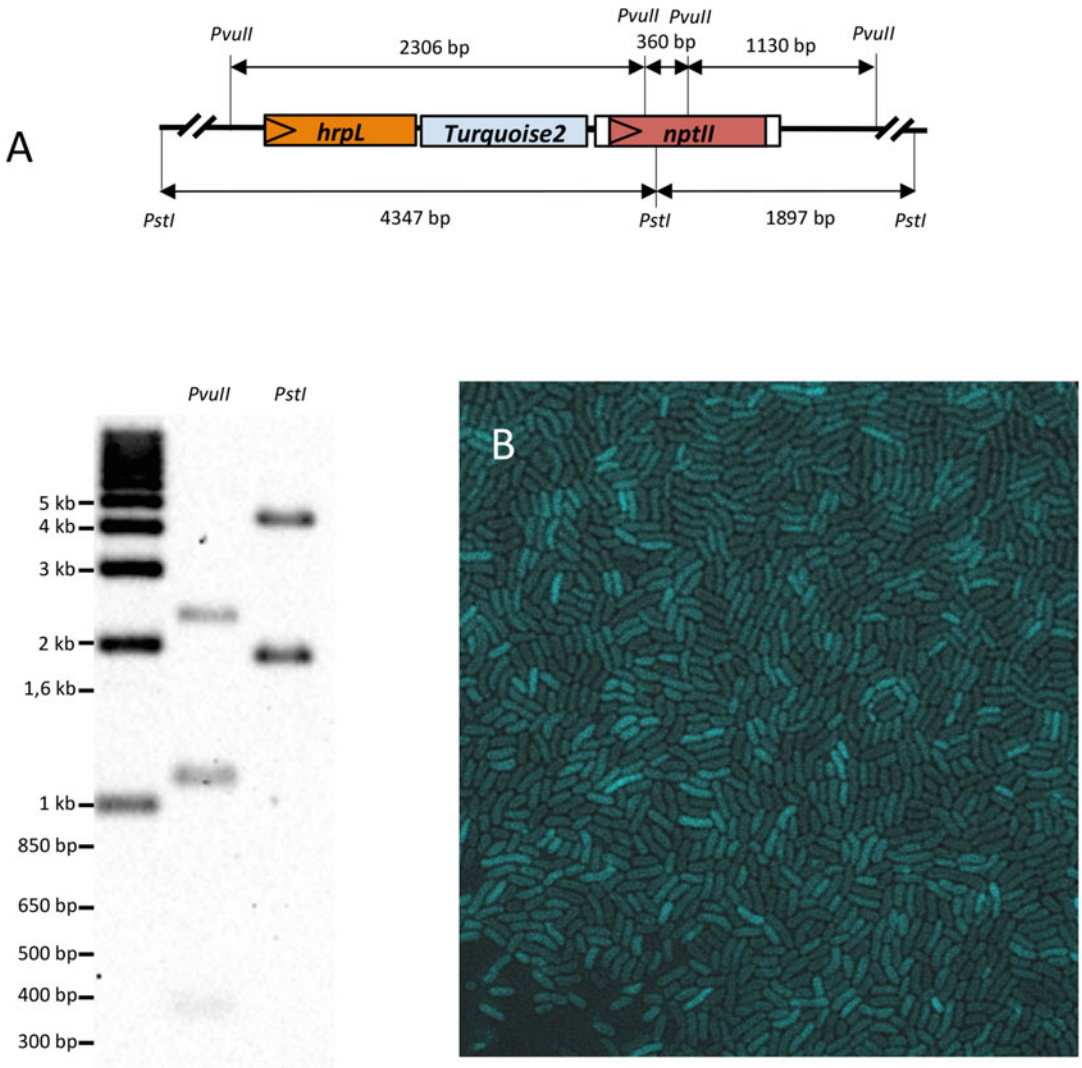

Fig. 4 Southern blot analysis and confocal microscopy analysis of *P. syringae* pv. tomato DC3000 carrying a *hrpL::mturquoise2*. (**a**) Southern blot analysis of genomic DNA from DC3000 derivative carrying the *hrpL::mturquoise2* transcriptional fusion. The diagram shows the organization and expected sizes of the construct upon digestion of genomic DNA using the indicated restriction enzymes. Below, blot of genomic DNA of the strain digested with either *Pvu*II or *Pst*I after probe hybridization, displaying bands with the expected sizes (**b**) Confocal microscopy image of *Arabidopsis* apoplast-extracted bacteria showing phenotypically heterogeneous activation of *hrpL::mturquoise2* expression

4 Notes

1. Optimize annealing temperature to the primers used.
2. For ligation of blunt-end fragments in pGEM-T, a regular A-tailing reaction must be performed, as follows: 1 μL of 10× standard buffer (with $MgCl_2$), 2 μL of 1 mM dATP, 0.2 μL of Taq DNA Polymerase, and 6.8 μL of your purified PCR fragment. Run the reaction for 30 min at 72 °C.

3. Any high-efficiency *E. coli* transformation protocol can be used. We obtained competent DH5α cells using a modification of the method described by Inoue and colleagues [23]. Briefly, a 1:1000 dilution of a saturated bacterial culture into 200 mL of SOB medium [19] was incubated with aeration at 22 °C cells reached an OD_{600} of 0.5. After chilling the culture in ice for 10 min, cells were collected by centrifugation (2500 × *g* 4 °C 10 min), suspended in 80 mL of ice-cold TB buffer and kept in ice for 10 min. The process was repeated twice with the cells suspended in 20 mL of TB after the second centrifugation step. After adding 1.5 mL of DMSO cells aliquots were kept at −80 °C prior to use. Transformation was carried out by heat-shock [19].
4. DNA plasmid extractions were carried out using the method described by [24], using isopropanol to precipitate DNA.
5. We have used *Eco*RI but any other 6-cutter restriction enzyme not cutting within the sequences of the 1 Kb A + B fragment generated flanking the fluorophore-antibiotic resistance reporter cassette can be used.
6. The plasmids containing the fusions of the fluorescent proteins to Km were generated as follows: The ORF of each fluorescent protein (Turquoise2, eYFP, mPlum, mOrange2, mCherry) was amplified using the primers Prot Fluor F and Prot Fluor-Km R or GFP$^+$ F and GFP$^+$Km R, in the case of GFP+ (Table 1), and the corresponding plasmids (Table 2). The FRT-Km-FRT fragment was amplified using the primers Km-Prot Fluor F and P2. The PCR reactions were set up as in Subheading 3.1.2. Each fluorescent protein ORF was fused to Km by PCR using equal amount of each fragment (10–20 ng) and the primers Prot Fluor F and P2 (for Turquoise2-Km, eYFP-Km, mPlum-Km, mOrange2-Km and mCherry-Km fusions) or GFP$^+$ and P2 (for the GFP$^+$-Km fusion). The resulting fragments were cloned in pGemT (*see* Subheading 3.1.3) to generate plasmids pGT-Turquoise2, pGT-YFP, pGT-mPlum, pGT-mOrange2, pGT-mCherry, and pGT-GFP$^+$ (Table 2).
7. Percentage of agarose within the gel and electrophoresis conditions should be adjusted depending on the expected size of the fragments.
8. Antibiotic resistance genes different from *nptII* could be used and therefore the antibiotic for plate selection should be modified accordingly.
9. The ORF of the gene encoding the fluorophore must be in the same orientation of the gene to which is to be fused. The selection of the restriction enzymes to be used in the restriction analysis of the resulting plasmid must generate DNA fragments of sufficiently different sizes in each possible orientation as to allow unequivocally determination of the correct one.

10. Plates should be fresh as storage at 4 °C can reduce the efficiency of transformation.
11. Storage of *P. syringae* competent cells at −80 °C drastically reduces transformation efficiency.
12. An LB plate is used in order to make completely sure that the selected clone is not ampicillin resistant. An LB plate supplemented with kanamycin could be used as well.
13. Dilutions 10^{-4} and 10^{-5} should be sufficient to ensure isolated colonies that have lost the *nptII* gene are obtained.
14. Digestions should be designed using two enzymes: one that cuts (e.g., *Pvu*II) and another that does not cut within the probe, respectively, as long as the fragments generated are not larger than 5 Kb.
15. DNA transfer from the agarose gel onto the membrane was carried out using upward capillarity transfer, but other means could be used as well.
16. A probe containing the *nptII* gene flanked by two FRT sites was generated by PCR using chemiluminescent digoxigenin-dNTPs and DIG Labelling Mix (Roche, Germany), primers P1 and P2, and pKD4 as the DNA template.
17. *Arabidopsis* leaves were pressure infiltrated using a needleless syringe and approximately 100 μL of a 5×10^5 cfu/mL bacterial suspension in 10 mM $MgCl_2$. Three days post inoculation (dpi) bacteria were recovered from the plant by an apoplastic fluid extraction. The apoplastic fluid extraction was carried out by pressure infiltrating a whole leaf with 3 mL of a 10 mM $MgCl_2$ solution inside a 10 mL syringe. Following 5 cycles of pressure application, the flow-through was removed and placed in two fresh 1,5 mL tubes and centrifuged for 1 min at max speed. Pellets were resuspended into 50 μL of $MgCl_2$ and analyzed by confocal microscopy.
18. Images of apoplast-extracted bacteria were taken using the Leica SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Variable AOTF filters were used for the visualization of Turquoise2 (excitation 405 nm/emission 425 to 500 nm). Image processing was performed using Leica LAS AF (Leica Microsystems).

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Chapter 16

Introduction of Genetic Material in *Ralstonia solanacearum* Through Natural Transformation and Conjugation

Anthony Perrier, Patrick Barberis, and Stéphane Genin

Abstract

Ralstonia solanacearum is a soil-borne plant pathogen, responsible of the bacterial wilt disease. Its unusual wide host range (more than 250 plant species), aggressiveness, and broad geographic distribution have made of this bacterium the main plant pathogenic model in the beta-Proteobacteria class. Many *R. solanacearum* strains have the ability to internalize exogenous DNA through natural transformation. This property is widely used in reverse genetics studies to create mutants or reporter gene constructs, in the aim to study the molecular bases of pathogenesis of this bacterium. In this chapter, we describe three in vitro methods (natural transformation, electrotransformation, and conjugation) commonly used to produce recombinant *R. solanacearum* cells after introduction of exogenous DNA.

Key words Natural competence, Bacterial conjugation, Electroporation, Plant pathogen, Reverse genetics

1 Introduction

The *R. solanacearum* species complex is divided in four monophyletic groups, designated as phylotypes I to IV, which are generally associated with the geographic origin of the strains [1, 2]. In the present chapter we present protocols primarily used and optimized for the phylotype I strain GMI1000, but can be extended to some other strains from the species complex with variable efficiency [3, 4]. The transfer of exogenous DNA inside the bacterial cells is a prerequisite to all the basic molecular techniques used to study this pathogen including the generation of mutants or gene reporter fusions and the completion of genetic complementation assays [5–7].

Many *R. solanacearum* strains, but not all, are naturally competent and can be transformed in vitro [3]. Although both the *comA* gene product and type IV pili were shown to be required for natural transformation [8, 9], the precise mechanism of DNA uptake and transport remains unknown. Natural transformation is

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related to the development of a competent state, which depends on the physiological state of the cells. Only cells in exponential growth achieve the competent state, which declines rapidly during the log phase [7]. The competent state is induced by culturing the bacterial cells in a limiting growth medium [4, 10]. To do so, bacteria are grown in a minimal growth medium supplemented with 2% of glycerol as sole carbon source, as it is poorly metabolized by *R. solanacearum*. The limit in size of the exogenous DNA fragments incorporated is rather large since DNA blocks up to 30 kb were reported to be integrated after natural transformation [11]. Different types of DNAs can be used for natural transformation. Genomic DNA fragments carrying a selectable antibiotic resistance gene can be used for quick generation of deletion or disruption mutants (*see* for example ref. 12). Linearized plasmids containing two regions of homology are used for stable chromosomal insertion allowing trans-complementation or reporter gene fusion analysis [7]. Circular plasmids containing chromosomal homology region can also be incorporated during the competent state, such as the nonreplicative plasmid pK18*mobsacB* [13] used to generate chromosomal deletion or site-directed mutagenesis.

We detail in the present chapter the three main methods commonly used to introduce genetic material in *R. solanacearum* and generate modified strains.

2 Materials

1. *Escherichia coli* strains pRK2013, pRK2073. *R solanacearum* strain...
2. BG medium: 10 g/L bacto peptone, 1 g/L casamino acids, 1 g/L yeast extract. For agar plates, BG medium is supplemented with agar (15 g/L), glucose (5 g/L), and optionally with triphenyl-tetrazolium chloride (0.05 g/L) to ease phenotypic characterization.
3. Minimal medium: 0.125 mg/L $FeSO_4{\cdot}7H_2O$, 0.5 g $(NH_4)_2SO4$, 0.05 g/L $MgSO_4{\cdot}7H_2O$, 3.4 g/L KH_2PO_4. The pH is adjusted to 6.5 with KOH. This medium corresponds to one-quarter strength M63 [14]. Supplement with 2% glycerol when necessary.
4. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl. For agar plates, LB medium is supplemented with agar (15 g/L).
5. Antibiotics used at the following final concentrations: 25 mg/L kanamycin, 10 mg/L gentamycin, 10 mg/L tetracycline, 50 mg/L ampicillin, 25 mg/L chloramphenicol for *E. coli*. 50 mg/L kanamycin, 40 mg/L spectinomycin, 10 mg/L gentamycin, 10 mg/L tetracycline for *R. solanacearum*.

6. 10% glycerol in ultrapure water.
7. Nitrocellulose filter (pore size 0.45 μm).

3 Methods

3.1 Induction of the Competent State

1. Cultivate bacteria from −80 °C stock on BG agar plates with the appropriate antibiotics during 2 days at 28 °C.
2. Inoculate a single colony from the BG agar plate into 10 mL of minimal medium supplemented with 2% glycerol. Incubate 2 days under shaking at 180 rpm at 28 °C.

3.1.1 Natural Transformation

1. Transfer 50 μL of competent cells into a sterile 1.5 mL micro-centrifuge tube.
2. Add 5 μL of plasmidic DNA (300–500 ng) or 10 μL of genomic DNA (2–4 μg) to the competent cells. Mix gently.
3. Spot the entire volume into the center of a BG agar plate (*see* **Notes 1** and **2**).
4. Incubate at 28 °C for 2 days.

3.1.2 Selection of the Transformants

1. Transfer the bacteria with a sterile 10 μL inoculation loop into a sterile 1.5 mL micro-centrifuge tube containing 1 mL of liquid BG medium. Gently resuspend the cells by pipetting up and down.
2. Plate 100 μL of the resuspended cells on BG agar plate containing the appropriate antibiotics (*see* **Note 3**).
3. Centrifuge the 900 remaining microliters at 16,000 × *g* for 2 min. Discard the supernatant and resuspend the pellet with 200 μL of liquid BG medium.
4. Plate 100 μL of the resuspended cells on two agar plates containing the appropriate antibiotics (*see* **Note 4**).
5. Incubate the plates at 28 °C for 2 days. Identify growing colonies and streak on a fresh agar plate containing the appropriate antibiotics. Further steps such as the confirmation of a correct DNA recombination event in the genome start here.

3.2 Electro-Transformation of *R. Solanacearum* Cells

While most of the DNA types can be integrated in the cells with high efficiency by natural transformation, electrotransformation could be useful for the integration of high molecular weight plasmid such as pLAFR-derived replicative plasmids [15]. Electrocompetent cells are made by concentrating and washing cells cultivated overnight in rich BG medium (*see* **Note 5**). The application of an electrical field to the electro-competent chilled cells will increase their permeability and facilitate the incorporation of the charged DNA [16].

3.2.1 Preparation of the Electrocompetent Cells

1. Cultivate bacteria from −80 °C stock on BG agar plates with the appropriate antibiotics during 2 days at 28 °C.
2. Inoculate one colony from the BG agar plate into a desired volume of liquid BG medium (*see* **Note 6**). Incubate overnight under shaking at 180 rpm at 28 °C.
3. Centrifuge 1 volume of culture at 16,000 × *g* for 2 min.
4. Discard the supernatant. Resuspend the pellet with 1/2 volume of ultrapure water (*see* **Note 7**) and centrifuge at 16,000 × *g* for 2 min at 4 °C.
5. Discard the supernatant. Resuspend the pellet with 1/4 volume of ultrapure water and centrifuge at 16,000 × *g* for 2 min at 4 °C.
6. Discard the supernatant. Gently resuspend the pellet with 1/8 volume of ultrapure water supplemented with 10% glycerol and centrifuge at 16,000 × *g* for 2 min at 4 °C.
7. Discard the supernatant. Gently resuspend the pellet with 1/80 volume of ultrapure water supplemented with 10% glycerol.
8. Prepare 50 µL aliquots in 1.5 mL microcentrifuge tubes.
9. Add 5 µL (300–500 ng/µL) of dialyzed plasmid DNA to a 50 µL aliquot and mix gently.

3.2.2 Electro-transfomation

1. Transfer the mixture to an electroporation cuvette (1 mm).
2. Electroporate at 1.8 kV for 5 ms.
3. Resuspend the cells into 1 mL of BG medium.
4. Incubate 2 h under shaking at 180 rpm at 28 °C.

3.2.3 Selection of the Transformants

1. Plate 100 µL of the resuspended cells on BG agar plate containing the appropriate antibiotics.
2. Centrifuge the 900 remaining microliters at 16,000 × *g* for 2 min. Discard the supernatant and resuspend the pellet with 200 µL of liquid BG medium.
3. Plate the resuspended cells on two BG agar plates containing the appropriate antibiotics.
4. Incubate the plates at 28 °C for 2 days.

3.3 Triparental Mating

Triparental mating is a natural way to introduce circular plasmids into *R. solanacearum* cells, especially for those of high molecular size. For example, a 560 Kb plasmid was successfully delivered in a recipient strain using this technique [17]. In case of the absence of *tra* genes in the mobilizable plasmid (which is generally the case for all small replicative plasmids in *E. coli*), the conjugative transfer will require a helper plasmid providing the *tra* genes [18]. Thus, three different strains are required in this so-called "triparental mating": a

donor *E. coli* strain carrying a mobilizable plasmid of interest, a helper *E. coli* strain containing a conjugative plasmid (pRK2013 carrying kanamycin resistance or its derivative pRK2073 carrying spectinomycin resistance [18]) and a recipient *R. solanacearum* strain.

1. Cultivate the *E. coli* donor and helper strain from −80 °C stock on LB agar plates with the appropriate antibiotics during 1 day at 37 °C.
2. Cultivate the *R. solanacearum* recipient strain from −80 °C stock on BG agar plate with the appropriate antibiotics during 2 days at 28 °C.
3. Inoculate a single colony of the *E. coli* donor and helper strain from the LB agar plate into 5 mL liquid LB medium with the appropriate antibiotics. Incubate overnight under shaking at 180 rpm at 28 °C.
4. Inoculate one colony of the *R. solanacearum* recipient strain from the BG agar plate into 5 mL liquid BG medium with the appropriate antibiotics. Incubate overnight under shaking at 180 rpm at 28 °C.
5. Add 500 µL of the overnight *E. coli* cultures into 4.5 mL of LB medium. Incubate under shaking at 180 rpm at 37 °C until mid log phase (*see* **Note 8**).
6. Add 5×10^8 of both donor & helper *E. coli* cells for 10^8 recipient cells (with a volume of 500 µL per strain) in a sterile 2 mL micro-centrifuge tube.
7. Centrifuge at 16,000 × *g* for 1 min.
8. Discard the supernatant. Gently resuspend the pellet with 100 µL of sterile water.
9. Transfer the bacteria into the center of a nitrocellulose filter on a BG agar plate (*see* **Note 1**).
10. Incubate during 6–12 h at 28 °C (*see* **Note 9**).
11. Transfer the filter into a sterile 1.5 mL micro-centrifuge tube containing 1 mL of liquid BG medium.
12. Vortex during 30 s to resuspend the cells (*see* **Note 10**).
13. Plate 100 µL of the resuspended cells on BG agar plate containing the appropriate antibiotics.
14. Centrifuge the 900 remaining microliters at 16,000 × *g* for 2 min. Discard the supernatant and resuspend the pellet with 200 µL of liquid BG medium.
15. Plate 100 µL of the resuspended cells on two BG agar plates containing the appropriate antibiotics (*see* **Note 4**).
16. Incubate the plates at 28 °C for 2 days.

4 Notes

1. At this step the BG agar plate should not contain glucose and triphenyl-tetrazolium chloride.
2. Alternatively, the BG agar plate can be replaced by a minimal medium agar plate supplemented with 2% glycerol to increase the transformation efficiency.
3. When transforming with genomic DNA, the efficiency may vary a lot depending on the DNA purity and integrity. It is therefore recommended to also plate at ten times dilution of the resuspended cells to avoid a bacterial lawn in case of high-transformation efficiency.
4. As indicated in **Note 3**, this can result in a bacterial lawn, but in case of low-transformation efficiency it is preferable to do it as a backup.
5. Electro-competent cells of *R. solanacearum* cannot be stored reliably at −80 °C. In order to keep high transformation efficiency, we recommend preparing the cells extemporaneously.
6. 4 mL of overnight culture is necessary to make 1 aliquot of 50 μL of electrocompetent cells.
7. The ultrapure water and the glycerol should be prechilled at 4 °C and all the following steps should be done on ice.
8. It is important to use *E. coli* strains from exponential growth phase (OD600 = 0.5).
9. To keep high-transformation efficiency, this step should not be longer than 12 h as *R. solanacearum* can have a deleterious effect on the viability of *E. coli* cells.
10. At this step, if the recipient strain does not possess a selective marker to counter-select *E. coli* cells, we recommend to add some bacteriophage T4, which is specific for *E. coli*, to get rid of the donor strain.

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Chapter 17

In Vitro and In Vivo Secretion/Translocation Assays to Identify Novel *Ralstonia solanacearum* Type 3 Effectors

Fabien Lonjon, Nemo Peeters, Stéphane Genin, and Fabienne Vailleau

Abstract

Phytopathogenic bacteria have evolved multiple strategies to infect plants. Like many gram-negative bacteria, *Ralstonia solanacearum*, the causal agent of bacterial wilt, possesses a specialized protein secretion machinery to deliver effector proteins directly into the host cells. This type 3 secretion system (T3SS) and the bacterial proteins translocated, called type 3 effectors (T3Es), constitute the main pathogenicity determinants of the *R. solanacearum* species complex (RSSC). Up to 113 orthologous groups defining T3E genes have been identified among the RSSC strains sequenced to date. The increasing number of *R. solanacearum* genomic sequences available still expands the number of T3E candidates which require experimental validation. Here, we describe in vitro (type 3 secretion) and in vivo (type 3 translocation based on CyaA′ reporter gene) methods to identify and validate type 3-dependent delivery of proteins of interest highlighted as candidate T3Es. We also present protocols to generate dedicated vectors and *R. solanacearum* transformation to perform these experiments.

Key words Secretion, Translocation, *Ralstonia solanacearum*, Type 3 secretion system, Type 3 effector, CyaA′

1 Introduction

Ralstonia solanacearum species complex (RSSC), the causal agent of bacterial wilt, possesses a wide host range, infecting more than 250 plant species within 50 botanical families [1, 2]. It includes important crops such as tomato, potato, eggplant, or banana, but also the model plants *Arabidopsis thaliana* and *Medicago truncatula*. *R. solanacearum* has been ranked as the second most important bacterial plant pathogen based on scientific/economic criteria [3]. Many strains, with different geographic origins, have been isolated and characterized, each belonging to one of the four phylotypes (I, II, III, and IV), altogether representing the RSSC [4, 5]. Recent studies proposed the division of the RSSC into three species, clustering phylotype I and III strains [6, 7]. This soilborne bacterium enters into the plant via the roots, then spreads to the

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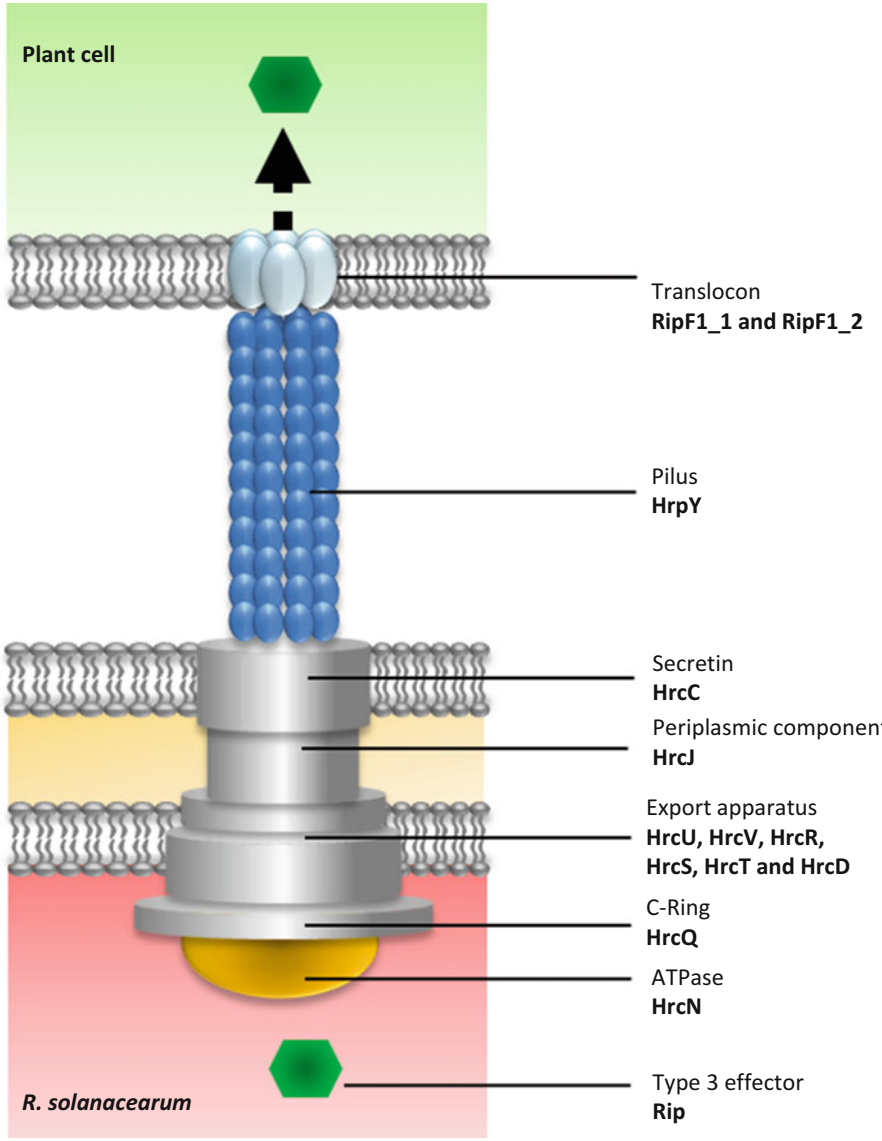


Fig. 1 Schematic representation of *R. solanacearum* type 3 secretion system. Black lines point out the names of the different structures forming the syringe. In bold are indicated the names of the *R. solanacearum* proteins composing these different structures. *Hrp* Hypersensitive response and pathogenicity genes; *Hrc* Hrp conserved, *Rip* Ralstonia injected protein

aerial parts of the plant through the xylem [8]. Among many molecular pathogenicity determinants, the type 3 secretion system (T3SS) and the translocated substrate proteins, called type 3 effectors (T3Es), constitute the main determinants of *R. solanacearum* virulence [9] (Fig. 1). Mutants defective for T3SS are unable to cause a hypersensitive response (HR) or disease symptoms on plants and are called *hrp* (HR and pathogenicity) mutants [8, 10]. The primary function of T3Es is to promote disease by targeting various plant pathways [11–13]. One particularity of *R. solanacearum* is its

large number of T3Es (up to 76 T3Es delivered by a single strain), corresponding to 113 T3Es genes, called Rip for Ralstonia injected proteins [14]. However, very few T3Es have been described as been required for bacterial pathogenicity, probably due to functional redundancy [15, 16]. Indeed, almost all single T3E mutants are not distinguishable in disease appearance from wild-type strains, neither on Arabidopsis Col-0 plants, nor on Marmande VR tomato cultivar. Stronger phenotypes were obtained using T3E polymutants [15, 17–19]. Some *R. solanacearum* T3Es can be recognized by plant resistance proteins, leading to plant resistance [20].

Thanks to next-generation sequencing (NGS) technology, many more *R. solanacearum* genomes are now available, giving rise to new putative T3Es. It is crucial to validate the type 3 secretion/translocation of any new T3E candidate, before trying to unravel its contribution to the virulence of a given strain. This can be performed in vitro (type 3 secretion) or in vivo (type 3 translocation). For secretion assays, strains carrying a candidate T3E fused to a triple HA tag (3HA) are grown in a secretion inducing media. Then bacterial pellets and supernatants are separated, and analyzed by immunoblot to detect secreted proteins [21, 22]. For translocation assays, strains carrying a candidate T3E fused to a CyaA′ domain (4–1197 N-terminal part) as a reporter gene. CyaA′ domain originates from a calmodulin-dependent adenylate cyclase domain of the cyclolysin toxin of *Bordetella pertussis* [23]. If the candidate T3E-CyaA′ fusion is injected into the host cytoplasm, we can detect high amounts of cyclic adenosine monophosphate (cAMP) *in planta* [24, 25] (Fig. 2). The *cyaA′* gene can also be

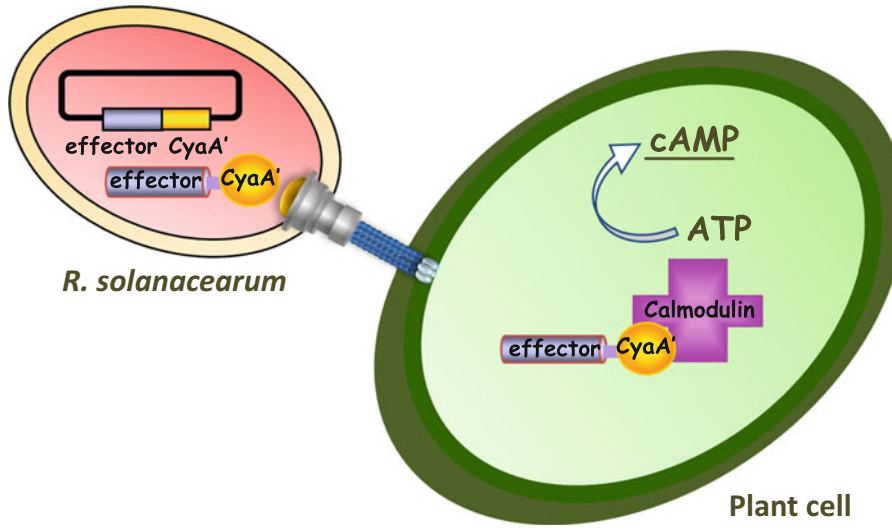


Fig. 2 Schematic representation of CyaA′ translocation assay in *R. solanacearum*. A candidate type 3 effector is fused to the CyaA′ 4–1197 N-terminal part in a replicative plasmid in *R. solanacearum*. If the fusion protein is injected inside the plant cell, CyaA′ can convert ATP into cyclic AMP (cAMP). Then cAMP concentration can be detected by enzyme-linked immunosorbent assay (ELISA)

used for the identification of a large repertoire of T3Es in a given strain using as transposon insertion for a functional screening approach [26].

Here, we provide a protocol for the identification and validation of putative *R. solanacearum* T3Es. The experimental procedures include the generation of the required vectors by Gateway™ cloning, their transformation in *R. solanacearum*, the in vitro secretion assays as well as the in vivo translocation assays based on the *cyaA'* reporter gene.

2 Materials

2.1 Strains

1. *Escherichia coli* strains DH5α and DB3.1 for cloning the *R. solanacearum* T3Es and for transformation of the plasmids carrying the Gateway™ ccdb cassette, respectively.
2. *Ralstonia solanacearum* wild-type strain and a type 3 secretion system mutant (*see* **Note 1**).

2.2 Media and Antibiotics

1. Luria-Bertani (LB) medium (for 1 L): 10 g tryptone, 5 g yeast extract, 10 g NaCl. 15 g/L of agar for solid medium.
2. BG medium (for 1 L): 10 g Bacto peptone, 1 g casamino acids, 1 g yeast extract. 15 g/L of agar for solid medium. Supplement with 0.5% glucose and 0.005% tetrazolium chloride when necessary.
3. MP medium (for 1 L): $1.25.10^{-4}$ g $FeSO_4.7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.05 g $MgSO4.7H_2O$, 3.4 g KH_2PO_4. pH adjusted to 7 with KOH [27]. Supplement with 2% glycerol when necessary.
4. Secretion medium: MP medium supplemented with 10 mM glutamate, 10 mM glucose and 0.1 g/L Congo Red (*see* **Note 2**).
5. Antibiotics used at the following final concentrations: 25 mg/L kanamycin, 10 mg/L gentamycin, 10 mg/L tetracycline, 50 mg/L ampicillin, 25 mg/L chloramphenicol for *E. coli*. 50 mg/L kanamycin, 40 mg/L spectinomycin, 10 mg/L gentamycin, 10 mg/L tetracycline for *R. solanacearum*.
6. 20% glycerol.

2.3 Vectors, PCR and Cloning

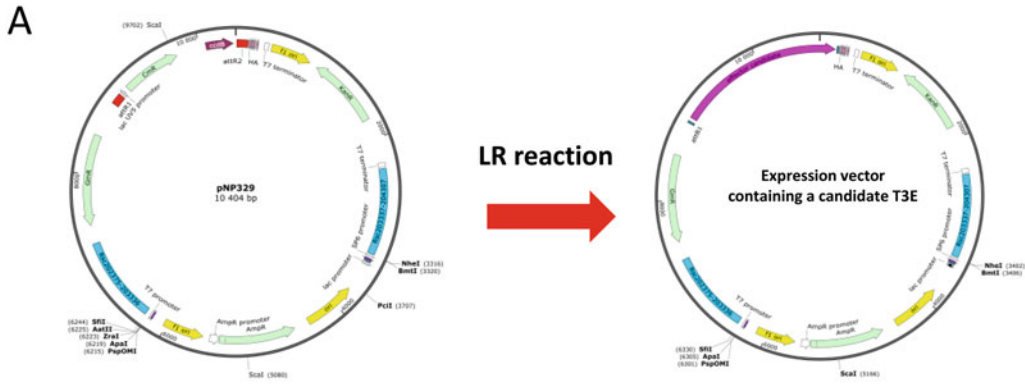
1. Phusion High-Fidelity PCR kit (New England Biolabs, USA).
2. Taq DNA polymerase.
3. dNTP Mix (10 mM each).
4. pNP329 destination vector (for secretion assays): a pRCG derivative [28] carrying the ripG7 promoter (from RSc1445146 to RSc1445497) upstream of the Gateway™ cassette, a triple HA tag, and the gentamycin and the kanamycin resistance genes. This is flanked by two sequences of

homology to the chromosome of GMI1000 strain (left: RSc202375 to RSc203336, right: RSc203337 to RSc204307) (*see* **Note 3**). Allowing stable recombination in *R. solanacearum* chromosome between *RSc0178* and *RSc0179* genes) (Fig. 3a) [21].

5. pFL12 vector (for translocation assays): a pLAFR6 derivative carrying the ripG7 promoter (from RSc1445146 to RSc1445497) upstream of the Gateway™ cassette and the CyaA′ 4–1197 N-terminal part [22].
6. Primers used at 10 μM concentration:
   - oNP611 (5′-GAAAGCACGCTGTTTCCGCTATTT-3′).
   - oNP612 (5′-GCGTAGTGCGCAAGACGAACAA-3′).
   - oNP613 (5′-GGCTCAAGGAGAAGAGCCTTCAGA-3′).
7. pENTR/SD/D-TOPO cloning Kit and Gateway™ LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific, USA).
8. pAM5 vector (a multicopy pLAFR6 derivative carrying HrpB under the control of its own promoter) [29].
9. 2 μg/μL proteinase K solution.
10. Polyethylene glycol (PEG)–KOH pH 13.3.
11. 10% glycerol.
12. Miniprep plasmid extraction kit.
13. Agarose.
14. Electrophoresis equipment.

2.4 Immunoblot

1. Laemmli buffer 4×: 500 mM Tris–HCl pH 6.8, 16% SDS, 80% glycerol, 40% β-mercaptoethanol, bromophenol blue.
2. Supernatant resuspension buffer: Laemmli 1× buffer and 0.2 M Tris–HCl pH 7.5.
3. Tris/Glycine/SDS buffer (running buffer) solution: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.
4. Transfer buffer: 1× Tris/Glycine/SDS buffer, 20% ethanol.
5. Tris-buffered saline (TBS): 10 mM Tris–HCl pH 7.5, 150 mM NaCl.
6. TBS Tween: 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20.
7. Blocking buffer: 5% milk in TBS.
8. Antibodies: anti-hemagglutinin-peroxidase, High Affinity from rat IgG_1; Anti-CyaA′ Antibody monoclonal, mouse IgG_1; Goat Anti-Rabbit IgG-HRP.



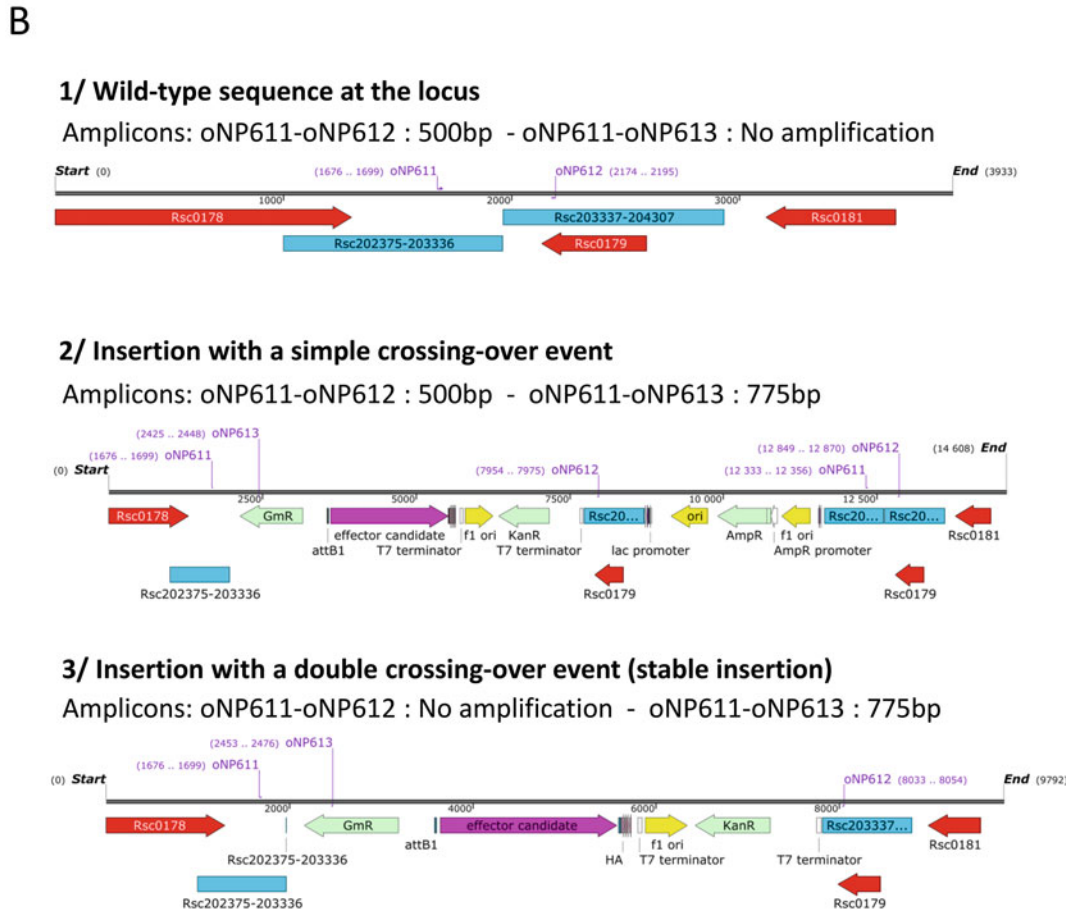


Fig. 3 Vectors used for secretion assays. (**a**) Schematic representation of the destination vector (pNP329) and the derivate expression vector containing a candidate type 3 effector (T3E). In black are highlighted the restriction sites that cut one time outside recombination region. (**b**) Schematic representation of the strategy used to validate the correct insertion in *R. solanacearum* chromosome. After transformation 3 events can occur: 1/ no transformation or insertion in the wrong place (wild-type sequence at the locus); 2/ insertion with a simple crossing-over event; or 3/ insertion with a double crossing-over event leading to a stable insertion. For each event is indicated the size obtained after PCR with oNP611-oNP612 and oNP611-oNP613 primers.

2.5 Secretion Assay

1. 10 mL syringe without needle.
2. Syringe filter unit 0.22 μm.
3. PVDF membrane, 33 mm.
4. 90% acetone solution.
5. 25% trichloroacetic acid solution (TCA).

2.6 Translocation Assay

1. cAMP Biotrak EIA Kit (GE healthcare life science, UK).
2. *Nicotiana tabacum* cv. Bottom Special.
3. 4 mm inox bead.
4. Mixer mill.
5. Liquid N_2.

3 Methods

Tobacco seeds are sawn on potting soil and then grown in greenhouse (20–22 °C; 16 h light) for 4 weeks.

3.1 Generation of Vectors

1. Mix 100 ng of pNP329, 100 ng of pENTR vector containing the putative T3E, 1 μL of Gateway™ LR Clonase™ II Enzyme Mix. Complete to 5 μL with ultra-pure water. Incubate for 1 h at 25 °C (*see* **Note 4**).
2. Add 1 μL of proteinase K solution and 4 μL of ultrapure water in the reaction mix. Incubate for 10 min at 37 °C.
3. Transform *E. coli* DH5α cells with 2 μL of the reaction. Plate the bacteria on LB medium + Ampicillin. Incubate the plate overnight.
4. Pick 3–5 colonies. Extract plasmid DNA using the miniprep kit. Check restriction profile to identify a correct clone.

3.2 Transformation of Ralstonia solanacearum

1. Inoculate 1 colony of the wild-type strain and 1 colony of a type 3 mutant in 5 mL of liquid MP medium supplemented with 2% glycerol for 2 days at 28 °C with shaking.
2. Linearize the pNP329 derivative carrying the candidate T3E. Find a restriction enzyme that cuts outside the recombination region (*see* **Note 5**). For one transformation, mix 1 μg of the vector, 1 μL of enzyme and 1.5 μL of 10× buffer. Complete to 15 μL with ultrapure water. Incubate for 3 h at 37 °C. Verify linearization by loading 5 μL on 1% agarose gel.

Fig. 3 (continued) For (**a**) and (**b**) green arrows indicate the antibiotic resistance genes, yellow arrows the origin of replication, white arrows the promoters, white boxes the terminators, red boxes the *attR* site for Gateway™ recombination, green boxes the *attB* site for Gateway™ recombination, pink arrow the candidate T3E, purple arrow the *ccdB* gene, blue boxes the homology region with *R. solanacearum* chromosome and red arrow *R. solanacearum* genes

3. In a sterile 1.5 mL Eppendorf tube, mix 100 µL of the strains grown for 2 days in MP medium with 2% glycerol (*see* **Note 6**) with 10 µL of the linearized vector. Gently mix, and deposit in the center of a 65 mm-diameter petri dish containing BG medium. Let it dry under a sterile hood for 10 min. Incubate for 2 days at 28 °C.
4. Resuspend the bacteria in 1 mL BG medium. Plate 100 µL and 900 µL on BG medium containing 0.5% glucose, 0.005% tetrazolium chloride, and gentamycin. Incubate for 3–5 days at 28 °C (until colonies appear).
5. Pick two colonies per transformation. Purify two times by picking and restreaking single colonies.
6. Analyze the transformants by colony-PCR. Pick one colony, resuspend it in 5 µL water. Add 45 µL of polyethylene glycol–KOH pH 13.3 (PEG-KOH). Incubate for 10 min at room temperature. This can be directly used as matrix for PCR.
7. Run two PCR colony reactions per transformant, and always include a nontransformed wild-type control colony per analysis batch: one using oNP611-oNP612 primers (amplifying the wild-type nonrecombined locus); and the other one using oNP611-oNP613 primers (amplifying the pNP329-recombined locus). For one PCR reaction mix 12 µL of ultrapure water, 1 µL of 10 µM oNP611 primer, 1 µL of reverse primer (oNP612 or oNP613), 1 µL of 10 mM dNTP, 5 µL of 5× Reaction Buffer, 0.5 µL of DMSO, 0.5 µL of DNA Polymerase, and 4 µL of PEG–KOH matrix. In a thermal cycler use the following conditions: 95 °C × 2.5 min; 30 cycles (95 °C × 30 s, 65 °C × 30 s, 72 °C × 1 min); 72 °C × 10 min.
8. Analyze the PCR product on 1% agarose gel. Bona fide transformant should not produce any band with oNP611-oNP612 primers and a 775 bp band with oNP611-oNP613 primers (Fig. 3b), and the other way around for the wild-type control. Inoculate the correct transformants on liquid BG medium with gentamycin. Incubate at 28 °C with shaking overnight. Store at −80 °C in 20% glycerol.

3.3 Electroporation of Ralstonia solanacearum

1. To allow for a better detection of candidate T3E in the culture supernatant we increase the expression of the transcription factor HrpB in the strain by transforming the strains generated in Subheading 3.2 with the pAM5 plasmid (*see* **Note 7**) [29].
2. Grow the strains carrying the candidate T3E fused to a triple HA tag (T3E-3HA) overnight on liquid BG medium with gentamycin.
3. In a 2 mL Eppendorf tube, centrifuge the overnight cultures for 2 min at 13,000 rpm (~16,000 × *g*) in a microcentrifuge.

4. From this step work on ice. Remove the supernatant. Gently resuspend the pellet with 2 mL ultrapure water. Centrifuge for 2 min at 13,000 rpm (~16,000 × *g*) at 4 °C in a microcentrifuge.
5. Remove the supernatant. Gently resuspend the pellet with 1 mL ultrapure water. Centrifuge for 2 min at 13,000 rpm (~16,000 × *g*) at 4 °C in a microcentrifuge.
6. Remove the supernatant. Gently resuspend the pellet with 500 μL 10% glycerol. Centrifuge for 2 min at 13,000 rpm (~16,000 × g) at 4 °C in a microcentrifuge.
7. Remove the supernatant. Gently resuspend the pellet with 45 μL 10% glycerol (*see* **Note 8**).
8. Transfer the cells to a 1 mm electroporation cuvette. Then add 2 μL of dialyzed pAM5 plasmid. Gently mix. Apply 1.8 kV to the electroporation cuvette and immediately resuspend with 1 mL liquid BG medium. Incubate for 2.5 h at 28 °C with shaking.
9. Plate 100 and 900 μL on BG medium containing 0.5% glucose, 0.005% tetrazolium chloride, gentamycin, and tetracycline. Incubate for 3–5 days at 28 °C (until colonies appear).
10. Pick two colonies per transformation. Purify it two times until you get single colonies. Inoculate a single colony on liquid BG medium with gentamycin and tetracycline. Incubate at 28 °C under shaking overnight. Store at −80 °C in 20% glycerol.

3.4 Secretion Assay

1. Inoculate the wild-type and the type 3 mutant strains carrying the T3E-3HA fusion and the pAM5 plasmid on 5 mL liquid BG medium with gentamycin and tetracycline.
2. Measure the optical density 600 nm ($O.D._{600}$) of the overnight cultures. Dilute it with sterile water in a 15 mL falcon tube to $O.D._{600} = 0.2$ (2.10^8 bacteria/mL).
3. Centrifuge for 10 min at 5000 rpm (~4300 × *g*) in a tabletop centrifuge. Remove the supernatant. Gently resuspend in 15 mL secretion media. Verify that the $O.D._{600}$ is still 0.2. Transfer to a sterile 50 mL Erlenmeyer flask.
4. Incubate at 28 °C with shaking for 8 h (*see* **Note 9**).
5. Measure the $O.D._{600}$. When running several samples, the lower $O.D._{600}$ is considered as the reference $O.D._{600}$. (Reference $O.D._{600}$ should be included within 0.5–0.8 to collect enough material for immunoblot). Dilute all the samples to this reference $O.D._{600}$ with secretion medium.
6. Centrifuge at 4000 rpm (~2700 × *g*) in a tabletop centrifuge. Transfer the supernatant in a new 15 mL falcon tube. Resuspend the bacterial pellet with 500 μL sterile water. Transfer 150 μL to a new 1.5 mL Eppendorf tube. Store at −20 °C.

7. Filter the supernatant with 0.22 μm syringe filter in a new 15 mL falcon tube.
8. Transfer 1 mL to a cold 2 mL Eppendorf tube containing 1 mL of 25% trichloroacetic acid as many times as needed. Incubate overnight at 4 °C.
9. Centrifuge at 13,000 rpm (~16,000 × *g*) for 15 min at 4 °C in a microcentrifuge. Discard the supernatant. Wash the pellet with 1 mL acetone 90%. Centrifuge at 4 °C 13,000 rpm (~16,000 × *g*) for 15 min in a microcentrifuge.
10. Repeat the washing step.
11. Dry the pellet 10 min under the chemical hood. Store at −20 °C up to several months.
12. Add 50 μL of Laemmli 4× buffer in the pellet samples. Resuspend the supernatant sample with 40 μL supernatant resuspension buffer. Incubate for 5 min at 85 °C. Analyze 20 μL of each supernatant and pellet samples by immunoblot, using an anti-HA antibody (1:5000 dilution) (*see* **Notes 10** and **11**).
13. T3Es should be detected in the supernatant of the wild-type strain, but not in the supernatant of the type 3 mutant (Fig. 4a).

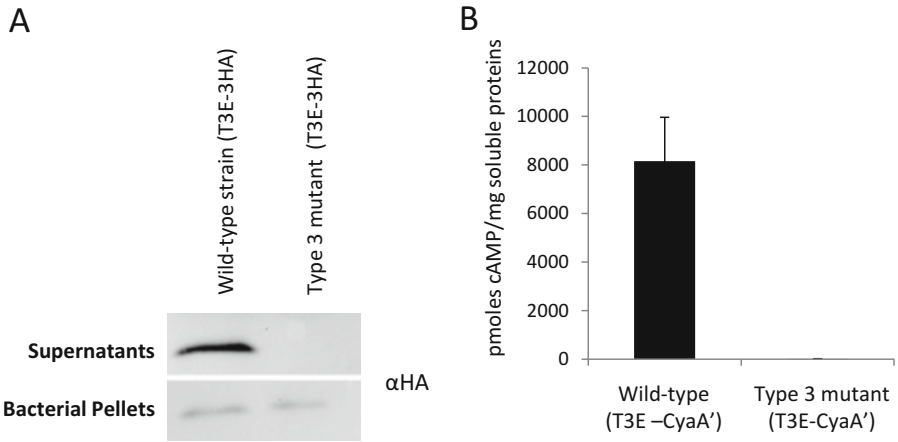


Fig. 4 Validation of a new type 3 effector in vitro and in vivo. (**a**) Secretion assays were performed and total proteins from bacterial pellets and from the supernatants were detected by immunoblot. We can observe production of the candidate type 3 effector (T3E) in both wild-type and type 3 mutant strains. However its secretion is type 3 dependent. (**b**) CyaA′ translocation assay of a candidate T3E in *N. tabacum*. Cyclic adenosine monophosphate (cAMP) levels were detected to determine the level of translocation of candidate T3E-CyaA′ fusion proteins in tobacco leaves for wild-type and type 3 mutant strains. We can see that high amount of cAMP is detected with the wild-type strain but not with the type 3 mutant, indicating that translocation is type 3 dependent. All these data demonstrate that the candidate T3E can be considered as a bona fide T3E

3.5 In Vivo Translocation Assay

1. To generate the vector, perform an LR reaction using pFL12 and a pENTRY clone carrying the candidate T3E following the instructions shown in Subheading 3.1.
2. Transform the pFL12 derivative carrying the candidate T3E in the wild-type strain and in a type 3 mutant by electrotransformation following the protocol shown in Subheading 3.3.
3. Grow the strains in liquid BG medium overnight at 28 °C with shaking (*see* **Note 12**).
4. Measure $O.D._{600}$. Centrifuge in a microcentrifuge for 5 min at 5000 rpm (~2300 × *g*). Resuspend the cells with water to get a final $O.D._{600} = 0.1$.
5. Infiltrate the bacterial suspension in a leaf of a *N. tabacum* plant. Highlight the infiltrated zone with a black pen (*see* **Note 13**). Incubate at 28 °C with light for 7 h.
6. Harvest five discs (8 mm) per infiltration in a 2 mL Eppendorf tube containing a 4 mm inox bead. Transfer it directly in liquid nitrogen. Store the tube at −80 °C.
7. Grind the leave disc with a mixer mill (30 Hz, 30 s). Invert the grinding tube racks and repeat the grinding one more time (30 Hz, 30 s). Add 150 μL of AB buffer (cAMP Direct Biotrak EIA kit).
8. Place the tube in boiling water for 5 min. Centrifuge for 10 min at 13,000 rpm (~16,000 × *g*) at 4 °C in a microcentrifuge. Transfer the supernatant to a new 2 mL Eppendorf tube. Dilute the positive control 5000 times and the negative control 10 times in AB buffer.
9. Measure the cAMP content by using the "cAMP Direct Biotrak EIA kit," nonacetylation protocol, following manufacturer's instructions.
10. For T3Es, you should detect high amount of cAMP within the wild-type strain samples, but not in the type 3 mutant samples (Fig. 4b).
11. Confirm the production of the fusion proteins by immunoblot using the CyaA′ antibody.

4 Notes

1. As a T3SS impaired mutant, we use the GMI1694 strain, corresponding to a *hrcV* mutant, but any *hrp* (*i.e* T3SS-defective) mutation can be used [30].
2. As the secretion medium contains Congo Red, it should be manipulated with care under a chemical hood. Congo Red is used to stabilize the proteins in the supernatant as we observe a lower amount of some secreted proteins when it is not added.

3. We routinely use pNP329 for secretion experiments (also used as a gene expression tool in *R. solanacearum*). A replicative (pLAFR6-based) plasmid can also be used to generate the triple HA fusions. pNP329 possesses homologous recombination sites for strains belonging to phylotypes I and III, not working for strains from other phylotypes (II and IV). However, a "strain-conversion" plasmid, modifying the recombination sequences in a phylotype II recipient strain is available [28], allowing thus for the usage of pNP329.
4. LR reactions can be incubated at 25 °C overnight to increase the number of transformants.
5. Whenever possible (not cutting inside the candidate T3E) we use the *ScaI* enzyme to linearize the vector.
6. For optimal transformation efficiency, $O.D._{600}$ should be within 0.1–0.6.
7. Usage of strains carrying pAM5 plasmid is crucial as we observe that most of the effectors are not detectable in the supernatants of the strains that do not carry the pAM5 plasmid.
8. *R. solanacearum* electrocompetent cells should be prepared freshly. Storage of *R. solanacearum* electrocompetent cells will dramatically decrease the transformation efficiency.
9. The incubation time can be prolonged to increase the secretion efficiency. For the analysis of weakly secreted proteins, cultures can be incubated up to 18 h.
10. Alternatively supernatant can be loaded onto SDS/polyacrylamide gel and send to mass spectrometry for whole supernatant detection [22].
11. One can compare protein loading in each well by staining a SDS/polyacrylamide gel with Pierce™ Silver Stain Kit. Also to check cell lysis, one can perform an immunoblot using antibody anti-cytoplasmic protein.
12. *R. solanacearum* used for plant inoculation should be streaked out from −80 °C on BG medium 3–4 days before inoculation. It is not recommended to use strains older than 1 week.
13. Bacteria should not be inoculated into very young or very old leaves. Because plant reactions may also vary depending on the infiltrated leaf, in an objective of randomization, it is recommended to invert sample positions on the different leaves infiltrated for each replicate. Leave at least 10 cm free between the different infiltrations in a same leaf.

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Chapter 18

Plant Pathogenicity Phenotyping of *Ralstonia solanacearum* Strains

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Abstract

In this chapter, we describe different methods for phenotyping strains or mutants of the bacterial wilt agent, *Ralstonia solanacearum*, on four different host plants: *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), tobacco (*Nicotiana benthamiana*), or *Medicago truncatula*. Methods for preparation of high volume or low volume inocula are first described. Then, we describe the procedures for inoculation of plants by soil drenching, stem injection or leaf infiltration, and scoring of the wilting symptoms development. Two methods for measurement of bacterial multiplication in planta are also proposed: (1) counting the bacterial colonies upon serial dilution plating and (2) determining the bacterial concentration using a qPCR approach. In this chapter, we also describe a competitive index assay to compare the fitness of two strains coinoculated in the same plant. Lastly, specific protocols describe in vitro and hydroponic inoculation procedures to follow disease development and bacterial multiplication in both the roots and aerial parts of the plant.

Key words *Ralstonia solanacearum*, Phenotyping on plants, In planta bacterial growth measurements, Tomato, *Nicotiana benthamiana*, *Arabidopsis thaliana*, *Medicago truncatula*

1 Introduction

Ralstonia solanacearum is the causal agent of bacterial wilt of more than 200 plant species and is responsible for one of the most devastating bacterial plant disease in the world [1]. *R. solanacearum* is a soilborne plant pathogen which naturally infects plants through root tips and lateral root cracks, invades the xylem vessels and spreads rapidly to aerial parts of the plant through the vascular system [2, 3]. In susceptible plants, *R. solanacearum* population can reach up to 10^{10} colony-forming units (CFU) per gram of fresh weight, inducing clogging of the vascular system and causing the wilting symptoms and eventual plant death [4].

Many pathogenicity determinants are required by *R. solanacearum* for successful infection of its hosts [4]. An

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essential pathogenicity determinant is the type 3 secretion system (T3SS) as the corresponding mutant strains produce no disease symptoms on plants and their multiplication in plant tissues is considerably reduced, up to 10^4-fold less than the wild-type strain [3]. The T3SS enables the translocation of type 3 effector (T3E) proteins into the plant cell [5]. A total of 74 T3Es have been identified in the sequenced strain GMI1000 [6]. Several of these T3Es are avirulence factors since they can induce plant defense response on some specific plant lines. This is the case for both RipP1 (formerly PopP1) and RipAA (formerly AvrA) on various *Nicotiana* spp. [7], for RipP1 on a petunia line [8] and for RipP2 (formerly PopP2) on some *Arabidopsis* ecotypes [9]. However, the contribution to the overall virulence of most of the T3Es is not clear as most single T3E mutant strains do not significantly differ from the wild-type strain. One notable exception is the strong contribution of RipG7 (formerly GALA7) for the virulence of *R. solanacearum* specifically on the model legume *Medicago truncatula* [2, 10, 11]. Furthermore, several multiple T3E mutants have been shown to be less aggressive than their wild-type strain, suggesting functional overlap between these T3Es [10, 12, 13].

The absence of a detectable difference between the virulence phenotype on plants of a mutant and a wild-type strain may reflect a lack of sensitivity of the experimental procedure used. A more sensitive assay to compare the phenotype on plants is to measure the capacity of the bacteria to multiply within the host plants. This can be achieved by counting the numbers of CFU in plant material upon serial dilution plating [13]. Determination of the bacterial multiplication in the plant material is also possible using a quantitative-PCR (qPCR) approach with *R. solanacearum* specific primers [9, 14]. More recently, a competitive index assay has been developed that allows quantifying a differential fitness of two *R. solanacearum* strains in planta [15]. In such assay, the wild-type and mutant strains are coinoculated in the same proportion but at very low concentration, in the same plant tissue. This inoculation method reduces the plant-to-plant variation and enhances thus the detection power of a differential fitness between the two strains. Using this approach, the contribution of several T3Es in *R. solanacearum* in planta fitness has been demonstrated, while previous experiments based on disease symptom development failed to reveal a role in virulence for these T3Es [15]. There are thus many methods for phenotyping *R. solanacearum* wild-type strains and mutants on plants. The method chosen depends on the objective of the experiment and on the required sensitivity to compare the phenotypes of several bacterial strains on different plants.

In this chapter, we describe seven different methods for phenotyping *R. solanacearum* strains and mutants on plants. It should be stressed that these methods can also be used to assay several

plant genotypes, variants or mutants with a wild-type *R. solanacearum* strain, to assess the contribution to susceptibility or resistance of specific plant genes. After description of the procedures for high volume and low volume inoculum preparation, this chapter describes methods for (1) inoculation of plants by soil drenching, (2) stem injection or (3) leaf infiltration and (4) measurement of bacterial multiplication in planta. It also describes (5) competitive index assays to compare the fitness in planta of two strains. Lastly, specific protocols are described to follow disease development and bacterial multiplication under (6) in vitro or (7) hydroponic conditions, two procedures that allow an access to the plant roots.

2 Materials

1. Glycerol stock of *R. solanacearum* wild-type strains or mutants stored at −80 °C.
2. Complete BG medium (for 1 L): 10 g Bacto peptone, 1 g Casamino acids, 1 g yeast extract. For solid BG medium, add 5 g glucose, 0.05 g triphenyltetrazolium chloride, and 15 g agar.
3. Antibiotics: Gentamycin (10 mg/L), Kanamycin (50 mg/L), Spectinomycin (40 mg/L), Tetracycline (10 mg/L). Half the antibiotic concentration for liquid cultures.
4. Fåhraeus medium [16]: Prepare stock solutions of macroelements and microelements. Make five solutions for macroelements (for 100 mL): 13.20 g $CaCl_2{\cdot}2H_2O$, 12 g $MgSO_4{\cdot}7H_2O$, 10 g KH_2PO_4, 7.5 g $Na_2HPO_4{\cdot}2H_2O$, and 0.5 g ammonium iron (III) citrate. Make five solutions at 1 mg/mL for each microelement: $MnCl_2{\cdot}4H_2O$, $CuSO_4{\cdot}5H_2O$, ZnCl, H_3BO_3, and $Na_2MoO_4{\cdot}2H_2O$. For 1 L of Fåhraeus medium: 1 mL of each stock solution of macroelements except for $Na_2HPO_4{\cdot}2H_2O$ (2 mL) and 100 µL of each stock solution of microelements; add 0.33 g/L $(NH_4)_2SO_2$; adjust to pH 7.5 and for solid medium incorporate 15 g Difco Agar before autoclaving.
5. Araponics medium: mix FloraGro and Floramicro solutions (General Hydroponics). For solid araponics medium, add 0.65% Difco Agar.
6. Plant materials:
   (a) 4-week-old tomato plants (*Solanum lycopersicum* cultivar Super Marmande VR) grown in a classic greenhouse, 1 plant per 7 × 7 cm pot or 16 plants per 30 × 30 cm tray containing 2 L potting soil (*see* **Note 1**).

(b) 4-week-old tobacco plants (*Nicotiana benthamiana*) grown in the green house in 7 × 7 cm pot, or 16 plants in a 30 × 30 cm tray.

(c) 4-week-old *Arabidopsis* plants (*Arabidopsis thaliana* Col-0 ecotype) grown in a growth chamber (22 °C, 70% humidity, 9 h light) in jiffy pots.

(d) 4-week-old *Arabidopsis* plants (*Arabidopsis thaliana* Col-0 ecotype) grown in hydroponic conditions (*see* Subheading 3) in a growth chamber (22 °C, 70% humidity, 9 h light).

(e) 10-day-old *Medicago truncatula* Gaertn. Genotype A17 (derived from cultivar Jemalong) grown in a growth chamber (22 °C, 70% humidity, 9 h light) in jiffy pots.

(f) 3-day-old *Medicago truncatula* Gaertn. Genotype A17 grown in square petri dishes (12 × 2 cm) containing Fåhraeus medium with an interface of CYG™ seed growth paper (Mega International, St. Paul, MN, U.S.A.) between plantlets and Fåhraeus agar medium.

7. Growth chamber.
8. 28 °C incubator.
9. Rotary shaker.
10. Spectrophotometer.
11. 7 mm diameter punch borer.
12. Disposable 1 mm diameter glass beads.
13. Ceramic beads.
14. 1 mL disposable syringes.
15. 25 µL Exmire microsyringe.
16. Mill grinder.
17. 13 cm aquarium air pump with a ceramic diffuser.
18. 10 L Plastic bins (Athena series, Fami storage system).
19. qPCR machine.
20. 95% anhydrous H_2SO_4.
21. 12% sodium hypochlorite.
22. 70% and absolute ethanol.
23. TRIzol reagent.
24. Chloroform–isoamyl alcohol (24:1).
25. 5 M NaCl.
26. RNAse free water.
27. Black 96-well plates (clear bottom).
28. Quant-iT Picogreen dsDNA Assay Kit (Thermo Fisher Scientific).

29. 2× SYBR Green master mix.
30. Scalpel and extra fine tip tweezers.

3 Methods

3.1 High Volume Inoculum Preparation

1. Under sterile conditions, streak out the strains on BG medium with the appropriate antibiotics from their −80 °C glycerol stock.
2. Incubate at 28 °C for 48 h.
3. Take an isolated colony and inoculate 50 mL liquid BG medium with the appropriate antibiotics in a 250 mL sterile Erlenmeyer flask.
4. Incubate at 28 °C under agitation at 180 rpm in a rotary shaker for more than 16 h.
5. Take 1 mL to measure the OD_{600nm} with a spectrophotometer.
6. Calculate the bacterial concentration, knowing that $OD_{600nm} = 1$ corresponds to 10^9 CFU/mL.
7. Dilute the bacterial suspension to 10^8, 5×10^7 or 10^7 CFU/mL. The dilution can be performed by mixing the original BG medium grown bacteria with water (for a large inoculum, room-temperature tap water can also be used).
8. Take 1 mL to check the dilution by measuring the OD_{600nm} (*see* **Note 2**).

3.2 Low Volume Inoculum Preparation

1. Under sterile conditions, streak out the strains on BG medium with appropriate antibiotics from their −80 °C glycerol-stock.
2. Incubate at 28 °C for 48 h.
3. Take an isolated colony and inoculate 10 mL liquid BG medium with the appropriate antibiotics in a 50 mL sterile Erlenmeyer flask.
4. Incubate at 28 °C under agitation at 180 rpm in a rotary shaker for more than 16 h.
5. Transfer 1.8 mL of the bacterial suspension in a 2 mL Eppendorf.
6. Centrifuge for 2 min at 13,000 rpm in a table top centrifuge.
7. Resuspend the pellet in 1.8 mL sterile water.
8. Take 1 mL to measure the OD_{600nm} with a spectrophotometer.
9. Calculate the bacterial concentration knowing that $OD_{600nm} = 1$ corresponds to 10^9 CFU/mL.
10. Dilute the bacterial suspension to 10^8 CFU/mL in a final volume of 1.8 mL.

11. Take 1 mL to check the dilution by measuring the OD_{600nm} which should be equal to 0.1 (*see* **Note 2**).
12. Depending on the bioassay considered, dilute the bacterial suspension to 10^6, 5×10^4 or 10^4 CFU/mL using the suspension dilution procedure in a final volume of 1 mL.

3.3 Soil Drenching Infection of *Arabidopsis* or *Medicago* Plants

1. Prepare a high volume inoculum (2 L per 50 plants to analyze) to a final concentration of 10^8 CFU/mL.
2. Place the *Arabidopsis*- or *Medicago*-containing jiffy pots in a large container to soak in the inoculum for 20 min (2 L of inoculum per 50 plants) (*see* **Note 3**).
3. Transfer the *Arabidopsis*- or *Medicago*-containing jiffy pots to a new tray (alternatively place on large sheets of disposable aluminum foil).
4. Pour enough fine potting soil onto the remaining inoculum in order to obtain a firm surface and place the plants back (*see* **Note 4**).
5. Incubate in the growth chamber for 10–20 days with the following conditions: 27 °C the day/26 °C the night, 80–85% relative humidity, 12 h light.
6. Once per day, score the wilting symptoms on a visual scale of 1–4 (Fig. 1) (*see* **Note 5**).
7. Repeat the experiment at least three times to draw conclusions (*see* **Note 6**).

3.4 Soil Drenching Infection of Tomato Plants and Measurement of Bacterial Multiplication In Planta

1. Transfer the tomato plants in the growth chamber (28 °C day/27 °C night, 80–85% relative humidity, 12 h light) 2 days before the inoculation.
2. Prepare a high volume inoculum (500 mL per strain to analyze, for a 16-plant tray or for ten individually potted plants) to a final concentration of 5×10^7 CFU/ mL (*see* **Note 7**).
3. Pour the inoculum onto the soil surrounding the tomato plants: 50 mL in pots with 1 plant or 500 mL in trays with 16 plants (*see* **Note 8**).
4. Incubate in the growth chamber for 10–20 days.
5. Once per day, score the wilting symptoms on a visual scale of 1–4 (Fig. 1) (*see* **Notes 5** and **6**).
6. Measure the bacterial multiplication in planta as follows: Sample 1 cm of the stem above the cotyledons and weight it (*see* **Note 9**). Sterilize the surface by putting it in 70% ethanol for 30 s, then rinse the stem in sterile water for 30 s (individual petri dishes can be used for the ethanol and water baths). Cut the stem in six parts (one centered longitudinal section and three transversal sections) with a sterile scalpel and transfer the

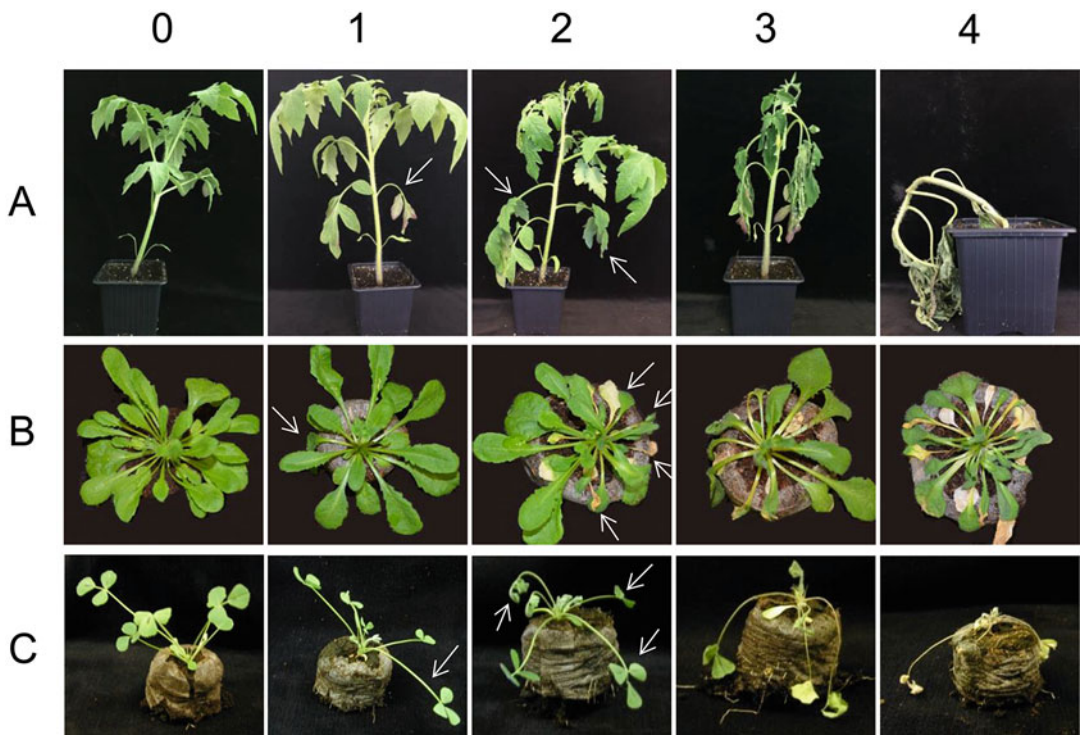

Fig. 1 Symptoms notation scale for (**a**) tomato (*Solanum lycopersicum*), (**b**) *Arabidopsis thaliana*, and (**c**) *Medicago truncatula*. (**c**) Symptoms notation scale is reprinted from Vailleau et al., 2007 [17], Characterization of the Interaction Between the Bacterial Wilt Pathogen *Ralstonia solanacearum* and the Model Legume Plant *Medicago truncatula*, Molecular Plant-Microbe Interactions Vol. 20: pp. 159–167. 0 = No wilting. 1 = First wilting symptoms appears. 2 = Wilting of half of the plant. 3 = Wilting of most of the plant. 4 = Complete wilting. White arrows show leaves that are wilting

sections into a 2 mL Eppendorf tube containing 1 mL sterile water. Incubate at room temperature for at least 30 min and less than 2 h so that the bacteria can diffuse from the stem sections into the sterile water. Generate serial 10× dilutions of the bacterial suspension and plate 100 µL of the appropriate dilutions on solid BG medium with antibiotics corresponding to the strain (*see* **Note 10**). Incubate at 28 °C for 48 h and count bacterial colonies. Calculate the bacterial concentration into the stem using the following formula:

Bacterial concentration (CFU/g fresh matter) = [(number of colonies × 10)/dilution factor]/weight of the sampled stem.

7. Repeat the experiment at least three times to draw conclusions, each experiment containing either one tray with 16 plants or 10 plants in individual pots. If the bacterial load is assessed over the course of the disease development rather than at the end of the wilting, randomly select individual tomato plants to assess their bacterial load.

3.5 Stem Injection of Tomato Plants and Measurement of Bacterial Multiplication In Planta

1. Transfer the tomato plants in the growth chamber (28 °C day/27 °C night, 80–85% relative humidity, 12 h light) 2 days before the inoculation.
2. Prepare a small volume inoculum (1 mL per ten plants to inject) to a final concentration of 10^6 CFU/mL.
3. Inject 10 μL of the bacterial suspension into the stem 0.5 cm above the cotyledons using a microsyringe. Between each strain or mutant, sterilize the microsyringe by pumping up and down 70% ethanol three times and then sterile water three times.
4. Incubate in the growth chamber for 6–10 days.
5. Once per day, score the wilting symptoms on a visual scale of 1–4 (Fig. 1) (*see* **Notes 5** and **6**).
6. Measure the bacterial multiplication in planta: sample 1 cm of the stem 1 cm above the inoculation point, weight it and follow the procedure to measure the bacterial multiplication in planta after soil drenching infection of tomato plants (*see* Subheading 3.4).
7. Repeat the experiment at least three times to draw the conclusions, each experiment containing either one tray of 16 plants or 10 plants in individual pots.

3.6 Assay for In Planta Growth of R. solanacearum by Leaf Infiltration of Nicotiana benthamiana Plants

1. Prepare a small volume inoculum (5 mL per plant to infiltrate) to a final concentration of 5×10^4 CFU/mL (*see* **Note 11**).
2. Chose a single leaf per plant and infiltrate the bacterial suspension with a blunt 1 mL disposable syringe (*see* **Note 12**). Gently wipe the excess inoculum from the abaxial and adaxial leaf surfaces.
3. Immediately after infiltration, sample four discs (7 mm diameter punch borer) from one half side of the inoculated leaf, in order to measure the original inoculum per square cm of leaf surface.
4. Incubate the plants in a growth chamber with the following conditions: 28 °C day/27 °C night, 80–85% relative humidity, 12 h light.
5. Put the leaf discs in a 2 mL Eppendorf tube and grind them with a fixed amount of disposable 1 mm diameter glass beads at 30 Hz for 30 s with a mill-grinder. Place the tubes in a centrifuge and do a short spin to pellet all plant debris stuck on the Eppendorf lids.
6. Add 1.8 mL sterile water, vortex and dilute it.
7. Plate the appropriate 10× dilutions on solid BG medium with the antibiotics corresponding to the mutant used.
8. Incubate at 28 °C for 48 h.
9. Count the bacterial colonies.

10. After 48–72 h in the growth chamber, depending of the experiment, sample again four discs of the other half of the same leaf with a punch borer and repeat **steps 5–9** (*see* **Note 13**).

3.7 Competitive Index Assays in Tomato Plants

1. Transfer the tomato plants in the growth chamber (28 °C day/27 °C night, 80–85% relative humidity, 12 h light) 2 days before the inoculation.
2. Prepare a small volume inoculum for each strain used for competitive index assay.
3. Mix 100 µL of a 10^8 CFU/mL suspension of strain A with 100 µL of a 10^8 CFU/mL suspension of strain B (Fig. 2) (*see* **Note 14**).
4. Serial-dilute the mixed inoculum to a final concentration of 10^6 CFU/mL and 10^4 CFU/ mL in 1 mL final volume.
5. Plate 100 µL of the 10^4 CFU/mL suspension on solid BG medium with the appropriate antibiotic for strain A and on solid BG medium with the appropriate antibiotic for strain B (Fig. 2) (*see* **Note 14**).

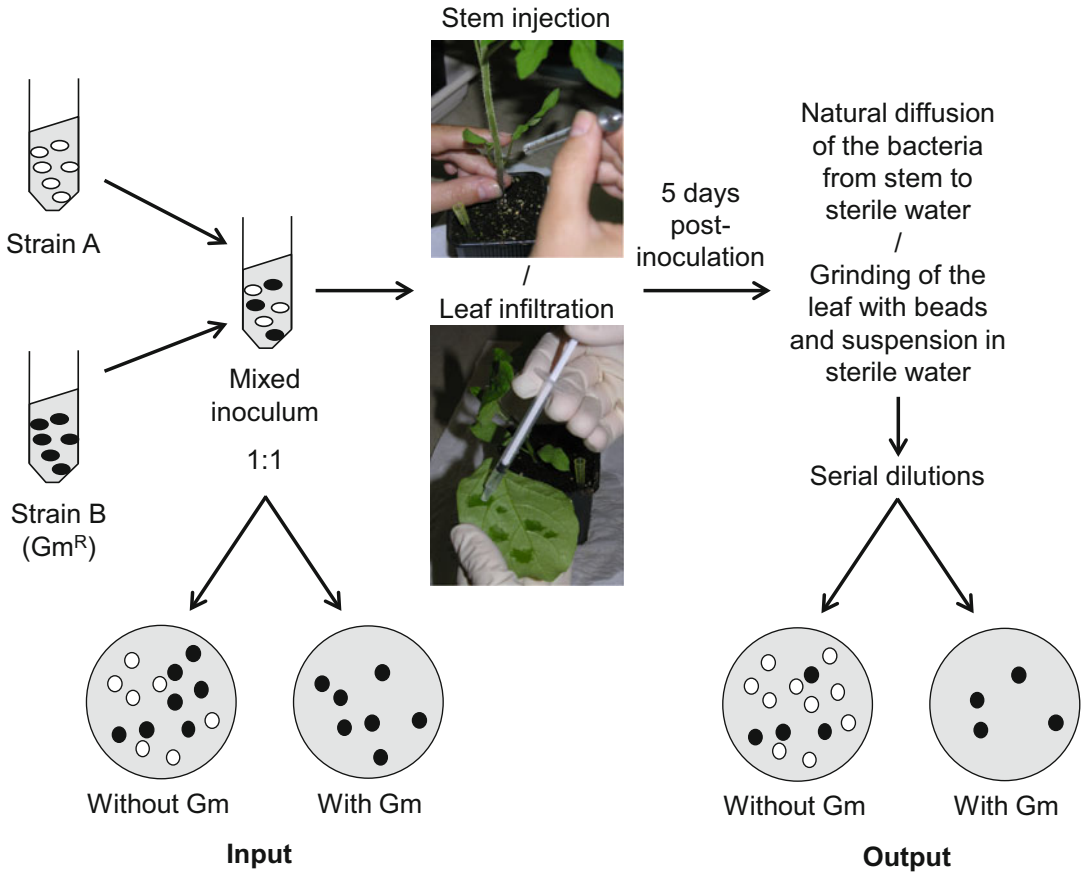

Fig. 2 Schematic representation of the competitive index assays (adapted from Macho et al. 2008 [18]). *Gm* gentamycin

6. Incubate at 28 °C for 48 h.
7. Count the bacterial colonies and calculate the ratio of strains A and B in the mixed inoculum which should be close to one (Input; Fig. 2).
8. For competitive index assays in tomato stem: inject 10 µL of the 10^6 CFU/mL mixed inoculum into the stem 0.5 cm above the cotyledons using a microsyringe. Between each mixed inoculum, sterilize the microsyringe by pumping up and down 70% ethanol three times and then sterile water three times. For competitive index assays in tomato leaf: infiltrate 50 µL of the 10^4 CFU/mL mixed inoculum into the leaf using a blunt syringe.
9. Incubate the plants in the growth chamber for 5 days.
10. After incubation, recover the bacteria in the inoculated plant material as follow (Fig. 2):

    For inoculated stems, sample the whole stem and petioles (without the leaves) 1 cm above the inoculation point. Sterilize the surface by putting the stem and petioles in 70% ethanol for 30 s, then rinse the stem and petioles by putting them in sterile water for 30 s. Truncate the stem and petioles into 1 cm segments with a sterile scalpel and put them in a Falcon tube containing 3 mL sterile water. Incubate at room temperature at least 30 min and less than 2 h so that the bacteria naturally diffuse from stem and petiole to sterile water.

    For inoculated leaves, sample four discs (7 mm diameter punch borer). Put the leaf discs in a 2 mL Eppendorf tube and grind them with a fixed amount of disposable 1 mm diameter glass beads at 30 Hz for 30 s with a mill-grinder. Place the tubes in a centrifuge and do a short spin to pellet all plant debris stuck on the Eppendorf lids. Add 1.8 mL sterile water and vortex.
11. Serial dilute the bacterial suspension and plate 100 µL of the 10^{-4}, 10^{-5} and 10^{-6} dilutions on solid BG medium with the appropriate antibiotic for strain A and on solid BG medium with the appropriate antibiotic for strain B (Fig. 2) (*see* **Note 14**).
12. Incubate at 28 °C for 48 h.
13. Count the bacterial colonies and calculate the ratio of strains A and B in the Output (Fig. 2).
14. Calculate the competitive index (CI) as follow:

    CI = [Strain B CFU/Strain A CFU (output)]/[Strain B CFU/Strain A CFU (input)].
    CI = 1: B fitness = A fitness; CI < 1: B fitness < A fitness; CI > 1: B fitness > A fitness
15. Repeat at least five independent experiments to draw the conclusions.

16. Calculate the mean CI with SD and SE and perform a Wilcoxon test to conclude on differences between mean CI values [19].

3.8 Disease Development on Medicago truncatula Plantlets After R. solanacearum Root Inoculation Under In Vitro Conditions

1. Scarify the *Medicago* seeds with concentrated anhydrous H_2SO_4 (95%) for 7 min, and wash three times in sterile water.
2. Under a hood, sterilize the seeds for 2 min with a 12% sodium hypochlorite solution, and rinse six times with sterile water. Keep the seeds 30 min in the last water bath for an imbibition step.
3. Sow the seeds onto 0.8% (wt/vol) water agar in petri dishes (around 50 seeds per plate), seal it with Parafilm, and put the plates upside down for germination in the dark for 3 days at 4 °C, then 24 h at 14 °C.
4. Transfer germinating seeds (6–10 plants) on slanted agar in square petri dishes (12 × 12 cm) containing Fåhraeus medium and CYG seed growth paper. For this, transfer carefully the plantlets with curved extra fine tip tweezers, putting the hypocotyls at the upper paper/agar delimitation (Fig. 3). Seal the plates with Parafilm (on three sides, leaving the upper part without Parafilm), and incubate with an 45° angle for 3 days with the following conditions: 23 °C, 75% humidity, 16 h light at 100 μmol/m^2/s.
5. Inoculate 3-day-old plantlets in square petri dishes with 300 μL of a bacterial suspension at 10^7 CFU/mL brought to the tip of the root. Seal plates with Parafilm like previously.
6. Incubate the plants with a 45° angle in the growth chamber for 10–20 days with the following conditions: 28 °C the day/28 °C the night, 75% humidity, 12 h light at 100 μmol/m^2/s.

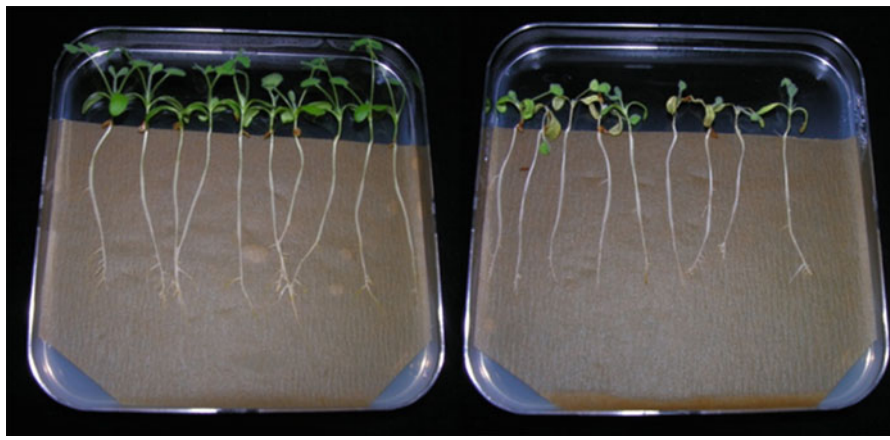

Fig. 3 Disease development on *Medicago truncatula* plantlets after *Ralstonia solanacearum* root inoculation under in vitro conditions. Inoculation with a virulent (right, chlorosis and wilting symptoms) or avirulent strain (left). Pictures taken 14 days after inoculation

7. Monitor disease development every day (chlorosis and wilting) from 10 to 20 days after inoculation (Fig. 3) (*see* **Note 6**).
8. Measure the bacterial multiplication in planta from 10 to 17 days after inoculation as follows: Surface sterilize at least three pools of three plantlets in a ethanol 70% bath for 1 min and rinse three times in sterile water baths for 1 min each. Per pool of three plantlets: weight, grind with a mortar and pestle and resuspend in 4 mL sterile water. Plate serial dilutions (10^{-1} to 10^{-5}) on solid BG medium with appropriate antibiotic. For statistical analyses, use a non parametric Mann Whitney test on series of biological repetitions to determine if the in planta bacterial multiplication of two strains can be considered different or not.

3.9 Inoculation of Arabidopsis Hydroponic Cultures

1. Surface sterilize *Arabidopsis* seeds. Mix 20 mg of seeds with 1 mL of 10% bleach solution in 95% ethanol solution in a 1.5 mL Eppendorf tube for 5–10 min, then wash twice with 95% ethanol and dry in the laminar flow hood for 1–2 h.
2. Prepare hydroponic culture units: cut the caps and 1 cm from the bottom of 0.5 mL Eppendorf tubes (Fig. 4a) and glue them on adhesive tape (Fig. 4a, b). Fill the culture units with half strength araponics solid medium diluted in water to a 1/4000 final concentration.
3. Transfer the hydroponic culture units in tip trays of a 1000 µL tip boxes filled with half strength araponics liquid medium diluted in water to a 1/4000 final concentration (*see* **Note 15**).
4. Sow 1–2 seeds in each hydroponic culture units. Close the tip box with transparent cover (Fig. 4c).
5. Grow *Arabidopsis* seedling for 10 days in a growth chamber with the following conditions: 22 °C, 70% relative humidity, 9 h light.
6. Transfer healthy seedlings (*see* **Note 16**) in pierced Plexiglas trays on 10 L bins filled with full strength araponics liquid medium diluted in water to a 1/2000 final concentration.
7. Grow *Arabidopsis* plantlets for 3 weeks in a growth chamber with the following conditions: 22 °C, 70% relative humidity, 9 h light. Change medium every 4 days.
8. Transfer the plants in the growth chamber for *R. solanacearum* inoculation with the following conditions: 27 °C the day/26 °C the night, 80–85% relative humidity, 12 h light. Replace araponics liquid medium by Fåhraeus liquid medium 7 h before inoculation.
9. Prepare a high volume inoculum to a final concentration of 10^8 CFU/mL in 10 L Fåhraeus liquid medium. The medium is aerated and the bacteria are kept in suspension using a 13 cm aquarium air pump with a ceramic diffuser (Fig. 4e).

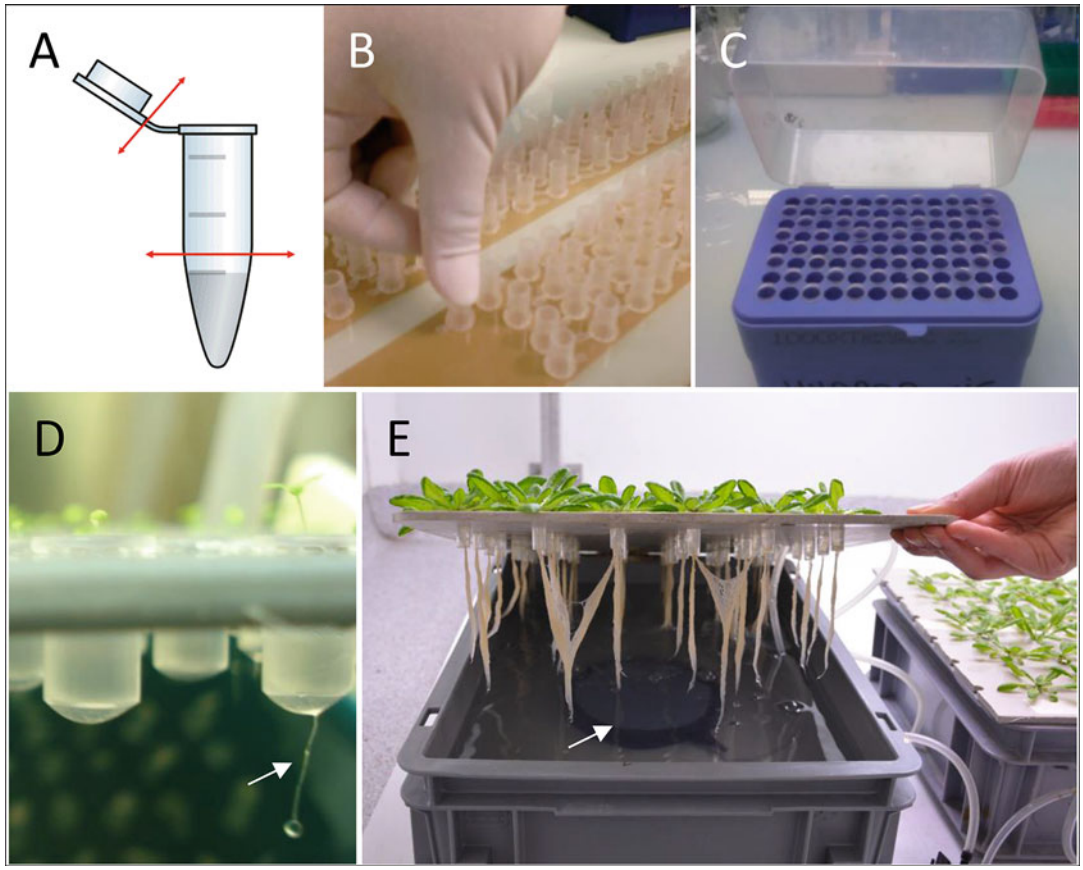

Fig. 4 *Arabidopsis* hydroponic culture setup and inoculation. 0.5 mL Eppendorf tubes are cut following red arrows (**a**) and glued on adhesive tape (**b**). Once filled with half strength araponics solid medium, hydroponic culture unit are displayed in trays of 1000 μL tip box filled with half strength araponics liquid medium (**c**). Healthy seedling for which root went through the solid medium (**d**; arrow) are transferred in pierced Plexiglas trays on 10 L bins filled with full strength araponics liquid medium (**e**). Inoculations are performed on 4-week-old *Arabidopsis* seedlings with a 10^8 CFU/mL bacterial suspension in Fåhraeus medium. Bacteria are kept in suspension using 13 cm aquarium air pump with a ceramic diffuser (e, arrow)

10. Incubate in the growth chamber for 10–20 days.
11. Once per day, assay wilting symptoms on a scale of 1–4 (Fig. 1) (*see* **Note 5**).

3.10 Measurement of Bacterial Multiplication in Roots Using a qPCR Method

1. Harvest the whole root system, wash the roots twice in two different sterile water baths for 1 min each, dry and transfer in a 2 mL Eppendorf tube containing a ceramic bead and 20 mg of sterile sand. Keep the samples frozen in liquid nitrogen until DNA extraction.
2. Generate two independent concentration ranges of bacteria (from 0 to 10^8 CFU/mL) by dilution from two independent overnight liquid cultures. Check the quantity of bacteria in each concentration range point by plating serial dilutions on

solid BG medium. Vacuum-infiltrate for 10 min 100 µL of each concentration range point in 100 mg fresh root tissue harvested from plant cultivated in hydroponic condition, in a 2 mL Eppendorf tube containing a ceramic bead and 20 mg of sterile sand. Centrifuge 10 min at 14,000 rpm in a tabletop centrifuge. Discard supernatant. Keep the samples frozen in liquid nitrogen until DNA extraction.

3. Grind the frozen samples with a Mill grinder (2 × 30 s at 30 Hz vibrational frequency) and extract the nucleic acids with TRIzol reagent using manufacturer's instructions. Perform an additional chloroform–isoamyl alcohol (24:1) extraction on 500 µL of aqueous phase. Add 1.25 mL absolute EtOH and 50 µL NaCl 5 M. Mix by inversion and incubate at −80 °C for 1 h. Precipitate nucleic acids by centrifugation at 13,000 rpm for 15 min at 4 °C in a refrigerated tabletop centrifuge. Discard supernatant and rinse pellet with 1 mL 75% Ethanol (diluted with RNase-free water). Vortex and centrifuge at 13,000 rpm for 30 min at 4 °C in a tabletop centrifuge. Discard supernatant, dry pellets at 65 °C for 2–5 min. Resuspend nucleic acids in 400 µL RNase-free water.
4. Measure DNA concentrations in black 96 well assay plates with clear flat bottom using quant-iT Picogreen dsDNA Assay Kit according to manufacturer's instruction. Adjust the DNA concentration of each sample to 4 ng/µL.
5. Perform qPCR in a 7 µL final volume containing 2 µL DNA, 36 pM final concentration of each primer, 3.5 µL LightCycler 480 SYBR Green 2× Master mix and 1.4 µL sterile water. Two primer pairs are used: EGL1ChF (5′-GCCGAAAGCA GACTACAACC-3′)/EGL1ChR (5′-TGGACAAATAGGGCT TGCT-3′) and AtEF1-F (5′-CTGGAGGTTTTGAGGCTG GTAT-3′)/AtEF1-R (5′-CCAAGGGTGAAAGCAAGAAGA-3′) (*see* **Note 17**). Perform qPCR in triplicate with the following conditions: 95 °C for 5 min; 40 × (95 °C for 10 s and 60 °C or 66 °C for 30 s for AtEF1-F/AtEF1-R or EGL1ChF/EGL1ChR primer pairs respectively) and a final dissociation step to perform melting curve analyses in order to assess the quality of qPCR products. The optimal cycle threshold (Ct) is determined by the LightCycler 480 Software version 1.5 provided by the manufacturer. Ct results obtained using EGL1ChF/EGL1ChR primers are normalized using results obtained with AtEF1-F/AtEF1-R primers.
6. Use the average qPCR Ct results from the two concentration ranges and the corresponding average bacterial quantity to establish a logarithmic regression curve. Use this curve to deduce the bacterial quantity in the root samples from the qPCR Ct results obtained from this root samples.
7. Repeat at least three independent experiments to draw the conclusions, each experiment containing 25 plants.

4 Notes

1. The 30 × 30 cm tray is not punctured for drainage and can thus contain the *R. solanacearum* inoculum.
2. It can be useful to dilute 100 µL of culture in 900 µL liquid BG medium to perform a accurate OD_{600nm} measurement with a spectrophotometer and plate the inoculum in order to have the exact bacterial concentration.
3. For some experiments, wounding of the root system is required. Cut the bottom third of the jiffy pots with scissors before soaking.
4. If assessing different strains, the minimal number of plants will depend on the difference in aggressivity among the reference and the tested strain. As a rule of thumb, when the relative aggressivity is not known or expected to be close, we perform each assay with a minimum of 20 plants. When different plant genotypes are assessed, we encourage randomizing the relative position of the different genotypes in order to do the visual scoring without knowledge of the relative position of the different plant genotypes.
5. The first symptoms should appear 3 or 4 days after inoculation for most experiments with optimal inoculation.
6. To compare the disease development of two given strains, the Kaplan–Meier survival analysis can be used with the Gehan–Breslow–Wilcoxon test done with the Prism version 5.00 (GraphPad Software) [11, 20, 21].
7. A higher concentration of 10^8 CFU/mL can be used if symptoms apparition seem delayed.
8. Be careful to correctly water the plants the day before inoculation as dry soil tends to be more difficult to soak leading to a nonhomogenous inoculation.
9. The bacterial load when assessed in the whole aerial part of the plant is well estimated by the bacterial load in this 1 cm stem section (unpublished data).
10. If wilting symptoms are observed, then plate the dilutions from 10^{-4} to 10^{-6}. If no wilting symptoms are observed, then plate the dilution 0 to 10^{-6} as important bacterial multiplication can occur before the first visual symptoms.
11. The wild type strain GMI1000 cannot be used for this experiment because *N. benthamiana* plants are resistant to this strain. The double mutant GRS473 (*popP1*::Ω *avrA*::Ω) has to be used [7].
12. Use the first big leaf starting from the top, as the older and younger leaves are sometimes difficult to infiltrate. If needed

poke a hole in the leaf with a needle to make the infiltration easier.

13. After 48 h, plate the dilutions 10^{-3} to 10^{-5}.
14. Competitive index assays are used to compare the fitness of two strains either into the stem or into the leaf of a plant. Usually, this approach is used to compare the fitness of a mutant and the corresponding wild-type strain. In order to differentiate the two strains, at least one of them must carry a specific marker such as an antibiotic resistance (gentamycin or spectinomycin resistance) or express of a fluorochrome (GFP or mCherry). When the two strains express different fluorochromes, it is possible to plate them on the same solid BG medium for the Input and the Output calculation (Fig. 2).
15. The bottom of the tubes must be soaked in the medium.
16. Healthy seedlings are seedlings with roots going through the solid medium and soaking in liquid medium (Fig. 4d).
17. The primer pair EGL1ChF/EGL1ChR, which amplifies a region of the unique endoglucanase gene (*EGL1*) localized on the *R. solanacearum* chromosome (EMBL AL646052), was designed in the present work with Primer3Plus online software. The primer pair AtEF1-F/AtEF1-R [9], which amplifies a region of the *AT5G60390* nuclear gene (GTP binding EFTu elongation factor), is used to normalize the results.

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Chapter 19

Methods to Quantify Biotic-Induced Stress in Plants

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Abstract

Plant pathogens such as fungi, oomycetes, viruses and bacteria infect important crops and account for significant economic losses worldwide. Therefore, it is critical to gain insights into plant–pathogen interactions at the cellular and molecular level. The outcome of the interaction between plants and pathogens greatly differs depending on the species, strains and cultivars involved as well as environmental factors, yet typically results in stress for the plant, the pathogen or both. These biotic-induced stresses can be monitored using a wide range of techniques, of which some of the most commonly used techniques are outlined in this chapter. One widely observed feature of biotic stress in plants is the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-). We describe the quantification of hydrogen peroxide by 3,3′-diaminobenzidine (DAB) staining and luminol-based assays, and of superoxide by nitroblue tetrazolium (NBT) staining. Other techniques detailed here include measurement of callose deposition by aniline blue staining, evaluation of cell death by trypan blue staining and analysis of the loss of membrane integrity by monitoring electrolyte leakage.

Key words Biotic stress, Plant pathogen, *Nicotiana benthamiana*, *Pseudomonas syringae*, ROS, DAB, Luminol, NBT, Callose deposition, Aniline blue, Cell death, Trypan blue, Electrolyte leakage

1 Introduction

Plant pathogens cause significant economic losses in important crops worldwide. The annual yield of cultivated species such as wheat, rice or tomato can be reduced dramatically by infection by pathogenic organisms, including fungi, oomycetes, viruses and bacteria [1–3]. In investigating how to limit these losses, it is of paramount importance to quantitatively analyze the processes involved in host responses to pathogen infection and host resistance. Here we provide a short introduction to some of the methods that are most commonly used to monitor biotic stress during plant–pathogen interactions.

To counteract pathogens, plants have evolved a sophisticated immune system that is able to block pathogen growth, which is commonly depicted as a two-branched immune system. Plants can perceive the presence of pathogens through detection of

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Pathogen/Microbe-Associated Molecular Patterns (PAMPs/MAMPs) or Damage-Associated Molecular Patterns (DAMPs) by extracellular Pattern Recognition Receptors (PRR). This recognition activates a defense response termed PAMP-Triggered Immunity (PTI). Many pathogens have evolved secreted effectors that are able to suppress PTI. In turn, plants have evolved an additional defense termed Effector-Triggered Immunity (ETI) activated by the recognition of pathogen effectors by plant resistance (R) proteins [4].

Generally, PTI responses include reactive oxygen species (ROS) production [5], callose deposition [6, 7] and ion fluxes [8], whereas ETI typically induces an additional hypersensitive response (HR), which can result in localized, programmed cell death and the production of antimicrobial compounds that limit pathogen growth [9, 10]. However, in compatible interactions, the pathogen is able to evade or suppress plant immunity and successfully invade plant tissues, thus causing disease. Nevertheless, even in compatible interactions, the pathogen induces stress to the plant, which can be quantified as well. Therefore, irrespective of the nature of plant–-pathogen interactions, the encounter between both organisms typically results in plant stress, which can be measured using the same techniques.

It should be noted that, instead of focusing on the effects that pathogens have on plants, other studies have concentrated on monitoring pathogen growth as a complementary approach to quantifying host–pathogen interactions. Furthermore, in recent years, with the increasing development and reduction in cost of "omic" approaches, many scientists are increasingly applying these cutting-edge techniques to study plant stress and defense responses. While some studies have focused on quantifying biotic stress through expression analyses of defense-marker genes by qRT-PCR, others have used microarrays, RNA sequencing and proteomics as tools to identify altered pathways in response to pathogen infection [11–15]. Other studies have concentrated on the analysis of differential accumulation of phytohormones such as salicylic acid (SA), methyl jasmonate (MeJA), abscisic acid (ABA) or ethylene [16], or of plant metabolites upon infection [17, 18]. Chlorophyll fluorescence has also been measured as a proxy for pathogen-mediated photosynthetic inhibition [19]. All of these techniques enable researchers to describe global changes in plant transcriptional and physiological responses upon infection with pathogens, but will not be addressed here.

This chapter will focus on several techniques routinely used in plant pathology to quantitatively, or semiquantitatively measure biotic stress in plants. These include detection and quantification of ROS molecules such as hydrogen peroxide (H_2O_2) by DAB (3,3-diaminobenzidine)- and luminol-based assays, or superoxide (O_2^-) by nitroblue tetrazolium (NBT) staining. Measurements of

callose deposition by aniline blue staining, evaluation of cell death by trypan blue staining and analysis of loss of membrane integrity by electrolyte leakage measurements are also discussed.

Because ROS molecules are highly reactive and unstable [5], the techniques used to quantify them are based on the detection of a final product generated upon reaction of ROS with a determinate compound. DAB is an organic compound derived from benzene that is oxidized to a brown alcohol-insoluble precipitate when it is in direct contact with hydrogen peroxide (H_2O_2) and peroxidase. Luminol is oxidized by horseradish peroxidase (HRP) in the presence of H_2O_2, releasing chemiluminescence that can be measured using a luminometer or imaged using a photon detecting imaging system. NBT reacts with superoxide (O_2^-) and forms a dark blue deposit (insoluble formazan) that can be visualized. The amount of staining (DAB or NBT) observed is proportional to the amount of hydrogen peroxide/superoxide present in the tissue; therefore, DAB and NBT staining are regularly used as a proxy for the amounts of cellular H_2O_2 or O_2^-, respectively. Likewise, the intensity and duration of the luminescence correlate with the levels of H_2O_2, allowing quantitative measurements of cellular H_2O_2.

Callose is a polysaccharide made up of β-1,3-linked glucose residues with some β-1,6-branches, which is deposited at the interface between the plasma membrane and plant cell wall in response to wounding and pathogen elicitors [6, 7]. Callose cell wall appositions can be detected and quantified by staining with aniline blue. This technique exploits the fact that aniline blue reacts with the callose and fluoresces under UV light.

During plant–pathogen interactions cell death can occur due to plant defense mechanisms or as a disease symptom. In incompatible interactions, plants commonly respond to pathogen infection with a hypersensitive response (HR), a localized cell death able to limit pathogen spread [9, 10], while in compatible interactions the pathogen can successfully disrupt or evade plant immune system, and cause disease and cell death. One of the classic techniques to detect and quantify cell death is trypan blue staining [20, 21]. In living cells, the plasma membrane is intact and therefore the dye cannot penetrate to the interior of cells. Contrarily, dead cells are permeable to the dye and it accumulates intracellularly, thus inviable cells are stained blue. It should be noted that trypan blue staining only detects cell death, and does not discriminate between different causes of cell death. Therefore, when studying cell death it may be necessary to understand the processes underlying the interactions and include the appropriate controls to be able to discriminate between programmed cell death (HR) and cell death as a disease symptom [22, 23].

An alternative method to assess membrane integrity in plants is the measure of electrolyte leakage. Plant cells with intact membranes are able to maintain electrolytes within the cell boundaries.

However, when challenged or under stress, many factors lead to membrane instability and loss of integrity, hence, electrolyte leakage [24, 25]. By measuring the electrolyte leakage from leaf discs floated on water, the degree of cell membrane instability and damage can be estimated.

The procedures described below have been optimized for foliar pathogens such as the model phytopathogen *Pseudomonas syringae*. Although all the protocols provided here have been detailed for the model plant *Nicotiana benthamiana*, they can be adapted to other model dicot species such as *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), or common bean (*Phaseolus vulgaris*), and to monocots such as rice (*Oryza sativa*). However, some of the protocols may require minor modifications to further optimize them for different plant species and tissues. The starting plant material for any of the protocols described here is infected tissue and infection protocols are specific to different pathogen systems. The choice of inoculation procedure and inoculum density used may depend on the nature of the interaction being studied. For information on how to prepare *P. syringae* cultures and *N. benthamiana* plants for infection please *see* Chakravarthy et al. [26] or Nguyen et al. [27]. For information on how to prepare *P. syringae* cultures and *Arabidopsis thaliana* plants for infection please *see* Yao et al. [28] or Katagiri et al. [29].

Importantly, environmental conditions such as light, temperature or relative humidity can affect both the host and the pathogen, and therefore, changes in these conditions can alter the outcome of the experiments. Similarly, variation in plant age, leaf age or development can alter plant responses to infection. Hence, it is important to perform the experiments maintaining all these factors as constant as possible to ensure consistent and reproducible results.

2 Materials

Common materials

1. Ultrapure H_2O.
2. Absolute, 75%, and 50% ethanol.
3. 50% and 20% glycerol.
4. 50 mL polypropylene tubes.
5. Aluminum foil.
6. Petri dishes.
7. Measuring cylinder.
8. Balance.
9. pH meter.
10. Shaker.

11. Water bath.
12. Tweezers.
13. Pipette and tips.
14. Cork borer (0.4 and 0.7 cm diameter).
15. Image capture device (camera, microscope, scanner, ...).
16. Plant material and pathogen cultures.
17. Vacuum pump and chamber.

2.1 Detection of Hydrogen Peroxide (H_2O_2) by DAB Staining

1. 1 mg/mL 3,3-diaminobenzidine–HCl (DAB–HCl) solution, pH 3.8 (DAB requires low pH to dissolve): measure 100 mL of ultrapure H_2O, adjust the pH to 3.8 using HCl and dissolve 0.1 g of DAB. Keep the solution in the dark (*see* **Note 1**).

2.2 Luminol-Based Detection of Hydrogen Peroxide (H_2O_2)

1. Dimethyl sulfoxide (DMSO).
2. 1 mM flg22 stock solution (molecular weight: 2272.52 g/mol): add 2.27 mg of flg22 to 1 mL of sterile, ultrapure H_2O and vortex vigorously for 15 s. The solution should appear transparent. Store at −20 °C where it is stable for up to 6 months. Flg22 peptide (QRLSTGSRINSAKDDAAGLQIA) [30].
3. 500× luminol stock solution (LSS): add 15 mg of luminol to 1 mL of DMSO and vortex vigorously for 15 s. The solution should appear light green. Store in the dark at −20 °C where it is stable for up to 3 months (*see* **Note 2**).
4. 500× horseradish peroxidase stock solution (HPSS): add 10 mg of horseradish peroxidase to 1 mL of ultrapure H_2O and vortex vigorously for 15 s. The solution should appear brown. Store at −20 °C where it is stable for up to 3 months.
5. 96-well plate, white and flat-bottomed (*see* **Note 3**).
6. Luminometer (e.g., Plate reader).

2.3 Detection of Superoxide (O_2^-) by Nitroblue Tetrazolium Staining

1. 1 M potassium phosphate monobasic (KH_2PO_4) stock solution: for 100 mL dissolve 13.61 g of KH_2PO_4 in ultrapure H_2O.
2. 1 M potassium phosphate dibasic (K_2HPO_4) stock solution: for 100 mL dissolve 17.42 g of K_2HPO_4 in ultrapure H_2O.
3. NBT staining solution: 0.1% NBT (Nitroblue tetrazolium chloride) in 50 mM potassium phosphate buffer (pH 7.8) and 10 mM NaN_3 (Sodium azide). This solution should be prepared freshly before use and covered with aluminum foil to protect it from light (*see* **Note 4**). Prepare as follows: add 4.6 mL of 1 M KH_2PO_4 and 45.4 mL of 1 M K_2HPO_4 stock solutions to 950 mL of ultrapure H_2O to obtain 1 L of 50 mM potassium phosphate buffer (pH 7.8). Dissolve 0.65 g of NaN_3 in 1 L of 50 mM potassium phosphate buffer (pH 7.8) to get a

final concentration of 10 mM sodium azide (NaN_3). Dissolve 1 g NBT in 1 L 50 mM sodium phosphate buffer (pH 7.8) containing 10 mM sodium azide to obtain the final 0.1% NBT staining solution.

2.4 Detection and Quantification of Callose Deposition by Aniline Blue Staining

1. Aniline blue working solution (0.1% Aniline blue in 150 mM K_2HPO_4, pH 9.5): dissolve 1 g of aniline blue (methyl blue) in 2 mL of ethanol absolute. Dissolve 26.13 g of K_2HPO_4 in 1 L of ultrapure H_2O, adjust the pH to 9.5 and add the dissolved aniline blue (*see* **Note 5**).
2. Microscope with UV lamp and DAPI filter (excitation filter 390 nm; dichroic mirror 430 nm; emission filter 460 nm) and with camera.
3. Microscope slides and covers.

2.5 Detection of Cell Death by Trypan Blue Staining

1. Trypan Blue (TB) stock solution: mix 100 g (or 93.46 mL) of phenol, 100 mL of lactic acid, 100 mL of glycerol, 100 mL of ultrapure H_2O and 0.2 g of trypan blue. The TB stock solution can be kept at room temperature (*see* **Note 6**).
2. Trypan Blue (TB) working solution: mix 1 vol. of TB stock solution with 2 vol. of ethanol absolute (e.g., 100 mL of TB stock solution + 200 mL of ethanol absolute). The working solution can be reused for up to three staining experiments and can be stored at room temperature.
3. Chloral hydrate solution: dissolve 1 kg chloral hydrate in 400 mL of ultrapure H_2O. To dissolve the chloral hydrate heat to 65 °C and stir it for 1 h until it is completely dissolved. This will yield approximately 1 L of solution, which can be stored at room temperature, in the dark, in a tightly sealed container (*see* **Note 6**).

2.6 Measurement of Electrolyte Leakage

1. Conductivity standard solutions.
2. Vials.
3. 10 and 25 mL pipettes.
4. Conductivity meter.

3 Methods

3.1 Detection of Hydrogen Peroxide (H_2O_2) by DAB Staining

1. Prepare the DAB solution just before starting the experiment. The solution needs to be freshly prepared to avoid oxidation of DAB (*see* **Note 7**). Cover the tube containing the solution with aluminum foil since DAB is sensitive to light.

2. Harvest the infected leaves and carefully transfer them to 50 mL polypropylene tubes. A maximum of two *N. benthamiana* leaves should be added per tube (*see* **Note 8**).
3. Add DAB solution to the tubes until the leaves are completely covered and incubate the samples in the dark for at least 8 h at room temperature and with mild agitation.
4. Discard the DAB solution and rinse the leaves with ultrapure H_2O to remove excess staining solution.
5. Add absolute ethanol to the tubes until the leaves are completely covered and incubate the samples in the water bath at 65 °C for 2 h to clear the chlorophyll.
6. Discard the ethanol absolute, replace with 75% ethanol and incubate for 1 h.
7. Discard the 75% ethanol, replace with 50% ethanol and incubate until the leaves have completely lost their green coloration (Fig. 1). It may take a longer or shorter period of time for the chlorophyll to be cleared depending on the plant species and leaf age (*see* **Note 9**). If the leaves remain green they can be incubated overnight in 50% ethanol at room temperature and with mild agitation.
8. Transfer the leaves to Petri dishes containing ultrapure H_2O or 20% glycerol and incubate for 5 min to enable rehydration. This will prevent the leaves from breaking easily and will make it easier to image them. Adding H_2O or 20% glycerol into the

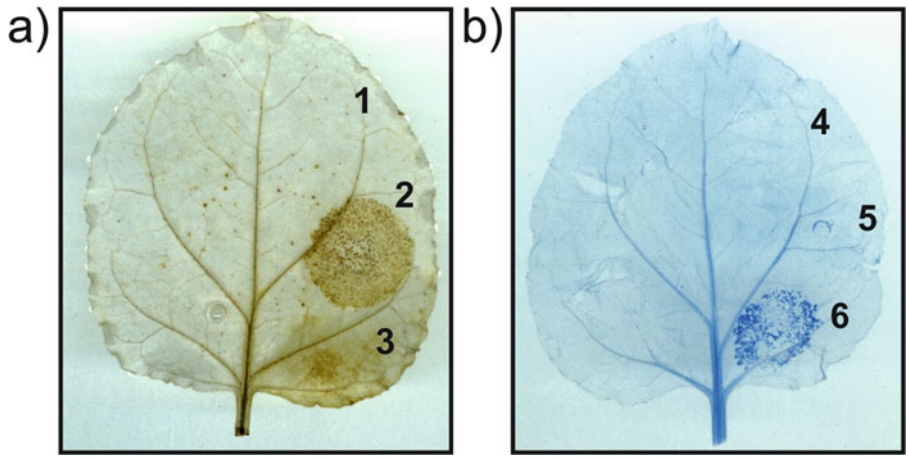

Fig. 1 Staining to detect plant defense responses and disease symptoms in *Nicotiana benthamiana* by (**a**) DAB staining or (**b**) trypan blue staining. *N. benthamiana* leaves were inoculated with H_2O (1, 4) or with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000; 2, 5), which elicits effector-triggered immunity (ETI), or a mutant strain of *Pst*DC3000 lacking the effector *hopQ1-1* (*ΔhopQ1-1*; 3, 6), which causes disease. Bacteria were inoculated at a density of 2×10^5 cfu/mL. At 30 h post-inoculation, leaves were stained by DAB staining (*see* Subheading 2.1) or trypan blue staining (*see* Subheading 2.5) and photographed

tube prior to transferring the leaves to Petri dishes may make it easier to extract leaves from tubes without breaking them.

9. Image the leaves according to the preferred method (*see* **Note 10**). Areas where H_2O_2 accumulates will be stained dark brown (DAB insoluble precipitates; Fig. 1).
10. Leaves can be stored in 50% ethanol at 4 °C.

3.2 Luminol-Based Detection of Hydrogen Peroxide (H_2O_2)

1. Add 200 μL of ultrapure H_2O to each well of a 96-well plate.
2. Punch out leaf discs using a cork borer (0.4 cm diameter). Be extremely careful to minimize damage to the leaf when obtaining the leaf discs (*see* **Note 11**).
3. Using tweezers carefully transfer the leaf discs to the wells with the adaxial side facing up (*see* **Note 12**).
4. Incubate the 96-well plate under continuous light for at least 16 h with mild constant rotation.
5. On the next day freshly prepare the working solutions. Calculate the amount of 500× LSS and 500× HPSS you need for a working solution of 1.25× of each component considering that for each well, including control wells, you will need 150 μL of luminol/peroxidase working solution. Prepare the luminol/peroxidase working solution (1.25× each) containing pathogen. For example, for 6 wells add 2.5 μL of 500× LSS and 2.5 μL of 500× HPSS to 1 mL of ultrapure H_2O containing the pathogen (*see* **Note 13**). As a positive control use luminol/peroxidase working solution (1.25× each) containing an elicitor such as flg22 (*see* **Note 14**). For example, for 6 wells add 2.5 μL of 500× LSS and 2.5 μL of 500× HPSS to 1 mL of ultrapure H_2O containing 100 nM to 20 mM concentration of flg22. If the desired final elicitor concentration is 100 nM, add 0.1 μL of 1 mM flg22 solution to 1 mL of H_2O, and then add 2.5 μL of 500× LSS and 2.5 μL of 500× HPSS. It is also useful to include controls into which H_2O_2 is spiked at a range of known concentrations to exclude false positive results.
6. Remove the 200 μL of ultrapure H_2O from the 96-well plate, carefully avoiding any damage to the leaf discs.
7. Add 150 μL of luminol/peroxidase working solution containing either pathogens or elicitors (*see* **Note 15**). If many wells are to be read it is important to check how long the reader takes per well or row and to add elicitor to the wells of the 96 well plate in the same order, and at the same time intervals as the wells will be read to obtain the most consistent and accurate results. Once the plate has been set up it is critical to rapidly transfer the plate to the luminometer.
8. Measure the luminescence for at least 30 min at 25 °C at 15 s intervals (*see* **Note 16**).

3.3 Detection of Superoxide (O_2^-) by Nitroblue Tetrazolium Staining

1. Prepare the NBT solution just before starting the experiment. The solution needs to be freshly prepared to avoid oxidation of NBT.
2. Harvest the infected leaves and carefully transfer them to 50 mL polypropylene tubes. For *Nicotiana benthamiana* a maximum of two leaves should be added per tube (*see* **Note 8**).
3. Add 0.1% NBT staining solution (in 50 mM potassium phosphate buffer (pH 7.8) and 10 mM sodium azide) until the leaves are completely covered.
4. Vacuum infiltrate the leaves by applying vacuum for 30–60 s and gently releasing it (*see* **Note 17**). To ensure complete leaf infiltration repeat the same procedure for two or three times or until they are completely infiltrated.
5. Incubate the leaves in the dark (wrap the tubes in aluminum foil) without vacuum for 2 h and at room temperature.
6. Discard the NBT staining solution and rinse the leaves with H_2O to remove the excess of staining solution.
7. Add absolute ethanol to the tubes until the leaves are completely covered, and incubate the samples at 65 °C in the water bath for 2 h to clear the chlorophyll.
8. Discard the ethanol absolute, replace with 75% ethanol and further incubate for 1 h until the leaves have completely lost the green coloration. It may take longer or shorter time for the chlorophyll to be cleared depending on the plant species and leaf age (*see* **Note 9**). If the leaves remain green incubate them overnight in 50% ethanol at room temperature and with mild agitation.
9. Transfer the leaves to Petri dishes containing ultrapure H_2O or 50% glycerol and incubate for 5 min to enable rehydration and prevent them breaking easily.
10. Image the leaves according to the preferred method (*see* **Note 10**). NBT reacts with the endogenous O_2^-resulting in the accumulation of dark blue stains. Therefore, the areas where O_2^- accumulates will appear stained dark blue.

3.4 Detection and Quantification of Callose Deposition by Aniline Blue Staining

1. Punch out leaf discs carefully to avoid damaging them (*see* **Notes 11** and **18**).
2. Transfer the leaf discs to 50 mL polypropylene tubes and add ethanol absolute to clear the chlorophyll.
3. Incubate the tubes overnight at room temperature and with mild agitation. If the leaf discs are not completely destained, further incubate the tubes at 60 °C until the chlorophyll is completely cleared.

4. Remove the ethanol absolute, replace with 70% ethanol and incubate for 15 min at room temperature.
5. Discard the 70% ethanol and rinse the leaf discs with ultrapure H_2O.
6. Immerse the leaf discs in 0.1% aniline blue in 150 mM K_2HPO_4 (pH 9.5) solution (*see* **Note 19**). Incubate the tubes at room temperature, with mild agitation and in the dark for 1.5 h (*see* **Note 20**).
7. Rinse the leaf discs with ultrapure H_2O and replace with 50% glycerol.
8. Mount stained leaf discs in 50% glycerol on a glass slide. Glycerol ensures longer preservation of the sample and reduces bubble formation.
9. Visualize callose deposition using fluorescence microscopy with UV lamp and DAPI filter. The optimal excitation and emission wavelength for aniline blue are 370 nm and 509 nm, respectively. Callose deposits appear stained bright blue.
10. To make the technique quantifiable, the total amounts of callose spots per microscope picture can be counted. Alternatively, the intensity of callose staining can be analyzed using image analysis software such as ImageJ or Callose Measurer [31] (*see* **Note 21**).

3.5 Detection of Cell Death by Trypan Blue Staining

1. Harvest the leaves and place a maximum of two *Nicotiana benthamiana* leaves in a 50 mL polypropylene tube (*see* **Notes 8** and **22**).
2. Add TB working solution (ethanol added) until the leaves are completely covered. Incubate the tubes in the water bath at 95 °C for 5 min (the leaves will become blue).
3. Remove the tubes from the water bath and incubate them overnight at room temperature and with mild rotation (*see* **Note 23**).
4. Remove TB solution, replace with chloral hydrate solution until the leaves are completely covered and incubate the tubes in the water bath at 65 °C for 2 h. Invert the tubes every 20 min.
5. Discard the solution, replace with fresh chloral hydrate solution and incubate the tubes overnight at room temperature and with mild rotation. The background blue coloration of the leaves will be reduced. If the leaves retain high blue coloration, incubate with chloral hydrate until this background coloration has completely disappeared (Fig. 1).
6. Discard the chloral hydrate solution and add 50% glycerol to preserve samples.

7. Image the leaves according to the preferred method (*see* **Note 10**). Dead cells will appear dark blue (Fig. 1).
8. The samples can be stored in 50% glycerol for several months.

3.6 Measurement of Electrolyte Leakage

1. Infect the plants with the desired pathogen following the preferred methodology (*see* refs. 16–18 for methods of infection). If infiltrating the pathogen to the leaf using a syringe, allow the surface of the leaves to dry before sampling them.
2. Extract ten discs per leaf of *N. benthamiana* using a cork borer (0.7 cm diameter) and place them in a Petri dish containing 25 mL of sterile ultrapure H_2O. Incubate for 1 h at room temperature with mild rotation (*see* **Notes 11** and **24**).
3. Transfer ten discs to individual vials containing 10 mL of ultrapure water (*see* **Note 25**). It is important that the vials are new since reused vials can contain contaminants that interfere with the results.
4. Measure conductivity over time using a conductivity meter. It is important to gently agitate and mix the samples before measuring the conductivity. The conductivity meter should be calibrated prior to use using conductivity meter standard solutions (*see* **Note 26**).

4 Notes

1. DAB may be carcinogenic, and thus gloves and appropriate protective gear should be worn when preparing and handling the DAB solution. The DAB staining solution needs to be disposed of as hazardous waste.
2. Luminol is an irritant, and thus gloves and appropriate protective gear should be worn when preparing and handling the luminol solution. The luminol solution should be disposed of according to your institution's standard procedure. Luminol is extremely sensitive to light; therefore aliquots should be covered with aluminum foil and stored in the dark.
3. It is critical that the 96-well plate is white, not black or clear, since black colored plates absorb the chemiluminescence and therefore reduce the level of light detected, while clear plates allow light to pass into adjacent wells. It is also important that the bottom of the plate is flat to allow the whole surface of the leaf disc to be in direct contact with the solution.
4. NBT is harmful, and thus gloves and appropriate protective gear should be worn. The NBT staining solution needs to be disposed of according to your institution's standard procedure.
5. Aniline blue is an irritant, and thus gloves and appropriate protective gear should be worn when preparing and handling

the aniline blue solution. The aniline blue staining solution should be disposed of according to your institution's standard procedure.

6. Chloral hydrate and phenol are toxic compounds, thus handle phenol, TB stock solution and chloral hydrate solutions in a fume extraction hood wearing gloves and the appropriate protective gear. Phenol, TB and chloral hydrate solutions should be disposed of according to your institution's standard procedure.
7. When freshly prepared (unoxidized) the DAB solution is light brown colored, whereas when oxidized it appears dark brown colored. There should be no visible precipitate in the stock solution. To achieve the best results it may be desirable to filter the fresh stock solution through a 0.2 μm microfilter prior to use.
8. The size of the tubes and volumes of solutions indicated in the protocol refer to the necessary amounts for *Nicotiana benthamiana* leaves. For other species such as *A. thaliana* or *P. vulgaris* the size of the tubes and volumes of solutions can be scaled according to the size and number of leaves. Long leaves, such as cereal leaves, may need to be gently folded into the tube. Importantly, leaves should have enough space in the tubes to ensure the whole surface is in direct contact with the solution. Leaves should be carefully inserted into the tubes to avoid damage since this can increase experimental background or interfere with the results. It is important to include both mock-inoculated and un-inoculated controls in experiments to be able to discriminate between pathogen-induced staining and staining resulting from damage to leaves during inoculation.
9. Older leaves generally require longer incubation times to be cleared with ethanol.
10. To ease the imaging process, place the leaves in Petri dishes containing H_2O and enable them to fully expand. Imaging can be performed using the preferred image capture device (e.g., scanner, camera, or microscope). If a scanner is used, place the Petri dish containing the leaves on top of the scanner and image it. If a camera is used ensure the Petri dish containing the leaves is placed in a suitable background (e.g., white paper). Alternatively, leaves can be smoothed onto laminated white card, which reduces reflection due to the Petri dish and inconsistencies in the contact of the leaf with the supporting surface. If a microscope is used, cut a small piece of leaf, mount it on a microscope slide and visualize it under the microscope. It is important to stain whole intact leaves rather than leaf discs or sections, as cutting plant tissue can cause damage to leaf tissue that results in high background. If leaf discs or sections are to

be visualized, stain whole leaves and after the staining procedure has been completed generate the leaf discs or sections from the whole stained leaves. The area stained can be quantified using image analysis software.

11. Be careful to minimize damage to the leaf discs when extracting them or when transferring them to the Petri dishes, tubes, or vials, as damaging the leaf discs can interfere with the results. To minimize damage when extracting the leaf discs, place the leaf on top of a tissue paper and press with the cork borer without moving it from side to side.
12. The leaf discs should be floated on H_2O to prevent dehydration, but they should not be submerged.
13. Depending on the pathogen and plant species, the concentration of pathogen can differ. For example, for *Pseudomonas syringae* pv. *tomato* DC3000 an appropriate concentration is $OD_{600} = 0.3$. *See* Smith and Heese [32] for information on how to prepare *P. syringae* solutions.
14. Other elicitors such as elf18 [33] can also be used. Ensure that your species, cultivar or ecotype of plant is able to respond to the elicitor of choice.
15. Make sure that the whole leaf disc is in direct contact with the luminol/peroxidase working solution containing either the pathogen or elicitors.
16. For details on how to measure luminescence read the device manufacturer's manual or *see* Bisceglia et al. [34]. It may be necessary to adjust the interval time depending on the plate reader.
17. Do not release the vacuum quickly as this can damage the leaves.
18. This protocol has been optimized for *N. benthamiana* leaves. For species with small leaves such as *A. thaliana* whole leaves can be stained.
19. When using tough and recalcitrant leaves an additional step of vacuum application lasting up to 30 min may be required. If necessary, this optional step can be applied to leaf discs that are immersed in aniline blue solution.
20. It is important to wrap the polypropylene tubes in aluminum foil to keep the solution and leaf discs in the dark.
21. The conditions at which plants are grown prior to the experiment can greatly influence the amount of callose that is deposited or the timing of callose deposition [35].
22. Other tubes can be used depending on the size of the leaves and volumes used. However, is important that tubes can be

hermetically sealed since the solutions used contain phenol and chloral hydrate, which are toxic.

23. Optional. Place the tubes inside a phenol compatible box or similar sealable container to avoid any phenol leakage to the environment during the overnight incubation.
24. The number of discs that can be extracted per leaf indicated in the protocol refers to *Nicotiana benthamiana* leaves. For other species such as *A. thaliana* or *P. vulgaris* a smaller or larger number of leaf discs can be extracted, respectively. Therefore, the number of leaves and leaf discs can be scaled depending on leaf size.
25. All the vials should contain ultrapure H_2O obtained from the same source. Various parameters such the model of the ultrapure water system or age of the filters can yield H_2O with different conductivity and pH values. It is important that all these factors remain as constant as possible to keep the conductivity of the H_2O in the vials homogeneous.
26. Calibrate the conductivity meter before the experiment according to the manufacturer's instructions. It is recommended to use two different standard solutions (e.g., 1413 and 84 μS standard solutions).

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Chapter 20

From Sample to Data: Preparing, Obtaining, and Analyzing Images of Plant-Pathogen Interactions Using Confocal Microscopy

Helen N. Fones and George R. Littlejohn

Abstract

This chapter describes the steps needed to inoculate host plants with a fungus of interest, and subsequently to visualize the infection using confocal microscopy. As an exemplar, we consider the interaction between wheat and the Septoria leaf blotch fungus, *Zymoseptoria tritici*. This method is easiest when a GFP- or other fluorophore-tagged strain of the studied fungus is available, but notes are also provided which describe possible staining techniques which may be employed if fluorescent fungus is unavailable in your system.

Key words Plant pathogenic fungi, *Zymoseptoria tritici*, GFP, Confocal microscopy, Image analysis

1 Introduction

This chapter describes the basic procedures needed to study infection of a host plant by a fungus through confocal microscopic observation of a fluorescent fungal strain. This is a powerful technique, allowing many approaches from the high-throughput collection of images of fungi on or in the plant leaf to detailed time-lapse videos of individual plant–fungal interactions [1, 2]. To obtain reliable, representative data using this technique it is important to consider carefully the experimental design and sampling strategy, both when selecting material to view and images to analyse. Here, we place these considerations centrally as we provide basic instructions for each step from plant infection to image analysis. There are a multitude of microscopy techniques available which are more or less well suited to answering specific kinds of biological questions. Practical considerations such as ease of use, local expertise, availability of equipment, and appropriately labeled biological materials all help determine the applicability of any particular method of imaging. Confocal microscopy is focussed on here because it is a commonly used technique. It allows the user to

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non-destructively "optically section" live biological samples. This allows for 3D information (normally fluorescent signals from selected fluorophores) to be collected for samples and assembled into accurate models of signal intensity in time and space. Firstly, tips are provided on how to choose a fluorescent protein (Subheading 3, **Notes 1** and **2**). Subheading 3.1 gives detailed instructions for leaf inoculation. Specific details are given for the commonly studied fungus *Zymoseptoria tritici* (*Zt*), but the methods are widely applicable. Plant growth conditions and inoculum concentrations are discussed in **Notes 3** and **4**; further tips and alternative inoculation methods given in **Notes 5** and **6**. Subheading 3.2 covers selection and preparation of samples for microscopy, while the essentials of image collection are covered in Subheading 3.3. Finally, Subheading 3.4 describes very basic image analysis using free software.

2 Materials

1. YPD Agar (for 1 L): 20 g yeast extract, 20 g peptone, 10 g dextrose, 12 g agar.
2. GFP-tagged fungal strain; here we have used GFP tagged versions of the *Z. tritici* strain, IPO323 [3–5].
3. Host seeds of a susceptible cultivar (when using *Z. tritici* IPO323, we use the varieties Galaxie, Riband, and Consort).
4. Miracloth.
5. Glass reagent sprayer: CAMAG.
6. Confocal microscope.
7. Glass slides and coverslips.
8. Perfluorodecalin.
9. Compost mix John Innes No. 2.

3 Methods

Before you begin—Choosing your imaging methodology

1. Before beginning your experiments, it is important to consider the imaging modality that you will use and how contrast will be achieved in the images you produce (*see* **Note 1**).
2. Fluorescence microscopy techniques, including laser scanning confocal microscopy as it is most commonly applied to biological samples, require fluorescent labeling of molecules, subcellular compartments or cells. In live-cell imaging, this is usually achieved using a fluorescent dye which has an affinity for a target of interest or by specific targeting of genetically encoded fluorescent proteins (FPs).

3. The use of an FP (e.g., GFP) tagged strain of your fungus or plant is appropriate where fusion proteins are to be localized, well-characterized proteins are used to provide compartment-specific labeling, protein–protein interactions are to be tested, or FP-based biosensors are to be used to determine the activity or concentration of a target ion or molecule.
4. Use of an FP tagged strain of your fungus can allow noninvasive labeling of the fungus or parts of the fungus, even when it is located deep within plant tissues. Staining with a fungal-specific, fluorescent stain (e.g., FITC-conjugated WGA, calcofluor white) is also possible (*see* **Note 2**).
5. Consider the best fluorophore for your work. GFP is used here. Choose a suitable GFP variant for your work (*see* **Note 1**).

3.1 Plant Inoculations for Imaging Plant–Pathogen Interactions

1. Grow plants (for *Zt,* use a wheat variety susceptible to your strain of interest) for 14 days; sow seeds on a standard compost mix (e.g., John Innes No.2) and maintain in stress-free conditions, optimally in a dedicated growth chamber (*see* **Note 3**).
2. Plate fungus onto appropriate agar (*Zt*: YPD) from frozen or other stock, using sterile toothpicks to serially spatially dilute spores and proceed as follows: Incubate (*Zt*: 18 °C) for 3–5 days, until abundant spores are formed. Create a spore suspension in dH_2O: transfer a scrape of conidiospores (*Zt*: yeast-like growth, which behaves similarly to pycnidiospores in this context) from the agar to a tube containing 1–2 mL dH_2O and vortex until homogeneous. Filter spore suspensions through two layers of sterile Miracloth to remove mycelial debris. Count spores using a haemocytometer; set colony forming units (cfu)/mL to 10^8 by diluting or adding spores. Using serial dilutions, obtain an appropriate cfu/mL spore concentration for your experiment (*see* **Note 4**). Transfer spore suspension to handheld reagent sprayer and spray evenly over leaves (*see* **Notes 5** and **6**). Return inoculated plants to growth chambers; cloche for 72 h post inoculation to ensure high humidity.

3.2 Sample Preparation

1. Sample selection should be random within treatments; use random number generation to select plant, leaf, and leaf section for analysis, if practicable.
2. Restriction of analysis to specific leaves (e.g., leaf 2 on each plant) and leaf areas may reduce variability in results, but could introduce bias. Think carefully about the questions you are trying to answer before restricting your dataset in this way.
3. Place selected leaf section on a glass slide; add water or perfluorodecalin (*see* **Note 7**) and coverslip. Tap gently to remove air bubbles.
4. Sample preparation and quality of mount have a very large effect on image quality.

3.3 Confocal Microscopy

1. Confocal microscopy involves careful balancing of imaging parameters so that excitation energy may be minimized and usable data may be obtained.
2. Firstly, decide on the spatial and temporal resolution and magnification required. This will be informed by the method of image processing or presentation later required. Select minimum spatial resolution.
3. Start with a low gain and laser power. Try to keep these low.
4. Select lens and set pinhole to 1 Airy Unit.
5. Image signal intensity may be increased by increasing the excitation (laser) power, increasing gain across the detector, increasing the bandwidth of light captures (this may decrease specific signal, so be careful) or increasing pinhole diameter.

3.4 Extracting Data

1. Save images as JPG or TIF (either single image or a z-stack projection).
2. Open in Image J [6].
3. Use Analyze → Set scale to set the scale of your images.
4. Select the part of the image you wish to analyse, then Edit → Clear Outside to isolate this.
5. Using the image thresholding tool (Image → Adjust → Threshold), select a feature or set of features. For example, using the HSB (hue-saturation-brightness) color space, it is possible to select all green (i.e., GFP-expressing) areas of your image.
6. Analyse → Measure will provide details of your selection, including area and maximum/average intensity of the selected pixels.
7. Be aware that intensity data are only comparable between images if all settings (e.g., laser power, detector gain) were constant during recording.

4 Notes

1. Choose a suitable fluorescent protein for the experiment you are conducting. Here, we use GFP-expressing fungus, which can be clearly seen against the red, chlorophyll autofluorescence of healthy leaves. However, if background fluorescence with short wavelength excitation is high (e.g., dead plant cells), an alternative such as red-fluorescent protein (RFP), which fluoresces at longer wavelengths, may be advisable. Similarly, longer wavelength radiation is more penetrating of tissue and may be a good choice for imaging deep in tissues. There are various forms of proteins such as GFP, whose characteristics differ. Some, for example, have greater initial fluorescence, while others may display slower rates of fluorescence decay [7]. It is also possible to obtain improved expression of GFP by codon-optimising according to the genome of the pathogen [8].

2. Fluorescent strains provide an advantage over fluorescent stains such as calcofluor white or FITC-conjugated lectin because they do not require the invasive and potentially damaging manipulation of the infected tissue that is required to achieve penetration of the dye into the internal cell layers or structures. Many staining protocols involve boiling, vacuum infiltration, or aggressive chemical treatments (e.g., phenol, acids) which may spatially disrupt the plant–pathogen interaction you wish to view, as well as usually resulting in the death of the stained tissue. FPs can often be fused to well-characterized proteins in order to be expressed in specific, well-characterized compartments of the fungal cell, or under specific conditions. It is possible with FPs to view the live fungus interacting with the plant tissue [4, 8]. On the other hand, expression of an FP is energetically demanding and may affect the *in planta* behavior of your fungus. Integration of the FP gene into the fungal genome may also have other unforeseen effects. Comparisons should be carried out to ensure virulence and pathogenicity are not compromised in your tagged strain, and appropriate controls incorporated into your experiments. Where possible, compare the behaviour of the wildtype strain using light microscopy or a fluorescent stain.
3. If working with multiple fungal strains, consider the layout of your growth chamber and any gradients of light or humidity. Blocking may be necessary in your experimental design to account for these. An example of best practice is inclusion of replicates of each treatment evenly divided between (and randomized within), for example, shelves on different vertical levels of a chamber where humidity varies from top to bottom. This may require extra plants, so must be considered early, along with the necessary level of replication in your work.
4. Fungal cells may interact in various ways, including autoinhibition of germination and during colonization of the leaf. In addition, high inoculation densities may trigger atypical reactions in the host, which may affect disease development. Leaves may become saturated with symptoms, preventing observation of small differences between strains. Thus, low inoculum densities may be preferable. Low-density inoculation methods are discussed in **Note 7** and in Fones et al. 2015 [9].
5. Handheld reagent sprayers give a very fine mist that does not run off hydrophobic leaves. Sprayed leaves have a silvery shimmer. Aim to spray each leaf from base to tip on the abaxial or on both surfaces; turn plants and vary the angle as you spray to achieve this. Do not overspray leaves, as this will cause runoff of your inoculum. Monocots grow from the base; use a marker pen to show where the base of the leaf was at the time of spraying, so that inoculated area is clear later. Ensure that

plant age, planting density, cultivar, soil, and time of day for inoculation are constant between experiments.

6. Alternative inoculation methods: **Brush inoculation**. Proceed with Subheading 3.1 as described, but instead of transferring the spore suspension to an atomizer, instead use a soft paintbrush to spread the inoculum onto the leaf. This allows specific areas to be inoculated but is time-consuming if large numbers of leaves are used. Brush inoculation transfers a high volume of inoculum and can lead to run off from the leaves. To mitigate this, a low concentration of surfactant (e.g., 0.1% Tween 20) can be added to the spore suspension. However, there is a risk that this may alter fungal behaviour if the surfactant can be metabolized. **High-density inoculation**. For some applications, it may be appropriate to pipette a known volume of high density inoculum onto the leaf. This method is described by Keon et al., 2007 [10]. **Precise, low density inoculation**. This method is extremely time-consuming but allows precise control of the number of spores transferred to the leaf. *See* ref. 9.
7. It is recommended that for deep imaging of plant tissues, especially over long time courses, perfluorocarbon mounting media are used to optically improve the mounted sample and reduce the physiological impact of imaging on the specimen [11, 12].

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Chapter 21

Screening of c-di-GMP-Regulated Exopolysaccharides in Host Interacting Bacteria

Jochen Schmid, Broder Rühmann, Volker Sieber, Lorena Romero-Jiménez, Juan Sanjuán, and Daniel Pérez-Mendoza

Abstract

Bacterial exopolysaccharides (EPS) often confer a survival advantage by protecting the cell against abiotic and biotic stresses, including host defensive factors. They are also main components of the extracellular matrix involved in cell–cell recognition, surface adhesion and biofilm formation. Biosynthesis of a growing number of EPS has been reported to be regulated by the ubiquitous second messenger c-di-GMP, which promotes the transition to a biofilm mode of growth in an intimate association with the eukaryotic host. Here we describe a strategy based on the combination of an approach to artificially increase the intracellular level of c-di-GMP in virtually any gram-negative bacteria with a high throughput screening (HTS) for the identification of monosaccharide composition and carbohydrate fingerprinting of novel EPS, or modified variants, that can be involved in host–bacteria interactions.

Key words Exopolysaccharide, c-di-GMP, High Throughput Screening, Carbohydrate Fingerprint, Biofilm

1 Introduction

Extracellular polysaccharides (EPS) are critical components for the bacterial adaptation to changing environments allowing the producers to thrive in very different niches and protecting them against osmotic stress, desiccation, toxic compounds or other abiotic and biotic stresses. EPS can also contribute to nutrient gathering and together with other macromolecules are central players in the formation and maintenance of biofilms, or can serve as valuable biogenic polymers in medical or technical applications. Biofilms are dynamic tridimensional structures where bacteria live in a self-produced matrix of extracellular polymeric substances.

EPS are also important biologic components in the interaction between bacteria and their eukaryotic hosts. They are key virulence factors in different plant and animal diseases through their capacity to mediate adhesion to surfaces, cellular recognition and helping to

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evade phagocytosis and different immune system compounds, thus improving their persistence in the host [1, 2]. In some of those diseases EPS are also important for their transmission, where mutants affected on EPS production are very poorly transmitted by and retained within insect vectors [3]. In this regard, different EPS degrading enzymes like glycoside hydrolases have been recently proposed as powerful tools to develop novel antibiofilm therapeutics [4, 5]. They are also involved in interactions with other different outcomes, including commensalism and mutualism. In rhizobia, for example, the EPS are also important for the establishment of effective symbiotic interactions with legume plants and can impacton their recognition specificity [6, 7].

Cyclic diguanylate (c-di-GMP, cdG) is currently considered a ubiquitous second messenger in bacteria. Best known for its role in controlling the transition from a planktonic/motile lifestyle to a sessile biofilm mode of growth, this nucleotide-based molecule influences a wide range of cellular processes, including cell-cell signaling, cell cycle progression and virulence [8]. Intracellular concentration of this cyclic dinucleotide can be modulated through various mechanisms, in accordance with environmental and physiological cues through complex signal transduction systems. It is synthetized from two molecules of GTP by diguanylate cyclases (DGC), and is degraded by specific phosphodiesterases (PDE). Bacterial genomes encode a variable number of those metabolizing enzymes, being bacteria with complex environmental lifestyles and which co-evolved with eukaryotes, the ones that normally possess a higher number of c-di-GMP related proteins [9, 10].

As a general rule, high levels of c-di-GMP promote bacterial decision to attach to a surface and form a biofilm community. In that sense, it has been proposed different c-di-GMP signaling proteins as possible targets to control biofilm formation and virulence in bacterial pathogens [11]. The formation of a biofilm allows a lifestyle that is entirely different from the planktonic state. The production of many matrix components, including bacterial EPS, is directly or indirectly activated by c-di-GMP [12, 13]. There are some exceptions, such as *Xanthomonas campestris* where biofilm formation is increased, but xanthan production is decreased by high levels of c-di-GMP [14]. Another contrary example is *Xylella fastidiosa* in which increased levels of c-di-GMP negatively influences biofilm formation [15]. This second messenger was discovered almost 30 years ago as an allosteric activator of bacterial cellulose synthase [16]. Up to date, nearly a dozen EPS are known to be regulated by c-di-GMP including several with relevance in the interaction of different bacteria with their respective hosts: e.g., the prevalent bacterial cellulose (BC) and poly-β(1-6)-N-acetyl-D-glucosamine (PNAG); the Pseudomonad alginate, Psl, and Pel; and the plant-associated bacteria mixed-linkage β-glucan (MLG) and unipolar polysaccharide (UPP) [12, 17]. As mentioned above,

microbial EPS are critical in understanding the physiology of many bacteria and their interaction with the host and for example, bacterial cells with the ability to produce EPS have proven to be more virulent in several animal models of infection than their isogenic nonproducing mutants [1].

Very often the study of these bacterial polymers is not a simple task due to different reasons: (1) one is the high inter- and intraspecies EPS variability. Among the common properties of bacteria that interact with eukaryotic host is the ability to secrete many different types of polysaccharides, ranging from highly diverse chemical structures to subtle modified versions [18, 19]. For example, isolates of *Burkholderia cepacia* complex may produce large amounts of EPS that endow the bacteria with a mucoid phenotype, thus facilitating bacterial persistence during infection [20]. *Salmonella* species produce several EPS components, some of them species specific, including cellulose, colanic acid, O-antigen capsule and Vi capsular polysaccharide which are based on different biosynthetic pathways [13, 21], with the two latter preventing complement-mediated clearance from the infected organism [22, 23]. An additional problem (2) is that the production of several of those EPS in host-interacting bacteria is cryptic in the absence of the eukaryotic partner and therefore difficult to identify under laboratory conditions. In that sense, artificially increasing the intracellular levels of the c-di-GMP by overexpressing the DGC the *pleD** has proven to be useful for uncovering novel and otherwise undetectable EPS in different plant-interacting bacteria [24, 25]. A third class of difficulties (3) includes the experimental approaches to precisely identify this plethora of bacterial EPS. These commonly imply nonquantitative or semiquantitative assays which complicate an accurate identification: e.g., direct visual observation of colony morphology on plates unstained or staining with colorants, viscosity, and carbohydrate precipitation. This can also be combined with other more quantitative approaches like the total carbohydrate content analysis [26]. Nevertheless, faster screening and analytical methods are required for the determination of the carbohydrate composition of novel EPS among a relative large number of different strains.

Here we describe a strategy which combines c-di-GMP EPS regulation with a high throughput screening (HTS) to find novel EPS or modified variants that can be involved in host–bacteria interactions. For this purpose, our recently reported mini-Tn*7* vehicles for expression of a highly active DGC [27] is used to artificially enhance c-di-GMP intracellular levels in a wide range of gram-negative bacterial species and thus likely revealing the production of novel cryptic polysaccharides under laboratory conditions. Tn*7* inserts into a specific site called *att*Tn*7* as single copy, with a determined orientation which is usually localized downstream of the *glmS* gene (encoding glucosamine-fructose-6-

phosphate aminotransferase) and where transposon insertions do not affect bacterial fitness [28–31]. The genetically modified recipient bacteria containing the DGC *pleD** are screened by our recently developed high-throughput workflow [26, 32] and compared with their respective reference strain for production of c-di-GMP activated EPS. This novel methodology could lead to uncover an unsuspected number of polysaccharide structures with relevance in the interaction of diverse bacteria with their eukaryotic host. Furthermore, with this approach it will be even possible to identify novel EPS variants with high potential for technical or medical applications which cannot be observed under normal laboratory conditions, due to non-activated EPS clusters in the genome [13]. Based on the altered monomer composition and total sugar content, strains with a significantly differing carbohydrate fingerprint represents very promising targets for further detailed analysis.

2 Materials

2.1 Culture Media and Growth Conditions

1. Luria–Bertani broth (For 1 L): 10 g tryptone, 5 g yeast extract, 5 g NaCl with 15 g of agar for solid media and supplemented with diaminopimelate (DAPA) to a final concentration of 0.3 mM and the antibiotic ampicillin (Amp) at 200 μg/mL (*see* **Note 1**).

2.2 Plate Format and Equipment

The protocol is described following a standard 96-well plate format and is designed to be performed manually with multichannel pipettes or could also be automatized with liquid handling workstations. In the case of 96-well plates, 2 mL deep-well plates (DWP) should be used, which are sealed with a breathable foil to allow active cell growth and prevent cross contamination.

As working station a liquid handling system (Tecan, Evo) and liquid handling station (Brand, LHS) might be used. In general, the working station should be able to realize 96-well pipetting in the volume from 5–1000 μL, centrifugation and incubation steps of DWP and microtiter plates (MTP) as well as absorption reads with a microplate reader.

Equipment:

1. Microtiter plate reader
2. Plate incubator
3. UHPLC-UV-ESI-MS (e.g., Ultimate 3000RS, Dionex, with degusser (SRD 3400), pump module (HPG 3400RS), autosampler (WPS 3000TRS), column compartment (TCC 3000RS), diode array detector (DAD 3000RS), ESI-ion-trap unit (HCT, Bruker)).

4. Special clamping device (tailor made steel clamp to avoid evaporation from 96-well PCR plates, sealed with silicon map). The clamping device was made from iron steel with 2 screws on the long side of the rectangle and 2 screws on the short side.

Material:

5. 96-well microtiter plates.
6. 96-well-PCR microtiter plate with TPE cap mat.
7. Gel-filtration plates (96-well Spin Column G-25).
8. A/B glass filter plate 1 μm.
9. HPLC filter plate Supor 0.2 μm.

2.3 Buffers and Solutions

1. Trifluoroacetic acid (TFA) 4 M.
2. 3.2% aqueous ammonia solution.
3. Control of neutralization solution: 0.05 g of phenol red in 50 mL of 20% ethanol.
4. HT-PMP-derivatization reagent: 125 mg 1-phenyl-3-methyl-5-pyrazolone (PMP), 7 mL methanol, 3.062 mL ddH_2O, and 438 μL of 3.2% aqueous ammonium solution. 19.23 mM acetic acid (0.962 mL 1 M acetic acid +49.038 mL ddH_2O).
5. Glucose standards for glucose assay: 500, 250, 100, 50, 25, 10, 5, 2.5, and 0 μM glucose.
6. Glucose-assay reagent mix: 4 mL 500 mM potassium phosphate (pH 5.7), 1.5 mL 50 mM 2.2-azinobis-(3ethylbenzthiazoline)-6-sulfonic acid, 2 mL 100 U glucose oxidase, 10 μL 1000 U horseradish peroxidase, and 42.49 mL ddH_2O.
7. Pyruvate assay: pyruvate-assay reagent mix (3 mL 1 mM N-(carboxymethylamino-carbonyl)-4.4′-bis(dimethylamino)-diphenylamine sodium salt (DA-64), 300 μL 10 mM thiamine pyrophosphate, 60 μL 100 mM magnesium chloride hexahydrate, 2.4 mL 500 mM potassium phosphate buffer (pH 5.7), 30 μL 100 U pyruvate oxidase, 12 μL 1000 U horseradish peroxidase, and 24.19 mL ddH_2O).

3 Methods

3.1 Insertion of Mini-Tn7 Vectors into Recipient Gram-Negative Bacteria

E. coli donor and helper strains are grown at 37 °C on LB. Triparental matings (donor, helper and recipient) are employed to deliver the mini-Tn*7* constructs into the genome of the different strains of interest (Fig. 1a; *see* **Note 2**). *E. coli* β2155 and β2163 [33] are used as mobilising donor strains for the pUC18T-mini-Tn7T derived constructs (Table 1). *E. coli* β2155 encodes *lacI*^{q} which keeps the expression of the diguanylate cyclase *pleD** low in the donor cell in the absence of the inducer Isopropyl β-D-1-thiogalactopyranoside (IPTG). Strain β2163 harbours the helper plasmid pUX-BF13

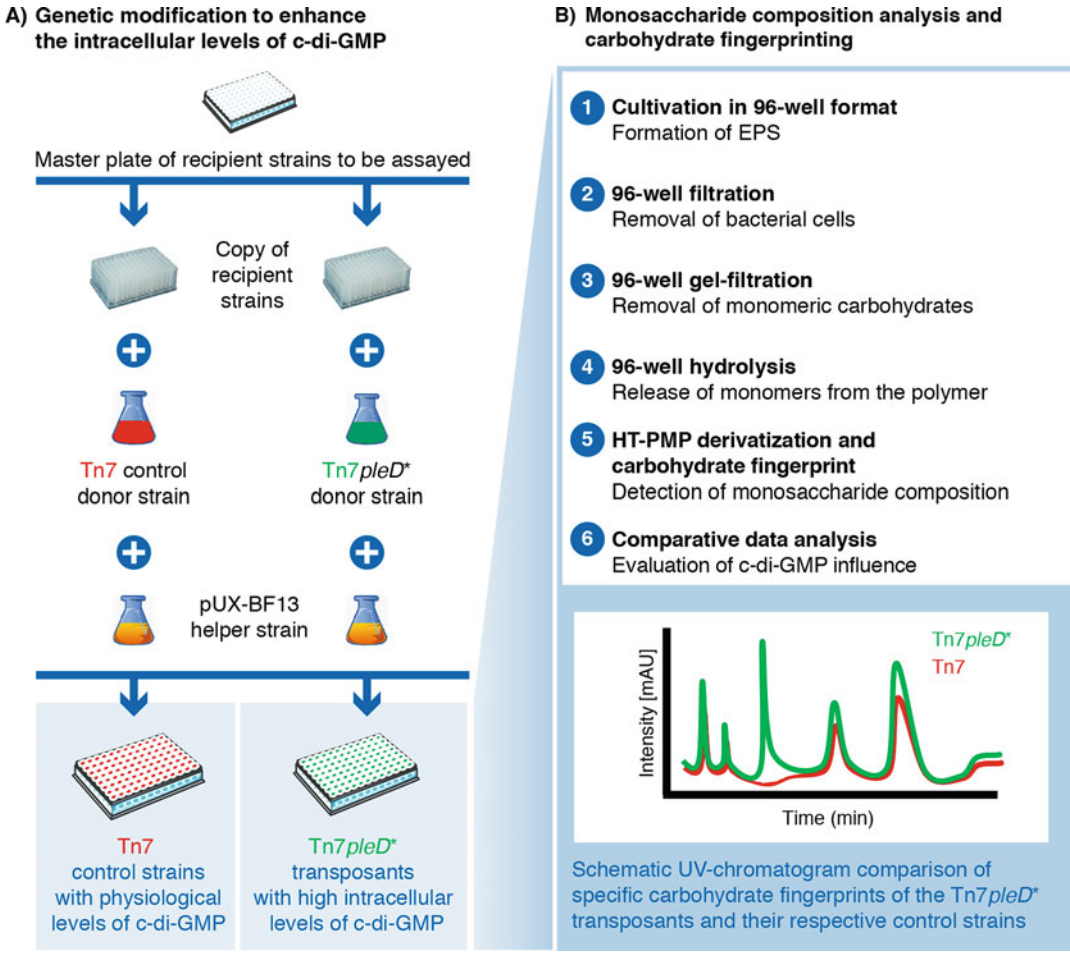

Fig. 1 Screening of c-di-GMP regulated exopolysaccharides. Overview of the high throughput approach for the genetic modification to increase the intracellular levels of c-di-GMP in the strains of interest (**a**), and the subsequent steps for the identification of monosaccharide composition and carbohydrate fingerprinting (**b**) of secreted c-di-GMP-regulated EPS that are not detected under physiological intracellular levels of this bacterial second messenger in laboratory conditions

providing the Tn*7* transposition functions required *in trans*. Both strains are *dapA* knockouts and therefore diaminopimelate auxotrophs, which facilitates the subsequent counter selection of these donor and helper strains even in rich recipient media [33].

1. Precultures of the donor strains containing the diguanylate cyclase *pleD** (Table 1, *see* **Note 3**), and helper strains are grown overnight at 37 °C in LB supplemented with ampicillin (200 μg/mL) and DAPA (0.3 mM). Precultures are diluted in 500 mL flasks containing 50 mL of the same media prewarmed at 37 °C to a final optical density at 600 nm (OD_{600}) of 0.05 and let them grow with shaking to late exponential phase ($OD_{600} = 0.5$).

Table 1
Plasmids for *pleD genetic modification. Amp^R, Kan^R, and Tet^R stand for resistance to ampicillin, kanamycin, and tetracycline, respectively**

Plasmids	Relevant characteristics	Reference
mini-Tn*7*pleD*Km	mini-Tn*7*pleD* with 1.2 kb KpnI fragment containing Km marker Amp^R, Kan^R,	[27]
mini-Tn*7*Km	mini-Tn*7*pleD*Km with a 1114 bp NcoI internal deletion of *pleD*Amp^R, Km^R,	[27]
mini-Tn*7*pleD*Tc	mini-Tn*7*pleD* with 1.3 kb KpnI fragment containing Tc marker Amp^R, Tet^R	[27]
mini-Tn*7*Tc	mini-Tn*7*pleD*Tc with a 1114 bpNcoI internal deletion, Amp^R, Tet^R	[27]
pUX-BF13	Helper plasmid providing the Tn*7* transposition functions in trans, Amp^R, mob+, ori-R6K	[29]
pJB3Tc19	Amp^R, Tet^R; cloning vector, Plac promoter	[34]
pJBpleD*	Amp^R, Tet^R; pJB3Tc19 derivate bearing a 1423 bpXbaI/EcoRI fragment containing *pleD**	[35]

2. Precultures of recipient strains to be assayed are generated from a master plate by inoculating a 2 mL 96-deep-well plate containing 250 μL of suitable recipient media using a 96-pin replicator and let them grow until an early stationary phase (approximately $OD_{600} = 1$) with a breathable sealing film in a microplate shaker (1000 rpm) equipped with an incubator hood.
3. The 50 mL of donor and helper cultures are separately transferred to a 50 mL reaction tube and washed two times (by centrifugation and subsequent resuspension) with fresh 37 °C prewarmed LB medium to completely remove the ampicillin (*see* **Note 4**).
4. Transfer 500 μL of the donor and 500 μL of the helper strain, to each well of the recipient plate to reach close to 1:1:1 ratio of donor–helper–recipient and mix them by pipetting up and down three times.
5. Prepare a 96-well microtiter conjugation plate by pouring 150 μL of melted LB agar supplemented with DAPA 0.06 mM in each well and let them solidify for 20 min under sterile conditions (*see* **Note 5**).
6. Spot 30 μL of each triparental conjugation mixture on the top of the agar of the conjugation plate, let them dry, cover them with a lid and incubate the plate for 24 h at a temperature suitable for the recipient strains (e.g., 30 °C).
7. Transfer 100 μL of suitable recipient media without DAPA to each well of the conjugation plate and resuspend the

conjugation mixture by pipetting up and down five times. Transfer 80 μL of the resuspended conjugation mixture to a new 96-deep-well plate containing 1.5 mL of suitable recipient media (e.g., EPS-screening media; *see* **Note 6**) without DAPA and supplemented with the appropriate antibiotic (Table 1). Let them grow at the same conditions described above until a late stationary phase.

8. Make a 1/100 fold dilution by transferring 10 μL of each well in a new 2 mL 96-deep-well plate containing 990 μL of fresh recipient media and let them grow in the same conditions described above until a late stationary phase.
9. Repeat **step 8** twice and then make a copy for storage by mixing 100 μL of each mini-Tn*7* transponsant with 40 μL of glycerol (50%) in a new 96-well microtiter plate sealed with an aluminum foil and store them at −80 °C (*see* **Note 7**).

3.2 Characterization of the Altered Carbohydrate Fingerprint Via UHPLC-UV-ESI-MS Analysis

1. The final culture highly enriched with the Tn7 transposants should be used for the analysis of the carbohydrate fingerprint (Fig. 1b) as described in detail by Rühmann et al. giving further hints for data interpretation [26, 32]. For high throughput the screening platform has to be separated into a fully automatized prescreening and only preselected hits should be analyzed in detail via carbohydrate fingerprint.
2. Analysis of the carbohydrate fingerprint starts with the removal of the cells via centrifugation. Therefore, transfer the main-culture DWP into the centrifuge (4300 × *g* for 30 min at 20 °C).
3. If cells were not completely removed, because of high viscosity, an adequate dilution of the main culture should be performed (e.g., 1:5 or 1:10).
4. Transfer 180 μL of the (diluted) main-culture supernatant to the filtration plate and centrifuge at 3000 × *g* for 10 min at 20 °C, to ensure complete cell removal.
5. Prepare equilibration of the gel-filtration plates by dispensing 150 μL of ddH_2O into all wells. Transfer the gel-filtration plate into the centrifuge (2000 × *g* for 2 min at 20 °C) and repeat this step two times and centrifuge the gel-filtration plate at 1000 × *g* for 2 min at 20 °C before further use.
6. Pipette 35 μL of filtrate into the center of the equilibrated gel-filtration plate and centrifuge the gel-filtration plate at 1000 × *g* for 2 min at 20 °C.
7. Prepare the glucose assay before hydrolysis: perform a 1:10 dilution by adding 45 μL of ddH_2O and 5 μL of gel filtrate and cover the MTPs with a silicone cap mat (*see* **Note 8**).

8. Prepare the pyruvate assay before hydrolysis: perform a 1:20 dilution by adding 95 μL of ddH_2O, transfer 5 μL of gel filtrate to each well, and seal the MTP with a silicon cap mat.
9. 96-well microhydrolysis (*see* **Note 9**). A special clamping device was developed to avoid evaporation during the small scaled hydrolysis step. Take a new PCR plate and transfer 20 μL of gel filtrate.
10. Add 20 μL of 4 M trifluoroacetic acid to each well. Then cover the PCR plate with a thermoplastic elastomer (TPE) cap mat and place the PCR plate in the special clamping device (*see* **Note 10**).
11. Mix the solutions via inverting the clamping device ten times. Put the PCR plate in a centrifuge and spin at 2000 × *g* for 2 min to collect all the liquid on the bottom. Put the PCR plate back to the clamping device and secure the device with screws.
12. Place the secured clamping device in the preheated sand bath and incubate for 90 min at 121 °C.
13. Remove the clamping device from the sand bath and let it cool down to room temperature.
14. Remove the screws and spin again in a centrifuge at 2000 × *g* for 2 min in order to collect all the condensate at the bottom of the wells and to prevent cross contamination during removal of the cap mat.
15. Add 3.2% aqueous ammonia solution to adjust the pH to 8 approximately. Cover the PCR plate with a TPE cap mat and shake it manually using the clamping device.
16. After neutralization centrifuge the PCR plate at 2000 × *g* for 2 min.
17. High-throughput-1-phenyl-3-methyl-5-pyrazolone (HT-PMP) derivatization of the carbohydrates [36]. Transfer 25 μL of the neutralized hydrolysate in a fresh PCR plate (*see* **Note 11**).
18. Add 75 μL derivatization reagent mix and cover the plate with a TPE cap mat.
19. After shaking the PCR plate in the clamping device centrifuge the plate at 2000 × *g* for 2 min to accumulate all the liquid at the bottom.
20. For derivatization place the PCR plate in a PCR cycler at 70 °C for 100 min followed by cooling down to 20 °C.
21. Transfer a 20 μL aliquot into a new MTP. Then add 130 μL 19.23 mM acetic acid to each well. Mix directly via aspirating and dispensing (minimum six times) and transfer all the liquid to a 0.2 μm filter plate.

22. Centrifuge the plate (1000 × *g* for 5 min), remove the filter plate, seal the MTP with a silicone cap mat and place the MTP into the UHPLC-UV-ESI-MS for the determination of the carbohydrate fingerprint.
23. Analyze the data of UV and MS/MS detection to determine differences of the monomer composition, and therefore altered EPS-compositions.
24. Analyze the results of the pyruvate assays to determine differences in pyruvate substitution of the produced polymers.

3.3 Preparation of the Glucose Assay

1. Perform a 1:10 dilution via adding 45 µL of ddH_2O and 5 µL of neutralized hydrolysate and cover the MTPs with a silicone cap mat. Add three times 50 µL of different glucose standards to a new MTP used for calibration.
2. Add 50 µL of glucose-assay reagent mix and cover the plates with a silicon capmat and incubate at 30 °C and 400 rpm for 30 min in an MTP incubator.
3. Directly after the incubation make an absorbance read at 418 and 480 nm in an MTP reader. For the calculation of the glucose concentration perform a linear calibration.

3.4 Preparation of the Pyruvate Assay

1. Perform a 1:20 dilution and add 95 µL of ddH_2O and transfer 5 µL of neutralized hydrolysate to each well.
2. Add 100 µL of pyruvate-assay reagent mix, cover the plates with a silicon cap mat, and incubate at 37 °C and 150 rpm for 30 min. Directly after the incubation measure absorbance in an MTP reader at 727 and 540 nm.

4 Notes

1. A suitable recipient media and growth conditions should be chosen for the recipient strains to be screened. Adequate antibiotics concentrations for the selection of the transposants should be determined for the different recipient strains and the following concentrations are just for a reference: kanamycin (Kan) 50 µg/mL, Tetracycline (Tet) 2.5 µg/mL
2. pUC18T-mini-Tn7 derived constructs listed in Table 1 are suitable for the insertion of the DGC *pleD** in a wide range of gram-negative bacteria [31]. Nevertheless, in case the mini Tn*7* transposition is not possible, or the insertion frequency in the selected recipient strains is very low, a mobilizable broad host range plasmid encoding the DGC *pleD** is also available (Table 1) and can be used following a similar procedure.

3. Here we describe the methodology to integrate the Tn*7 pleD** into the desired recipient strains. Repeat the same procedure (Subheading 3.1, **steps 1–9**) with the respective control donor (Tn*7* without *pleD**, Table 1) and a sibling recipient plate to generate the appropriate control of each recipient strain.
4. If the addition of antibiotics to the receptors culture media is required, it should be also removed and washed by centrifuging the recipient 96-deep-well plate and resuspending the cellular pellet with fresh prewarmed receptor medium twice before mixing them with the donor and helper strain.
5. DAPA concentration is reduced ten times to avoid cross-contamination in subsequent steps.
6. In the case of glucose used as C-source, different glucose assays should be incorporated to reduce background signals in the analysis. Additionally, no complex carbon sources such as yeast or malt extract should be used for culturing of the transposants.
7. At least three dilution-and-culture steps under selective antibiotic pressure should be performed in order to enrich the culture with the Tn*7* transposants.
8. For the correct determination of the glucose value of the polymer, measure the glucose content before hydrolysis and subtract it from the glucose content quantified after the hydrolysis step (*see* Subheading 2.3, glucose assay).
9. Heat up the incubation oven (including a sand bath) to 121 °C for at least 1.5 h before using.
10. Caution: Trifluoroacetic acid is a corrosive acid and toxic. Ammonium solution is corrosive. Handle both chemicals only under a fume hood.
11. After taking all aliquots, check the neutralization of the remaining liquid in the hydrolysis plate via adding 12.5 μL phenol red indicator. All wells that do not turn into pink color (pH 8) are not correctly derivatized.

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Chapter 22

Primary Characterization of Small RNAs in Symbiotic Nitrogen-Fixing Bacteria

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Abstract

High-throughput transcriptome profiling (RNAseq) has uncovered large and heterogeneous populations of small noncoding RNA species (sRNAs) with potential regulatory roles in bacteria. A large fraction of sRNAs are differentially regulated and rely on protein-assisted antisense interactions to *trans*-encoded target mRNAs to fine-tune posttranscriptional reprogramming of gene expression in response to external cues. However, annotation and function of sRNAs are still largely overlooked in nonmodel bacteria with complex lifestyles. Here, we describe experimental protocols successfully applied for the accurate annotation, expression profiling and target mRNA identification of *trans*-acting sRNAs in the nitrogen-fixing α-rhizobium *Sinorhizobium meliloti*. The protocols presented here can be similarly applied for the characterization of *trans*-sRNAs in genetically tractable α-proteobacteria of agronomical or clinical relevance interacting with eukaryotic hosts.

Key words RACE, Northern hybridization, mRNA target, Genetic reporter assay, GFP, Riboregulation

1 Introduction

Besides its agronomical and ecological relevance, the nitrogen-fixing rhizobia-legume symbiosis has long served as model experimental system to investigate the molecular bases of eukaryotic chronic infection by microbes. The outcome of legume infection by rhizobia is the formation of symbiotic nodules mostly in the plant root, where bacteroids, the morphologically differentiated form of invading bacteria, are intracellularly accommodated to reduce inert dinitrogen to plant-usable ammonia. Successful symbiotic associations primarily depend on the capacity of free-living rhizobia to survive stress (e.g., oligotrophy, acidity, salinity, or predation) and establish competitive populations in soil and plant rhizosphere. Subsequently, the formation of a nitrogen-fixing root nodule is a complex multistep process coordinated by the exchange of diverse signals between both symbiotic partners [1, 2]. Rhizobial

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responses to this variety of abiotic and plant signals involve continuous reprogramming of gene expression throughout the symbiotic interaction, whose regulation can be exerted at both transcriptional and posttranscriptional levels. Whereas protein-mediated transcriptional control of rhizobial gene expression has been the focus of intensive research, the posttranscriptional adjustment of gene product levels has remained overlooked until recently.

One of the most outstanding consequences of the use of next generation high-throughput sequencing technologies for transcriptome profiling (RNAseq) in bacteria has been the discovery of unexpectedly large and heterogeneous populations of 50–350 nt-long non-protein-coding RNA species (sRNAs) that await functional characterization [3]. Many of them are differentially transcribed from independent promoters within intergenic regions in response to environmental cues and rely on limited base-pairing with *trans*-encoded target mRNAs to posttranscriptionally control translation and/or stability of the message [4]. In nitrogen-fixing symbiotic bacteria, research on RNA-mediated gene regulation has been capitalized in the genetically tractable α-rhizobium *Sinorhizobium meliloti*, the symbiotic partner of alfalfa and related *Medicago* species [5, 6]. In this chapter we compile detailed protocols successfully applied for the primary molecular and functional characterization of *trans*-sRNAs expressed by *S. meliloti*. These include: precise determination of transcript boundaries by Rapid Amplification of the 5′/3′-cDNA Ends (RACE), sRNA detection and expression profiling by Northern blot hybridization, and experimental validation of putative target mRNAs. sRNA candidates subjected to this characterization must be previously selected from available catalogs of noncoding transcripts derived from either genome-wide mapping of transcription start sites (TSS) based on differential RNAseq (dRNAseq) or in silico prediction of conserved *trans*-sRNAs in phylogenetically related bacteria [7–11]. Therefore, these protocols constitute a workflow suited for the characterization of *trans*-sRNAs in diverse phylogenetically close α-proteobacteria of agronomical or clinical relevance interacting with eukaryotic hosts such as *Sinorhizobium*, *Rhizobium*, *Agrobacterium*, or *Brucella* species.

2 Materials

The following protocols require standard equipment in molecular biology, e.g., incubators, gel electrophoresis devices, refrigerated centrifuges, and hybridization oven. Working with RNA demands specific cautions to avoid degradation by RNases. Therefore, keep an extremely clean working area in the laboratory, including the glassware (cylinders, hybridization tubes, ...) and use pipettes only for RNA work, when possible. Clean glass vessels, equipment,

gloves and work surfaces with an RNase-free agent for the complete removal of RNase contamination. Always use sterile ultrafiltered water, filter tips, gloves, and RNase-free plastic tubes and chemicals.

2.1 Bacterial Cultures

1. Tryptone yeast (TY) medium [12] (For 1 L): 3 g yeast extract, 5 g tryptone, 0.65 g $CaCl_2 \cdot H_2O$. Add 15 g agar when necessary. Autoclave at 121 °C 20 min.
2. Vitamins solution (For 100 mL): 20 mg biotin, 100 mg thiamine. Sterilize by filtration.
3. Defined minimal medium (MM) [13] (For 1 L): 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.006 g $FeCl_3$, 0.05 g NaCl, 1.1 g sodium glutamate, 10 g mannitol. Autoclave at 121 °C 20 min and add in sterile conditions 1 mL vitamins solution.
4. LB medium (For 1 L): 5 g yeast extract, 10 g tryptone, 5 g NaCl. Add 15 g agar when necessary. Autoclave at 121 °C 20 min.
5. Antibiotics are added to the media when required at the following final concentrations: streptomycin (Sm) 100 mg/L for *Rhizobium* and 600 mg/L for *Sinorhizobium* strains; nalidixic acid (Nx) 10 mg/L; tetracycline (Tc) 10 mg/L; gentamycin (Gm) 40 mg/L; and kanamycin (Km) 50 mg/L for *E. coli* and *Rhizobium* and 180 mg/L for *Sinorhizobium* strains.
6. IPTG.
7. NaCl.
8. H_2O_2.
9. Pure nitrogen (N_2).
10. HCl and NaOH.
11. 2,2′ dipyridyl.
12. Ethanol.
13. Nitrogen-free mineral solution: 1.5 mM KH_2PO_4, 0.81 mM $MgSO_4 \cdot 7H_2O$, 2.7 mM KCl, 0.70 mM $CaSO_4 \cdot 2H_2O$, 72.2 μM Fe-EDTA, 16.5 μM $Na_2MoO_4 \cdot 2H_2O$, 10.7 μM $MnSO_4 \cdot 2H_2O$, 8.0 μM $CuSO_4 \cdot 5H_2O$, 10.4 μM $ZnSO_4 \cdot 7H_2O$, 0.29 μM H_3BO_3, 0.59 mM $CoCl_2$ $4H_2O$ [14].
14. Luteolin.
15. Rhizobial strain *Sinorhizobium meliloti* 1021.

2.2 Cell Harvest and RNA Isolation

1. Liquid nitrogen.
2. TE buffer 10×: 10 mM Tris–HCl (pH 7.4), 1 mM EDTA (pH 8.0). Adjust pH 8 and store at 4 °C.
3. 0.1% sarcosyl in TE buffer.

4. Lysis solution: 1.4% SDS, 4 mM EDTA, 50 μg proteinase K.
5. 5 M NaCl.
6. Ethanol.
7. RNase-free DNase set.
8. RNase OUT Recombinant Ribonuclease Inhibitor (40 U/μL).
9. Phenol (pH 4.5)–chloroform–isoamyl alcohol solution (25:24:1, v/v).
10. 3 M sodium acetate.
11. NanoDrop spectrophotometer or similar.
12. Components for DNA electrophoresis: 6× loading dye, 10× TAE buffer, and agarose.

2.3 RACE

1. Tobacco Acid Pyrophosphatase (TAP) and 10× TAP buffer.
2. 5′-RNA adaptor (5A): 5′-GCUGAUGGCGAUGAAUGA ACACUGCGUUUGCUGGCUUUGAUGAAA-3′.
3. 3′-RNA adaptor (3A): 5′-P-UUCACUGUUCUUAGCGG CCGCAUGCUC-idT-3′ (idT, inverted deoxythymidine).
4. Calf intestinal alkaline phosphatase (CIP).
5. T4 RNA ligase and 10× ligase buffer.
6. Phenol (pH 4.5)–chloroform–isoamyl alcohol solution (25:24:1, v/v).
7. RT-PCR Kit (e.g., ThermoScript System, Invitrogen).
8. Random hexamers.
9. RNase H.
10. Vector for cloning of PCR products.
11. T7 and SP6 universal primers.
12. Taq polymerase and buffer.
13. 3′-RACE Reverse Transcription Primers: (3RT) 5′-AGCATG CGGCCGCTAAGAAC-3′; 3′-RACE Outer Primer (3′-OUT), 5′-ATGCGGCCGCTAAGAACAGT-3′; 3′-RACE Inner Primer (3′-INN), 5′-CGGCCGCTAAGAACAGTGAA-3′; 5′-RACE Outer Primer (5′-OUT), 5′-GCTGATGGCG ATGAATGAACACTG-3′; 5′-RACE Inner Primer (3′-INN), 5′-GAACACTGCGTTTGCTGGCTTTGATG-3′.
14. 5′ outer and inner primers, 5′-O and 5′-I, respectively (to be designed according to the sequences of the target transcript).
15. Calf-intestinal alkaline phosphatase (CIP) and buffer.

2.4 Northern Hybridization

1. 10× TBE: 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA (pH 8.0).
2. Polyacrylamide–Urea solution: 6% acrylamide–bisacrylamide solution, 7 M urea.

3. 10% Ammonium persulfate (APS, prepare fresh or store aliquots at −20 °C).
4. Tetramethylethylenediamine (TEMED).
5. 2× RNA loading buffer: 97.5% formamide, 10 mM EDTA (pH 7.5), 0.3% xylene cyanol, 0.3% bromophenol blue.
6. RNA molecular weight marker.
7. 3 MM Whatman paper.
8. Positively charged nylon membrane.
9. Semidry electroblot transfer apparatus.
10. Ethidium bromide (EtBr).
11. Hybridization buffer: 0.5 M sodium phosphate buffer pH 7.2, 7% SDS, 10 mM EDTA.
12. Hybridization probes: 20–25mer oligonucleotides (50 pmol/μL) complementary to the sRNA under study and to the 5S.
13. T4 phosphonucleotide kinase (PNK), provided with 10× reaction buffer.
14. 10 mCi/mL ɣ-[^{32}P]ATP.
15. Sephadex G-25 spin column.
16. Hybridization tubes and oven.
17. Glass hybridization tubes.
18. 20× SSC: 3 M NaCl, 300 mM trisodium citrate (adjusted to pH 7.0 with HCl).
19. 1% SDS.
20. Phosphorimager cassette, screen, scanner, and image analysis software.
21. Ultraviolet lamp.

2.5 sRNA Overexpression and Target Validation

1. DNA oligonucleotides (100 pmol/mL) to PCR amplify and clone the sRNA and the regions of interest of target mRNA.
2. Reagents for conventional PCR: Phusion High-Fidelity DNA polymerase or an alternative proofreading polymerase, 5× Phusion HF Buffer, 10 mM dNPTs.
3. Primers: sinR_NdeIF (CATATG GCTAATCAACAGGCTG TC), TSS3_28bp_b_sinIR (GTAGCGATGCTGTCAGGCTC), PCR1 (CGGGCCTCTTCGCTATT) and PCR2 (TTAGCTCA CTCATTAGG).
4. PCR and plasmid DNA purification kits.
5. Vectors pR_EGFP and pSRK_C [15] or pSRK derivative [16] and pK18 *mobsac* [17].
6. Restriction enzymes.
7. T4 DNA ligase, 5000 U/mL and 10× T4 DNA ligation buffer.

8. Rubidium chloride.
9. Competent *E. coli* DH5α (cloning strain) and S17.1 (strain for conjugation by biparental mating).
10. Fluorimeter or flow cytometer to measure culture fluorescence.

3 Methods

The following protocols have been successfully implemented for the characterization of the *S. meliloti trans*-sRNA AbcR2 [10, 15, 18], among others.

3.1 Bacterial Cultures

1. Rhizobial strains are grown in TY or MM medium at 30 °C and *E. coli* strains in LB medium at 37 °C.
2. When necessary, IPTG is added to a final concentration of 0.5 mM to exponential cultures ($OD_{600} = 0.3–0.4$).
3. For stress induction, exponential cultures ($OD_{600} = 0.6$ in TY) are usually modified as follows and harvested 1 h after stress exposure. Heat and cold shocks: temperature shift of from 30 °C to 40 °C or 20 °C, respectively; high salt stress: addition of NaCl to a final concentration of 50–400 mM; oxidative stress: addition of H_2O_2 to a final concentration of 10 mM; microoxia: culture flush with pure nitrogen (N_2) for 1 min; acidic and alkaline stresses: cultures are centrifuged and the cell pellet resuspended in modified media by addition of either HCl to pH 5.6 or NaOH to pH 8.5, iron starvation: cultures are growth in TY with 300 μM 2,2′ dipyridyl, outer membrane stress: growth in TY supplemented with 2% EtOH.
4. To mimic symbiotic conditions rhizobia are grown in TY broth to the log phase, washed, and resuspended in nitrogen-free mineral solution to be subjected to either of the following treatments: addition of plant inducing flavonoids (e.g., luteolin to a final concentration of 20 mM) or host-plant inoculation for mature nodule harvest.

3.2 Isolation of Total RNA

Purified DNA-free total RNA is necessary for downstream applications such as RACE mapping of sRNA ends, Northern blot, (quantitative) RT-PCR or microarrays. The following protocol is designed to purify approximately 25 μg total RNA per sample, including sRNAs (*see* **Note 1**).

1. Obtain the bacterial culture in the desired condition.
2. Centrifuge at 6000 rpm for 3 min in a table top centrifuge.
3. Wash 1–2 times with 0.1% sarcosyl in 1× TE (*see* **Note 2**). Vortex.

4. Wash 1–2 times with 1× TE (*see* **Note 1**). In the last wash, split cells equivalent to OD_{600} = 3 (e.g., 5 mL culture of OD_{600} = 0.6) in 1.5 mL microtubes (several microtubes may be needed for each strain/condition, especially in stationary phase).
5. Remove the supernatant and freeze pellets in liquid nitrogen for 10 min (*see* **Note 3**). If required, pellets can be stored at −80 °C indefinitely.
6. Gently resuspend each bacterial pellet in 300 μL of lysis solution.
7. Incubate for 10 min at 65 °C. Mix regularly.
8. Chill on ice and add 125 μL of 5 M NaCl to each 1.5 mL microtube.
9. After 10 min on ice, centrifuge samples (15 min, 13,000 rpm, 4 °C on a refrigerated table top centrifuge).
10. Transfer aqueous (upper) phase to a new 1.5 mL microtube containing 1350 μL of cold 100% EtOH. Mix by inverting tubes.
11. Store at −80 °C for at least 1 h. When required, samples can be stored at this point overnight or several days.
12. Thaw samples on ice and centrifuge (30 min, 13,000 rpm, 4 °C on a refrigerated table top centrifuge).
13. Remove accurately all EtOH and resuspend the pellets in 42.5 μL of water (at this point, pellets belonging to the same sample can be pooled together).
14. Add 8.5 μL of DNAse I reaction mix (7 U Kunitz DNAseI, 40 U RNAse Out, 1× DNAse I buffer RDD).
15. Spin samples shortly and incubate for 1 h at 37 °C.
16. Add 500 μL of cold phenol–chloroform–isoamyl alcohol (25:24:1, v/v).
17. Vortex and separate the organic and inorganic phases by centrifugation (15 min, 13,000 rpm, 4 °C on a refrigerated table top centrifuge).
18. Transfer the aqueous (upper) phase to a new microtube.
19. Add 20 μL 3 M NaAc (pH 5.2) and 600 μL EtOH. Mix by inversion.
20. Repeat **steps 10** and **11**.
21. Remove supernatant and wash the pellet with 700 μL cold 70% EtOH (do not pipette or vortex).
22. Centrifuge samples (30 min, 13,000 rpm, 4 °C on a refrigerated table top centrifuge).
23. Remove the supernatant carefully.
24. Air-dry samples at room temperature with open lids for 10 min.

25. Resuspend the RNA pellets belonging to the sample in 25 μL of water.
26. Measure the RNA concentration with a NanoDrop (expected yield, ~1 μg/μL).
27. Check the RNA quality on an agarose gel.
28. Store at −80 °C.

3.3 RACE Mapping of sRNA Ends (5′-RACE and 3′-RACE)

This protocol discriminates between primary 5′ triphosphate (5′-PPP) ends and monophosphate processing sites (5′-P) of bacterial transcripts. The latter enable the RNA to be ligated to an oligoribonucleotide adaptor, and therefore, the amplification of the 5′-end sequence subsequent to reverse transcription (Fig. 1).

3.3.1 5′-RACE

1. Convert primary 5′-PPP ends to 5′-P by TAP treatment of total RNA previously isolated by adding 15 μg RNA, 5 U TAP and

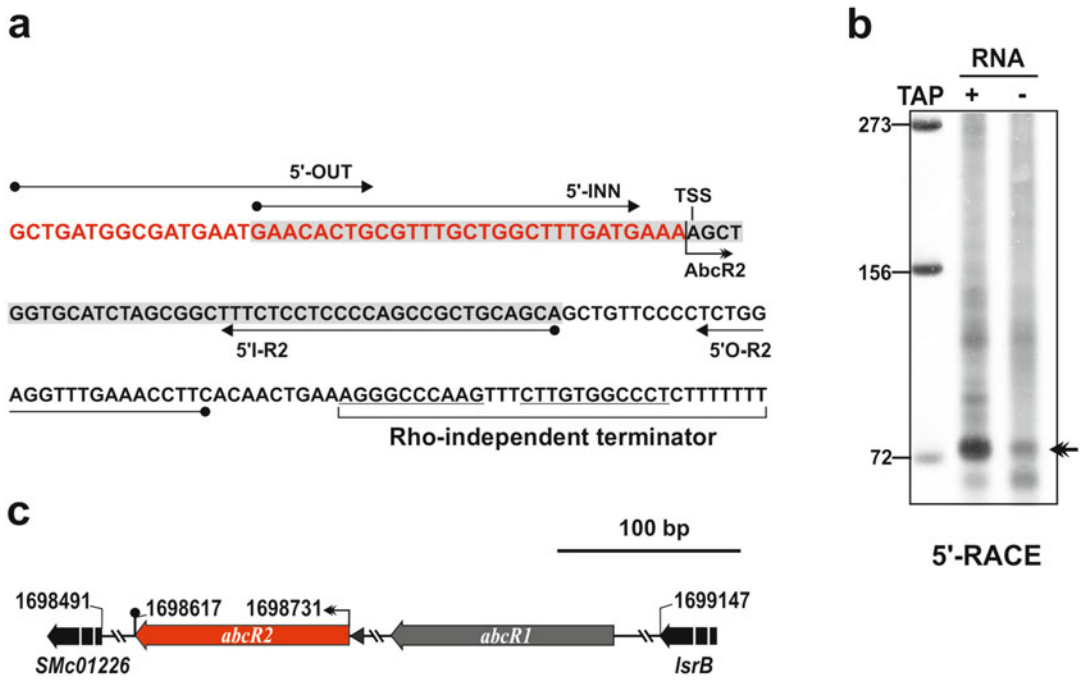

Fig. 1 Determination of the transcription start site (TSS) of the *S. meliloti* AbcR2 sRNA by 5′-RACE. (**a**) Sequence of the ligation product of the 5′-RNA adaptor (in red) and the full-length primary AbcR2 transcript (in black). Primers used in the nested PCR for amplification of the AbcR2 5′-end are indicated by arrows. The sequence of the second PCR product obtained with the pair of inner primers is shadowed. The Rho-independent transcriptional terminator is indicated by a bracket, with the inverted repeat sequences forming the stem of the hairpin of the terminator underlined. (**b**) Silver stained polyacrylamide gel of the AbcR2 5′-end amplification product (73 bp) from TAP-treated RNA samples. Note that, as expected from a primary transcript, the same product was undetectable in mock-treated RNA. (**c**) Annotation of the AbcR2 gene in the *S. meliloti* genome based on experimental determinations (5′-RACE) and computational predictions (Rho-independent terminator). Numbers indicate nucleotide positions in the chromosome of the reference Rm1021 strain

10× TAP buffer in a final volume of 10 μL during 1 h at 37 °C. Scale up the reaction if necessary (up to 50 μL). Use a mock-treated RNA sample as control throughout the protocol.

2. Stop reactions by phenol–chloroform extraction followed by EtOH NaAc precipitation as described in Subheading 3.2. Dissolve the precipitated RNA in 10 μL water.
3. Ligate the 5A oligoribonucleotide adaptor (ribonucleotide sequence can be modified at convenience) to the control and TAP-treated RNA; 1 μL of precipitated RNA, 100–300 pmol 5A, 30 U T4 RNA ligase, and 1 μL 10× ligase buffer in a final volume of 10 μL during 1–2 h at 37 °C.
4. Stop reactions by phenol–chloroform extraction followed by EtOH NaAc precipitation. Dissolve the precipitated RNA in 10 μL water.
5. Reverse transcribe the ligated RNA (2 μL) using a commercial RT-PCR system and random hexamers as primers. Usually cDNA synthesis from highly structured sRNA molecules is more efficient if performed at high temperatures. A typical reverse transcription is performed in three subsequent 20 min steps at 55, 60 and 65 °C, followed by inactivation of the enzyme 5 min at 85 °C and RNase H treatment.
6. Amplify products of reverse transcription (typically 1 μL of the RT reaction) with *Taq*-DNA polymerase by nested PCR with primer pairs (20 pmol each primer) specific to the adaptor (5′-OUT and 5′-INN) and the target sequence (Fig. 1a). If possible the expected size of the PCR products should be 100–300 bp.
7. Analyse PCR products in 2% agarose gels. Alternatively fragments shorter than 100 bp can be separated in nondenaturing 10% polyacrylamide gels (Fig. 1b). Note that amplification of primary 5′-ends from mock treated samples should render no or barely detectable PCR products.
8. Ligate the PCR products of interest to a PCR-product cloning vector, screen bacterial colonies obtained upon transformation by colony PCR with universal T7 and SP6 primers and sequence plasmids (6–12 clones) carrying the inserts of the appropriate size to determine and map the TSS of the sRNA (Fig. 1c).

3.3.2 3′-RACE

Transcription of *trans*-sRNA genes usually ends at hairpin structures specified by Rho-independent transcriptional terminators, which can be easily predicted in bacterial genomes by a number of publicly available bioinformatics tools (e.g., TransTermHP; http://transterm.cbcb.umd.edu/). In those cases that there is no recognizable Rho-independent terminator at a reasonable distance from the experimentally determined 5′-end, the 3′-end of the transcript can be mapped by RACE as follows.

1. Dephosphorylate total RNA with CIP to remove 5′-P ends from processed RNA and minimize adaptor ligation to the 5′-end of these transcripts; 15 μg RNA, 3 U CIP, 1 μL 10× CIP buffer in a final volume of 10 μL during 1 h at 37 °C.
2. Stop reactions by phenol–chloroform extraction followed by EtOH NaAc precipitation as described. Dissolve the precipitated RNA in 10 μL water.
3. Ligate the 3A oligoribonucleotide adaptor (ribonucleotide sequence can be modified at convenience) to the CIP-treated RNA; 5 μL of precipitated RNA, 100–300 pmol of 3A, 30 U T4 RNA ligase, and 1 μL 10× ligase buffer in a final volume of 10 μL during 1–2 h at 37 °C.
4. Stop reactions by phenol–chloroform extraction followed by EtOH NaAc precipitation. Dissolve the precipitated RNA in 10 μL water.
5. Reverse transcribe the ligated RNA (2 μL) as described above with 50 pmol of the 3RT primer, which is complementary to the 3A sequence.
6. Amplify products of reverse transcription (1 μL of the RT reaction) with *Taq*-DNA polymerase by nested PCR with primer pairs (20 pmol each primer) specific to the adaptor (3′-OUT and 3′-INN) and the target sequence.
7. Analyse, clone and sequence the PCR products as described for the 5′-RACE.

3.4 Northern Blot Analysis of sRNA Expression

Northern blot hybridization is essential to validate the sRNA(s) under study and to gain information about the RNA size, possible processing events, stability and relative abundance in different conditions. The technique involves an acrylamide gel electrophoresis to separate RNA samples by size, membrane blotting and detection with a complementary probe (Fig. 2).

1. Prepare polyacrylamide–urea solution (*see* **Note 4**).
2. Add 10 μL of 10% APS and 1 μL of TEMED per mL of gel solution.
3. Pour immediately the gel mixture in between two glass plates, separated by 1-mm spacers, and insert a comb (*see* **Note 5**).
4. Cast the electrophoresis device, add the necessary 1× TBE and remove the combs.
5. Run a pre-electrophoresis at 150 V (10 × 8 cm gels) to warm up the gel.
6. Mix 5–15 μg total RNA 1:1 with 2× RNA loading buffer (*see* **Note 6**).
7. Include a gel lane with 1 μg RNA molecular weight marker (MWM).

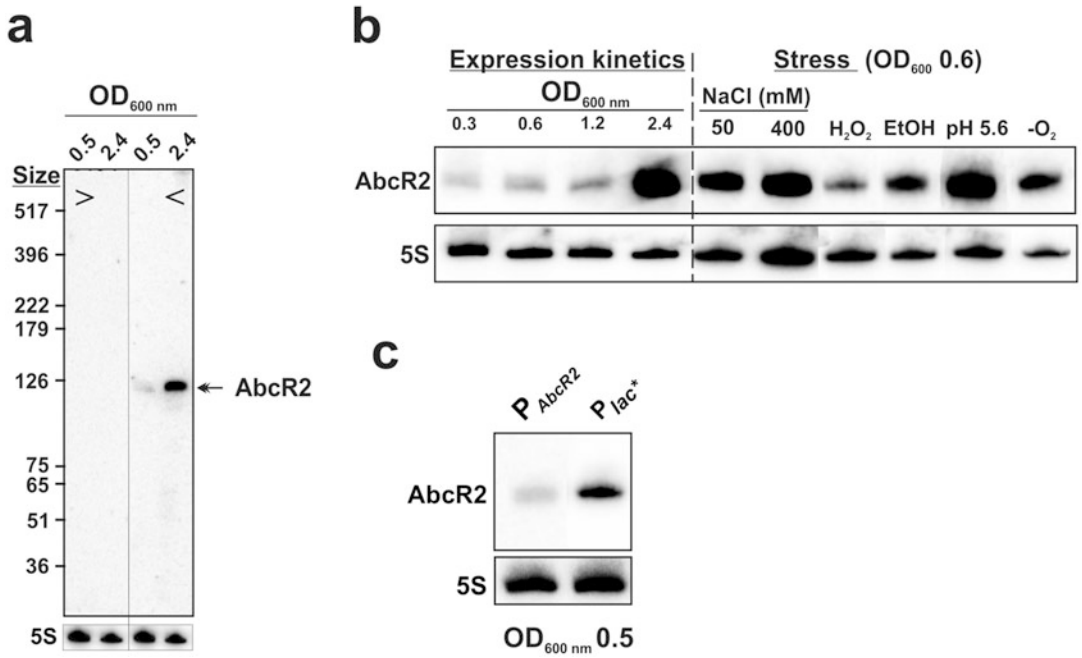

Fig. 2 Northern blot detection of AbcR2. (**a**) Full-length membranes showing the hybridization signal of an oligonucleotide probe complementary (<) to the 115 nt-long AbcR2 sRNA. Note that a primer complementary to the one used as probe (>) did not yield such a signal. RNA was extracted from bacteria grown in TY to exponential (OD_{600} 0.5) and stationary (2.4) phases as indicated on top. (**b**) Expression profiling of AbcR2 as revealed by Northern hybridization. Total RNA was obtained from bacteria grown in the conditions indicated on top. (**c**) Detection of AbcR2 when transcribed from either its chromosomal locus (P_{AbcR2}) or the constitutive *lacZ*-derived promoter (P_{lac*}) in plasmid pSRK-R2. RNA was obtained in both cases from exponentially growing bacteria in TY medium. In all cases the 5S rRNA was probed as loading control

8. Denature the samples by heating for 5 min at 95 °C and cool them on ice.
9. Stop the pre-electrophoresis and clean the gel wells by flushing 1× TBE with a syringe to remove the urea and unpolymerized acrylamide.
10. Spin samples down and load them into the wells.
11. Run the electrophoresis at 150 V (10 × 8 cm gels, *see* **Note 7**). After the electrophoresis, cut the lane with the MWM and stain it separately with EtBr.
12. Cut out a nylon membrane slightly larger than the size of the acrylamide gel.
13. Wet five Whatman papers with 1× TBE by immersion.
14. Place the dry Whatman paper onto the gel to carefully remove it from the glass.
15. Lay the membrane and three wet Whatman papers over the gel (*see* **Note 8**).

16. Turn around them and lay two prewetted blotting paper over gel.
17. Place everything on the electroblot transfer device.
18. Transfer RNA according to the manufacturer's instructions and link it to the membrane covalently by 5 min exposure to UV light (*see* **Note 9**).
19. Radioactive label the oligonucleotide as follows: 5 μL of ddH_2O, 1 μL of oligonucleotide (50 pmol/mL), 1 μL of 10× reaction buffer, 1 μL of T4-PNK and 2 μL of γ-[^{32}P] ATP. Incubate for 1 h at 37 °C.
20. Place the cross-linked nylon membrane in a glass hybridization tube (RNA must face the inside) containing 10–20 mL hybridization buffer. Incubate at 42 °C with rotation for 30–120 min in a hybridization oven.
21. Prepare the Sephadex G-25 spin columns to purify the oligonucleotide probe by eliminating the unincorporated γ-[^{32}P] ATP. First, vortex the columns to resuspend the resin. Break the bottom of the column and open the lid. Centrifuge the column in a microcentrifuge with a collection tube (2 min, 3000 rpm) to eliminate the storage buffer.
22. Bring the labeling reaction to 25 μL by adding ddH_2O and add the mixture to the center of the column resin.
23. Transfer the column to a new 1.5 mL microtube and centrifuge (2 min, 3000 rpm) in a microcentrifuge.
24. Heat the eluted purified RNA probe at 95 °C for 5 min.
25. Add the radiolabeled RNA probe into the hybridization bottle (on the hybridization buffer, not directly on the membrane) and hybridize overnight at 42 °C.
26. Discard the hybridization solution (*see* **Note 10**). Wash twice for 5 min with the 2× SSC/0.1% SDS solution and twice for 15 min with the 1× SSC/0.1% SDS solution. Perform all the washes at 42 °C.
27. Dry the membrane with Whatman paper and wrap it with plastic. Expose onto a Phosphorimager system overnight.
28. Scan the phosphorscreen with the scanner.
29. Detection of the abundant 5S rRNA as loading control in the same membrane is recommended. To remove the labeled oligonucleotides, place the membrane back in the hybridization oven, add boiling 0.1% SDS and shake it twice for 15 min. Repeat the protocol starting from **step 18** using a 5S rRNA probe(~120 nt).
30. For quantitative comparison of hybridization signal intensities, use an image analysis software.

3.5 Approach to Function by sRNA Overexpression

The biological function of sRNAs can be first approached by deletion or gain-of-function strategies. The former can be achieved by conventional double crossing over mutation of the *trans*-sRNA locus using plasmid pK18*mobsacB* [17]. Given that sRNAs function commonly relies in gene expression modulation rather than strength control of expression [19], sRNA deletion mutants do not normally result in severe phenotypes under laboratory conditions. Nevertheless, sRNA deletion mutants constitute useful recipient strains for further determinations, e.g., target validation by a genetic double plasmid assay.

Measurable phenotypes have been more frequently found in bacterial recombinants overexpressing *trans*- and asRNAs [20–22]. Most plasmids have been designed for overexpression of coding genes, and lack restriction sites to clone a sequence to be expressed precisely from its TSS. Torres-Quesada et al. [15] generated plasmid pSRK_C for constitutive sRNA expression by engineering the mid-copy pSRK Km [16] (Fig. 2c). To overcome problems with pleiotropic effects or weak overexpression, Robledo et al. [21] combined the IPTG-inducible native system of pSRK (Km, Gm) vectors with the well-known *S. meliloti sinR-sinI* genes involved in quorum sensing [23], to provoke strong pulse overexpression of the sRNA under study. This is achieved by cloning of the full-sequence of *sinR*, coding for the transcriptional regulator of the *N*-acyl homoserine lactone autoinducer *sinI*, under the IPTG-inducible P_{lac} promoter followed by the native *sinR-sinI* intergenic region. Then the full-length sRNA is fused to the TSS of *sinI*, leading to its indirect activation upon addition of IPTG (Fig. 3, upper diagrams). The second system has been proved to be more accurate for sRNA overexpression and target identification. Both strategies for overexpression of sRNAs are described in detail below:

1. Constitutive (over)expression of the full-length sRNA of interest in pSRK_C (Fig. 3, upper diagram). Design primer pairs that incorporate *Bam*HI and *Sac*I sites to the 5′- and 3′-ends of the fragments, respectively. After PCR amplification using genomic DNA as template, digest the obtained fragments and the purified vector with the aforementioned enzymes and ligate them.
2. IPTG-induced overexpression of the sRNA with the indirect *sinR-sinI* based system. Amplify the complete sequence of the *sinR* gene and the *sinR-sinI* intergenic region containing the *sinI* promoter with forward primer sinR_NdeIF including a *Nde*I site (underlined) and TSS3_28bp_b_sinIR reverse primer containing the fusion region using genomic DNA as template. Amplify the sRNA in parallel with a forward primer containing the reverse complementary sequence of TSS3_28bp_b_sinIR followed by the first ~20 nt of the sRNA from the TSS and a reverse primer incorporating a unique restriction site from the

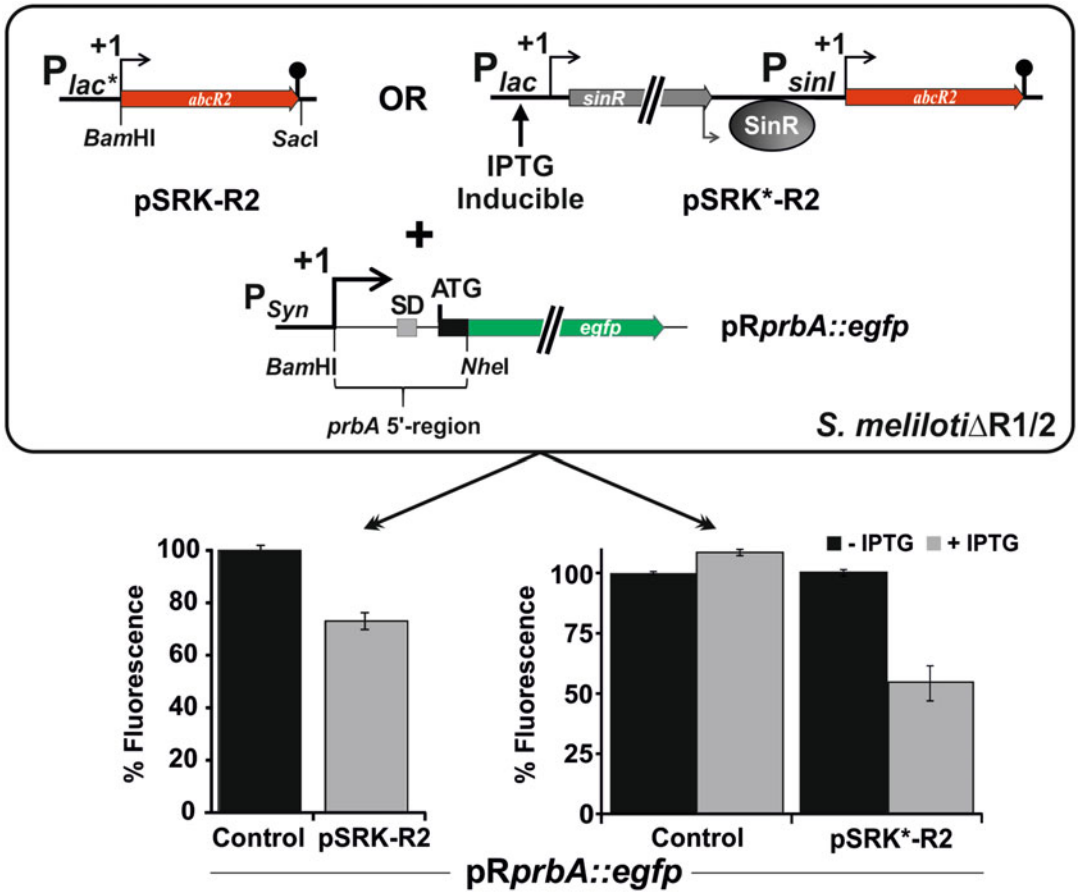

Fig. 3 Genetic reporter assay for the experimental validation of regulatory sRNA-mRNA antisense interactions. The sRNA (e.g., AbcR2) and a translational fusion of its putative target mRNA (e.g., *prbA* mRNA) to *egfp* are coexpressed from compatible plasmids in the same bacterial cell (*S. meliloti* lacking AbcR1 and AbcR2 *loci*). Fluorescence of the reporter strains is then scored quantitatively to assess sRNA-mediated posttranscriptional regulation of the target mRNA, using as controls the empty pSRK_C or pSRK plasmids. See text for details. P_{Syn} is a constitutive promoter with a consensus σ^{70} signature

plasmid downstream *Nde*I (e.g., *Xba*I) from genomic DNA. Fuse both overlapping PCR products in a second PCR with sinR_NdeIF and the sRNA reverse oligo and insert the resulting fragment into pSRK (Km, Gm) (Fig. 3, upper part).

3. Transform competent *E. coli* DH5α cells with the ligation products (*see* **Note 11**). Verify successful integration of both inserts by colony PCR using flanking reverse primer PCR1 and forward PCR2.
4. Purify, sequence and subsequently transform correct plasmids into *E. coli* S17.1 cells for biparental mating with the corresponding rhizobia strain (*see* **Note 12**).

5. Verify cells overexpressing or lacking a functional copy of the sRNA gene under study by Northern blot (Fig. 2c). Monitor growth, morphology, motility, and plant phenotypes, among others related with sRNA predicted function. As first approach to infer putative direct or indirect sRNA target genes, characterize the cellular responses by microarray-based transcriptome [21] and/or proteome profiling [15].

3.6 Prediction and Experimental Validation of mRNA Targets

The biological role of sRNA is frequently related with its mRNA targets. In this section we will describe in detail the methods routinely used to search for and experimentally confirm sRNA mRNA targets. Several computational algorithms have been developed to in silico predict *trans*-RNAs targets. We recommend those developed by the Freiburg Bioinformatics Group, which are freely accessible at http://rna.informatik.uni-freiburg.de. They include several RNA-related tools for prediction of sRNA-mRNA antisense interactions (IntaRNA, CopraRNA) [24, 25], homologs identification (GLASSgo) and multiple alignment of RNA molecules (LocARNA) [26], among others.

These in silico predictions of sRNA-mRNAs interactions result in dozens of potential targets that depend upon subsequent in vivo validation. *S. meliloti* has pioneered the sRNA target confirmation in rhizobacteria by a genetic double-plasmid reported assay that can be broaden to other related α-proteobacteria because it relies on broad host-range vectors [6, 15]. This assay relies in the cotransformation of bacterial cells (*see* **Note 13**) with a plasmid that (over) express the selected sRNA and a compatible reporter plasmid harboring a translational fusion of the 5′ region (starting from the native TSS) of the predicted mRNA target to the EGFP reporter under the control of a constitutive promoter. We use the low-copy IncP pJB3Tc19 derivative vector pR_EGFP containing P_{syn} promoter as a reporter plasmid as described below. Target recognition and sRNA-dependent translational control of the fusion protein can be then assessed by fluorescence measurement of the relevant sets of reporter strains. To further validate the interactions, sRNA-mRNA combinations carrying mutations disrupting or compensating the predicted base-pairing can be tested with the same system (*see* **Note 14**).

1. Once the 5′ and 3′ of the sRNA under study are known, interrogate the host genome with IntaRNA (Interacting RNAs) using the full-length sRNA sequence as query to predict its putative mRNA targets and interaction domains and whether there is a functional enrichment among them in our organism of interest (an NCBI RefSeq genome accession number is needed).
2. The CopraRNA (Comparative prediction algorithm for small RNA targets) tool integrates phylogenetic information to

predict conserved sRNA-mRNA interactions in different organisms. CopraRNA can be used if the sRNA under study has homologs in at least three different organisms with genome accession number. To search for sRNA homologs use first GLASSgo (GLobal Automated sRNA Search go) tool.

3. Putative targets with a p-value ≤ 0.01 or those belonging to a certain enriched functional group are interesting for further analysis. Default parameters show mRNA target predictions based on sequences 200 nt upstream and 100 nt downstream of the annotated start codons. Most known sRNAs base pairing is located at the RBS, however, they can also block or activate translation when the pairing region is located upstream the RBS or deep into the coding sequence [19]. A low energy score and a high number of perfectly paired bases in the seed region are also indicative to select good potential target candidates.
4. Experimentally check the elected set of sRNA mRNA target candidates by double plasmid assay. To obtain the reporter EGFP-fusions of the mRNA of interest in pR_EGFP, perform PCR with primer pairs to amplify the native 5′ region of the target mRNA containing the predicted sRNA interaction site (*see* **Note 15**). This commonly spans from the TSS of the mRNA to one of the initial codons of the coding sequence (typically 10–20). Oligonucleotides must incorporate *Bam*HI and *Nhe*I sites to the 5′- and 3′-ends of the fragments to be translationally fused to EGFP in plasmid pR_EGFP digested with the same restriction enzymes (Fig. 3, upper part).
5. Conjugate correct constructs into cells overexpressing the sRNA under study or control cells (*see* **Note 16**).
6. Select at least three transconjugants for fluorescence determinations, preferentially by a plate reader or a cytometer. When using the former, it is very important to adjust cultures to comparable optical densities (e.g., control vs. sample or IPTG-induced vs. uninduced culture), even if fluorescence values are normalized by OD (Fig. 3). Include strains harboring empty pR_EGFP to determine background fluorescence.

4 Notes

1. Most commercial kits for RNA isolation do not recover RNA molecules smaller than ~200 nt. Total RNA including the small RNA fraction (50–250 nt) can be also isolated using the miRNeasy mini Kit (Qiagen) as it has been previously described [21].
2. This detergent washing step is optional but strongly recommended to eliminate the polysaccharides and facilitate cell break in mucoid strains ($ExpR^+$).

3. Plant nodules were previously covered with liquid nitrogen and ground to powder in a mortar to isolate the bacteroids.
4. Slightly heat the mix to completely dissolve urea and filter the solution. This mix can be stored at 4 °C for subsequent gels.
5. Avoid introduction of air bubbles and do not insert the combs too deeply into the glass plates (5 mm is sufficient). Polymerization takes 1 h approximately, but at this point the cast gel can be stored overnight at 4 °C.
6. The required amount of total RNA for Northern blot depends on the target RNA abundance and the detection method sensitivity. Do not load more than 20 μL samples. If total RNA concentration is low, let samples dry in a vacuum concentrator and add 10–20 μL 1× RNA loading buffer.
7. Running time depends on the expected sizes of the target RNA. Use the loading buffer dyes to monitor run. In 6% polyacrylamide gels, xylene cyanol has a relative mobility of 110 nt and bromophenol blue of 25 nt. Typically, samples are separated until bromophenol blue is close to reach the bottom part of the gel.
8. Prevent introduction of air bubbles between the gel and the membrane.
9. When required, the membrane can be stored at this point indefinitely.
10. All waste containing radioactive material should be discarded in the radioactive waste.
11. Blue/white selection of transformants with IPTG and X-gal is not possible in pSRK_C derivatives.
12. Plasmids containing the SinR-SinI system are preferably transferred to *sinR-sinI* less derivatives to avoid background expression without IPTG.
13. Ideally, markerless deletion mutant of the sRNA under study should be used as recipient strain to avoid interference with the mRNA target translation.
14. To introduce compensatory mutations in the sRNA and mRNA sequence, perform PCR using the corresponding plasmid DNA as template and with internal complementary oligonucleotides carrying the mutations as described before in detail elsewhere [27].
15. For *S. meliloti* most TSSs have been already experimentally determined [8]. If these data is not available in the strain under study, the 5′UTR gene length should be estimated.
16. Either cells harboring empty pSRK_C or overexpression plasmids or vectors carrying a control sRNA can be used. SmelC812, an antisense RNA of insertion sequence ISRm19, has been proposed as control in *S. meliloti* [21].

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Chapter 23

A New, Nondestructive, Split-Root System for Local and Systemic Plant Responses Studies with Soybean

Ángeles Hidalgo, José E. Ruiz-Sainz, and José M. Vinardell

Abstract

Plants use long-distance signaling mechanisms to coordinate their growth and control their interactions, positive or negative, with microbes. Split-root systems (SRS) have been used to study the relevance of both local and systemic plant mechanisms that participate in the control of rhizobia–legume symbioses. In this work we have developed a modification of the standard split-root system (SRS) used with soybean. This modified method, unlike previous systems, operates in hydroponics conditions and therefore is nondestructive and allows for the continuous monitoring of soybean roots throughout the whole experiment.

Key words Soybean, Split-root system, Nodulation, Autoregulation of nodulation, Hydroponic culture

1 Introduction

Rhizobia are soil bacteria that establish specific symbiotic relationships with leguminous plants resulting in the formation of nitrogen-fixing nodules in plant roots. This symbiotic process is initiated and maintained by a complex exchange of signal molecules between both symbionts [1]. Because both nodulation and symbiotic nitrogen fixation require high energy consumption by the plant, the latter tightly controls the initiation of the nodulation process as well as the final number of nodules formed [2]. Diverse evidences show that this control can be exerted through both systemic and local mechanisms [3]. One of the methodologies more used to study the plant control of nodulation is the so-called "split-root system" (SRS). In this method the main root of the plant is excised and two appropriate lateral roots (hereafter called root-A and root-B) that emerge from the seedlings after root-tip removal are confined into separate compartments. This kind of approach allows to study whether treatments applied in one of the compartments (root-A) have an effect on the root system located in the other compartment (root-B).

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Soybean is one of the best studied legumes due to its enormous economic and agricultural importance worldwide [4]. SRS have been employed for symbiotic studies with soybean from the 1980s [5] until nowadays (*see*, for example, refs. 6, 7). However, the SRS used with soybean have the common inconvenient that both roots are buried into solid substrates such as vermiculite. These systems are "destructive" because scoring nodulation in any root requires the removal of the plant from the experiment.

In this work we have devised a new SRS for soybean that, unlike previous systems, operates in hydroponics conditions (Figs. 1 and 2).

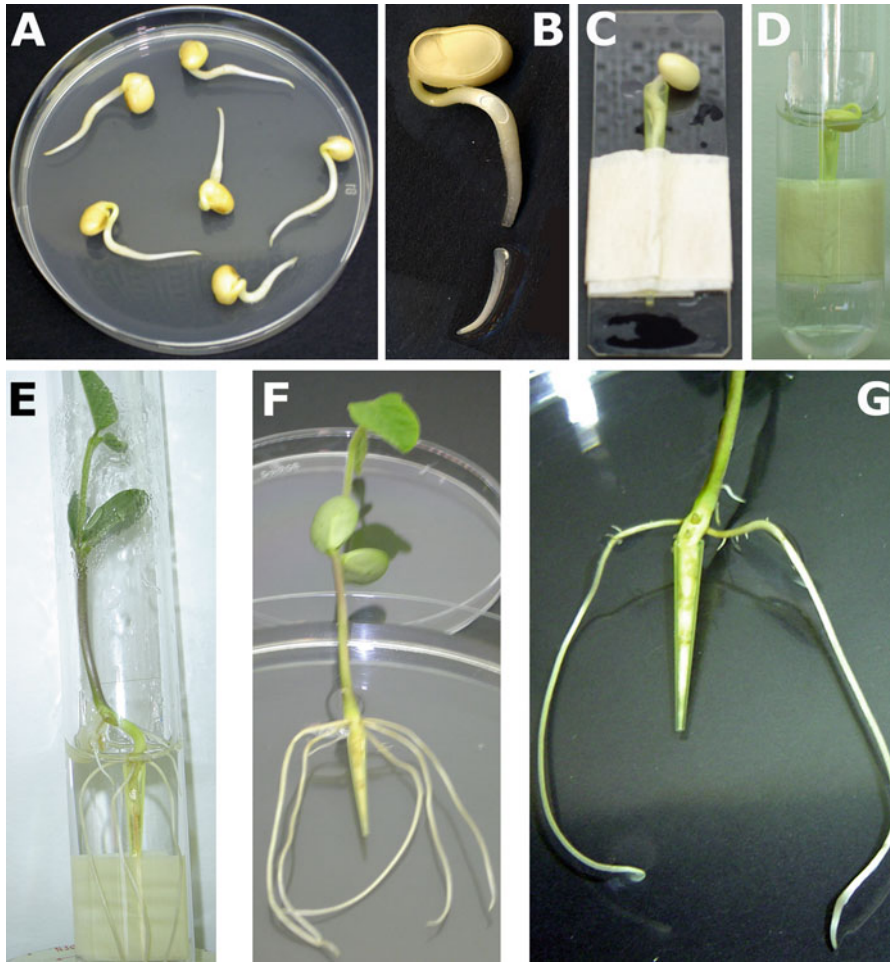

Fig. 1 Early steps of the split-root system setup. (**a**), germinated soybean seeds in agar–water plates. (**b**), soybean seedling whose main root has been truncated. (**c**), soybean seedling introduced by its truncated root into a pipette tip attached to a glass slide. (**d**), introduction of the "soybean seedling-glass slide" set into a glass tube containing Fåhraeus nutritive solution. (**e**), after 5 days of incubation in a plant growth chamber, soybean seedlings have developed various lateral roots. (**f**), a soybean seedling which has developed "highly symmetrical" lateral roots. (**g**), a soybean seedling with two "highly symmetrical" lateral roots after the excision of all other lateral roots

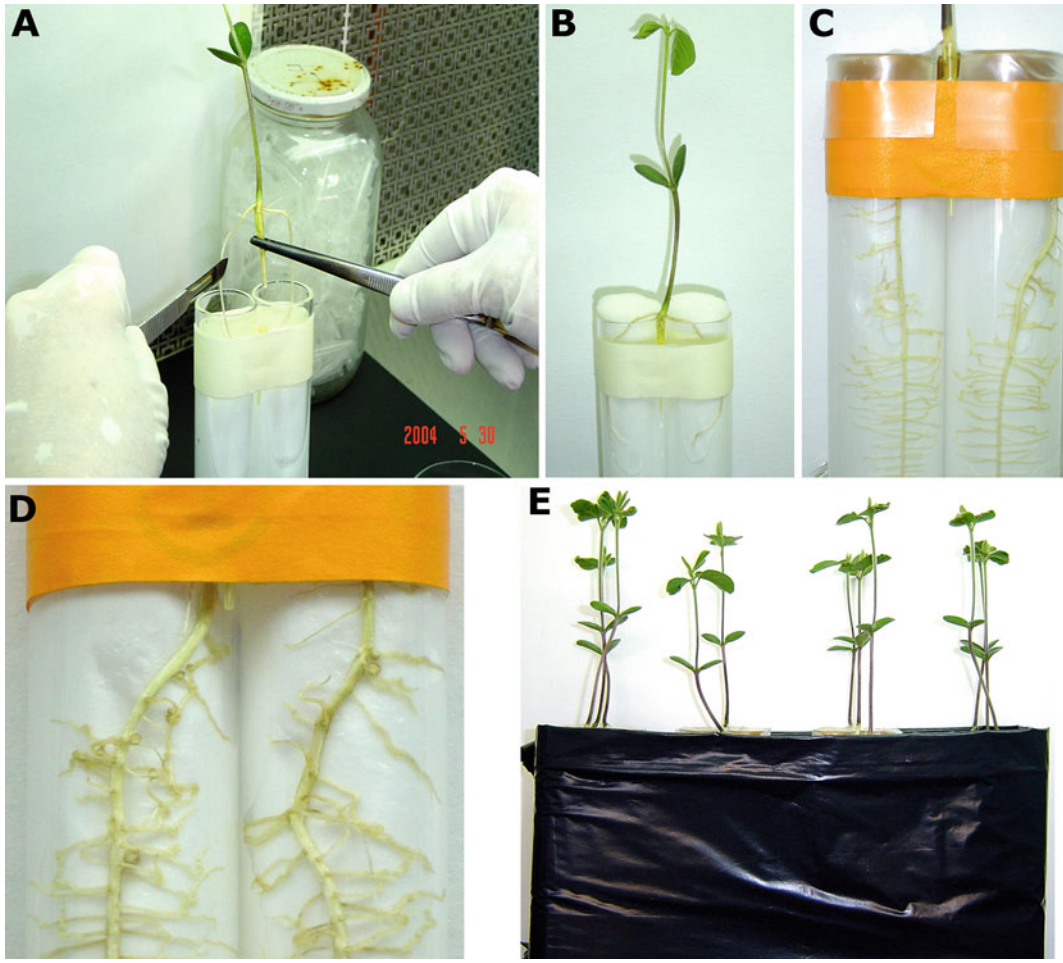

Fig. 2 Late steps of the split-root system setup. (**a**), Transfer of a soybean seedling with two "highly symmetrical" lateral roots to a twin-tube system (TTS). (**b–d**), aspect of a soybean plant grown in a TTS. Note that roots are located between the glass tube and a cylindrical sheet of filter paper that is in contact with the inner face of the glass tube. Several nodules are visible in panel **d**. (**e**), a tube-rack holding various twin-tubes containing soybean plantlets. Note that the tube-rack has been covered with black plastic in order to prevent illumination of roots

Therefore, this new method is nondestructive and allows for the continuous monitoring of soybean roots throughout the experiment (for a detailed description of the methodology employed, see the following sections). Briefly, the root tips of 2-day-old soybean seedlings are excised and the manipulated seeds are incubated for 120 h in liquid Fåhraeus nutritive solution in order to allow the development of lateral roots. Only seedlings having two lateral roots of similar length and position are selected. After the excision of the remaining lateral roots, seedlings are transferred to a double-tube system containing Fåhraeus nutritive solution, and each lateral root is

Table 1
Soybean responses to inoculation with *Sinorhizobium fredii* HH103 (*Sf* HH103) and/or *Bradyrhizobium japonicum* USDA110 (*Bj* USDA110) in SRS experiments in which only root-A or both roots-A and -B are inoculated

	Nodules at 29 dpi		pH at 29 dpi	
Inoculants Root-A/Root-B	**Root-A**	**Root-B**	**Root-A**	**Root-B**
Sf HH103/none	10.17 ± 1.83	0	5.66 ± 0.42	5.78 ± 0.34
Sf HH103/*Sf* HH103	7.50 ± 3.51	10.83 ± 4.79	5.14 ± 0.52	5.41 ± 0.36
Bj USDA110/none	14.67 ± 2.34	0	5.13 ± 0.73	4.98 ± 0.61
Bj USDA110/*Bj* USDA100	13.50 ± 3.08	11.50 ± 3.02	4.45 ± 0.81	4.48 ± 0.57

The table shows the number of nodules and pH in roots-A and -B when only root-A or when both roots A and B are inoculated with *Sinorhizobium fredii* HH103 (*Sf* HH103) or with *Bradyrhizobium japonicum* USDA110 (*Bj* USDA110) Numbers are mean values of 12 twin tubes, each tube containing one plant. All the roots-A and -B were inoculated at the same time. Nodulation was scored 29 days after inoculation

placed in a tube. Depending on the experiment, one or the two radical systems of each plant are inoculated with the appropriate rhizobial strain(s) and development of nodules in the roots can be monitored as frequently as required during the whole experiment.

We have carried out several experiments in order to investigate whether soybean nodulation assays using this new SRS generates consistent and reproducible results and whether they are in agreement with those previously reported using traditional destructive SRS.

We have analyzed the kinetics of nodulation of soybean cv. Osumi when only the root-A or both lateral roots (A and B) were inoculated with rhizobia. For this purpose, two sets of experiments with different two soybean-nodulating rhizobia, *Sinorhizobium fredii* HH103 and *Bradyrhizobium japonicum* USDA110, were carried out. Results are presented in Table 1 and Fig. 3. Nodules on inoculated roots (with *B. japonicum* USDA110 or *S. fredii* HH103) were observed 11 days after inoculation. Interestingly, the number of nodules and the kinetics of nodulation of inoculated root-A were independent of inoculation, or not, in root-B. As a consequence, the number of nodules formed by plants inoculated only in the root-A was approximately 50% of that scored in soybeans in which root-A and root-B were simultaneously inoculated. Since root-A and root-B had approximately the same number of nodules, we can conclude that nodulation in the root-A is not influenced by the simultaneous nodulation process that is occurring in the root-B as far as both lateral roots were inoculated at the same time. Similarly, the kinetics of acidification of the nutritive solution was very similar in both A and B roots, regardless of inoculation of only one or both lateral roots.

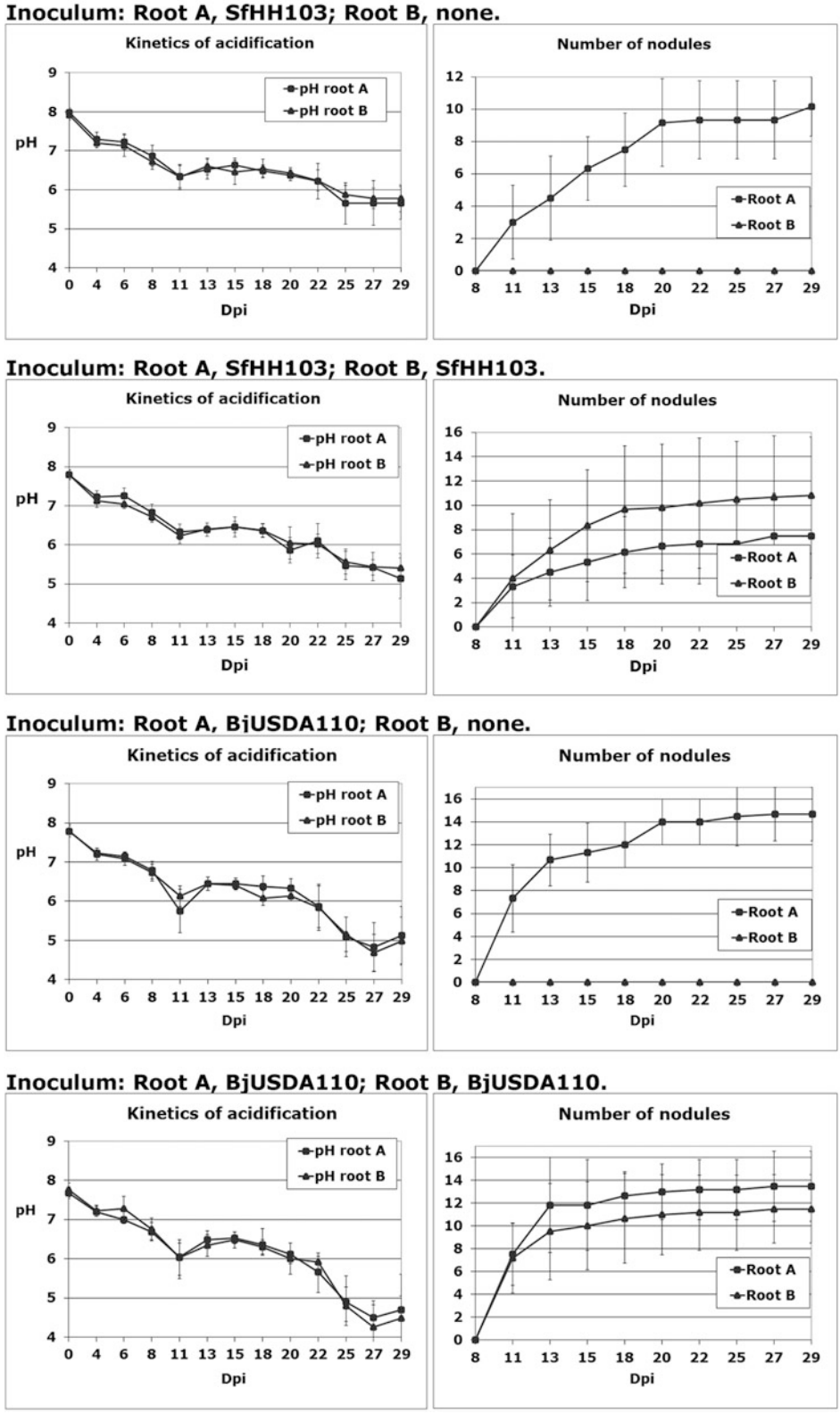

Fig. 3 Kinetics of acidification of nutritive solution (on the left) and of nodulation (on the right) of roots A and B when one (A) or both lateral roots (A and B) have been inoculated with *Sinorhizobium fredii* HH103 (*Sf* HH103) or *Bradyrhizobium japonicum* USDA110 (*Bj* USDA110). When both lateral roots were inoculated, the inoculum was added at the same time

We have also studied the kinetics of nodulation of root-A and root-B when inoculated at different times. This type of experiment is usually carried out to investigate how nodulation in root-B is reduced if root-A is inoculated hours or days before. The reduction of nodulation observed in the delayed inoculated root-B is attributed to the onset of a plant-controlled programme called "Autoregulation of Nodulation" (AON). AON is activated by the presence of symbiotically compatible rhizobia and provokes a progressive inhibition in the formation of subsequent new nodules, so that the total number of nodules formed in the legume root finally reaches a plateau instead of increasing indefinitely [5]. In these experiments, root-A is inoculated at day 0, and root-B is inoculated at 0, 6, 9 or 12 days after inoculation (dai) of root-A. As shown in Table 2, when root-B is inoculated 6 days later, the number of nodules developed is significantly lower than that formed in root-A. This difference in the number of nodules formed was much more evident when inoculation of root-B was delayed 9 or 12 days.

In summary, in this work we present a new type of SRS that is adequate for legumes of moderate size (such as soybean) and, because it is nondestructive, allows for continuous observation of the roots of any particular plant along the whole experiment. In addition to nodulation studies with rhizobial strains (such as those presented in this work), this kind of system is appropriate for a high variety of experiments devoted to the study of local and systemic plant responses.

Table 2
Autoregulation of nodulation (AON) of soybean plants grown in SRS experiments in which inoculation of root-B is delayed with respect to root-A

Inoculation of root-B[a]	Number of nodules at 32 dpi		Comments[b]
	Root-A	Root-B	
0	9.4 ± 3.7	13.3 ± 5.8	No significant differences ($p = 0.607$) between roots A and B were detected
6	23.3 ± 8.0	7.8 ± 3.1	Significant differences ($p = 0.003$) were detected. All root-B were nodulated
9	12.7 ± 5.3	3.2 ± 3.6	Root-B: Only 4 plants out of 6 were nodulated
12	25.3 ± 4.5	2.6 ± 3.9	Root-B: Only 2 plants out of 7 were nodulated

Numbers are mean values of 12 twin tubes, each containing one plant
[a]Days of delay respect to inoculation of root-A
[b]Treatments were compared by using the Mann–Whitney nonparametric test

2 Materials

Prepare all solutions using distilled water and analytical grade reagents. All the material needs convenient sterilizing. All manipulations must be done under aseptic conditions (the employment of a laminar flow cabinet is strongly recommended). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 Seed Germination

1. Petri dishes containing 25 mL of agar–water (2% w/v).
2. Ethanol 100% (v/v).
3. Sodium hypochlorite 12% (v/v).

2.2 Excision of the Main-Root Meristem and Formation of Lateral Roots

1. Liquid, nitrogen-free Fahräeus plant nutritive solution [8]: 0.7 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 20 μM ferric citrate, 5 μM H_3BO_3, 10 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, and 0.6 μM Na_2MoO_4, pH 6.5–7.0. If required, combined nitrogen (NH_4NO_3 or KNO_3) can be added to the desired concentration.

   Stock solutions of macronutrients, micronutrients, and ferric citrate are prepared separately. For 1 L of macronutrients, dissolve 0.1 g of $CaCl_2{\cdot}2H_2O$, 0.12 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of KH_2PO_4, and 0.075 g of $Na_2HPO_4{\cdot}2H_2O$ in distilled water (*see* **Note 1**). A stock solution of ferric citrate is prepared by dissolving 5 g of ferric citrate in distilled water (final volume of 1 L) with heat to facilitate ferric citrate dissolution. For the micronutrients solution (also called Gibson's solution), add 2.86 g of H_3BO_3, 2.08 g of $MnSO_4{\cdot}H_2O$, 0.22 g of $ZnSO_4{\cdot}7H_2O$, 0.08 g of $CuSO_4{\cdot}5H_2O$, and 0.13 g of Na_2MoO_4 to a final volume of 1 L using distilled water. For preparing 1 L of Fahräeus plant nutritive solution: add 1 mL of micronutrients solution and 5 mL of ferric citrate stock solution to 900 mL of macronutrients solution, mix, adjust pH to 6.5–7.0, complete with distilled water up to a final volume of 1 L and sterilize in the autoclave (121 °C, 20 min).
2. Truncated 200 μL plastic micropipette tips (yellow tips).
3. Microscope glass slides.
4. Water-resistant adhesive tape.
5. Large glass tubes (25 mm diameter, 245 mm long).
6. Razor-blade or scalpel.

3 Methods

3.1 Seed Germination

In this step soybean seeds are surface-sterilized and their germination is induced.

1. Surface sterilization of soybean seeds. The appropriate number of soybean seeds (typically fourfold the final number of plants required for the experiment) are transferred to a sterile flask and treated with absolute ethanol for 30 s (*see* **Note 2**), rinsed with water, treated with sodium hypochlorite 12% (v/v) for 6 min, and rinsed five times with sterilized water.
2. Seed germination. After the last washing, seeds are carefully transferred with sterilized forceps to petri dishes containing approximately 25 mL of agar–water (2% w/v) (*see* **Note 3**). These plates are incubated at 28 °C for 42–50 h in the dark.

3.2 Excision of the Main-Root Meristem and Formation of Lateral Roots

In this step the root tips of selected seedlings are removed in order to induce the formation of lateral roots.

1. Root-tip removal. Those seedlings having straight radicles of at least 3-cm long are selected (Fig. 1a) and their root tips (approximately 0.5–1.0 cm, Fig. 1b) are excised with a razor blade or an scalpel.
2. Induction of lateral roots formation. Cut off the tip (approximately 0.5 cm) of 200 μL plastic micropipette-tips (*see* **Note 4**). Attach truncated tips to glass slides with strong (water-resistant) adhesive tape. The truncated main-root of each seedling is introduced into the truncated micropipette tip (Fig. 1c), taking care that the upper part of the root is over the edge of the micropipette tip. Each "seedling-truncated micropipette tip-glass slide" set is transferred to a glass tube (*see* **Note 5**) containing Fåhraeus plant-nutritive solution, taking care that the whole radicle is immersed in the solution (Fig. 1d). Each tube is covered with sterile cotton and placed into a rack covered with a material that protects roots from light (e.g., cover the tube rack with black plastic bags). These tubes are incubated for 5 days in a plant growth chamber with a 16 h photoperiod at 25 °C in the light and 18 °C in the dark.

3.3 Transference of Seedlings to the Split-Root System

In this step, appropriate seedlings are selected and transferred to the split root system.

1. Selection of appropriate seedlings. After 5 days most seedlings have developed various lateral roots (Fig. 1e). Those seedlings having two highly symmetrical lateral roots (Fig. 1f; *see* **Note 6**) are selected for further work. The rest of the lateral roots are excised with a sterilized razor blade or a scalpel, so that each seedling had only two, highly symmetrical lateral roots (Fig. 1f).
2. Split-root system (SRS) setup. Twin-tubes systems (TTS) are prepared for each plant to be used in the SRS. Each TTS is composed of two 25 mm diameter, 245 mm long glass tubes.

Twin tubes are strongly attached in their upper parts with strong water-resistant adhesive tape. A truncated 200 μL micropipette tip is attached with adhesive tape to the upper part of the TTS. Each truncated pipette tip will contain only one soybean seedling.

3. Transfer of selected seedlings to the split-root system. Each selected seedling is transferred to a TTS. Each tube, containing a cylinder of sterilized filter paper closely attached to the inner side of the tube, is filled with 100 mL of Fåhraeus plant-nutrient solution. The pipette tip containing the seedling radicle is detached from the glass slide and inserted into a second, nontruncated, 200 μL pipette tip that is attached to the upper part of the TTS (Fig. 2a). This nontruncated pipette tip is placed in such a way that each individual lateral root is placed inside each twin tube just in between the filter paper and the crystal wall (Fig. 2b). In this way, the whole roots are visible along the experiment, allowing easy scoring of the appearance of nodules (Fig. 2c, d). Each tube is covered with a sterile sponge (*see* **Note 7**) and Parafilm to prevent excessive evaporation (Fig. 2b, c).
4. Inoculation of the split-root system and carrying out of the experiment. TTS are placed into a rack covered in such a way that roots are protected from light (Fig. 2e), and incubated for 2 days in a plant growth chamber with a 16 h photoperiod at 25 °C in the light and 18 °C in the dark. When lateral roots need inoculating, 1 mL of a late exponential phase bacterial culture (OD_{600}~1.0, approximately 10^9 cells/mL) is added in aseptic conditions to the corresponding glass tube (*see* **Note 8**), which corresponds to a final inoculant size of approximately 10^7 cells per mL of plant nutrient solution. After inoculation, TTS are placed again in the plant growth chamber and are incubated in the same conditions described above. The number of nodules can be scored at any desired time.

4 Notes

1. It is advisable the addition of $CaCl_2$ to the medium after autoclaving and just before use, since it may coprecipitate with phosphate ions. In this case, dissolve 100 g of $CaCl_2 \cdot 2H_2O$ in 1 L of distilled water, sterilize, and add 1 mL of this solution to the Fåhraeus solution.
2. During the treatments with ethanol and sodium hypochlorite and also during the washings with water, seeds have to be totally covered with the liquid. Flasks containing the seeds are subjected to gentle manual shaking during all the steps of chemical disinfection and subsequent washing.

3. Typically, place 10–12 soybean seeds per plate.
4. The micropipette tips employed are the yellow ones (commonly used for pippeting 10–200 μL volumes).
5. We use 25-mm diameter, 245-mm long glass tubes.
6. We refer to two lateral roots having similar lengths, emerging at the same distance from the truncated root tip, and being located at opposite sites on the root (Fig. 1f).
7. We typically use the sponges (Fig. 2a) which are included as protective agents in the enzymes or other molecular biology reagents packages supplied by companies. Alternatively, sterile cotton can be also used, but it would be less convenient since cotton caps tend to disaggregate when they are repeatedly placed in the glass tubes, since periodic reposition of Fåhraeus solution is required. In addition, chemical analyses of the plant nutrient solution could be carried out at any time of the experiment.
8. Usually, the inoculum is added directly onto the root. For this purpose, the filter paper that pushes the root against the inner side of the glass tube is separated (using sterilized forceps) from the glass at the vicinity of the seed to create a space in which the inoculum is deposited. After inoculation, the space is closed by pushing the filter paper against the glass wall.

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Chapter 24

Methods for the Characterization of Plant-Growth Promoting Rhizobacteria

Md. Motaher Hossain and Farjana Sultana

Abstract

A detailed description of methods most frequently used for the identification and characterization of beneficial microbial strains is presented in this chapter. The methods include microbiological, biochemical, and molecular approaches. Microbiological and biochemical methods comprise a broad range of techniques that are based on the analysis of phosphate solubilization, nitrogenase activity, indole-3-acetic acid production, bacterial motility, presence of catalase and nitrate reductase enzyme, Gram's staining of the cell wall, siderophore production, and microbial chemotaxis. The molecular methods involve a range of techniques that are based on the extraction and analysis of microbial DNA. The extracted nucleic acid can be specifically amplified using polymerase chain reaction (PCR), and subsequently cloned and sequenced. The sequencing of conserved genes such as internal transcribed spacer (ITS) region or 16S rRNA in a microbial genome is used extensively in resolving taxonomic identity of microbial strains. These methods are highly sensitive and allow for a high degree of specificity.

Key words Phosphate solubilization, Nitrogenase activity, Indole-3-acetic acid production, Bacterial motility, Catalase, Nitrate reductase enzyme, Gram's staining, Siderophore production, Microbial chemotaxis

1 Introduction

Boosting crop yield is an increasing demand in an age of rapid population growth and climate change without a concurrent increase in resource utilization. Modern crops are largely being selected in conjunction with application of economically and environmentally costly chemicals to produce high yields. Alternative solutions are required to maintain and increase crop yields sustainably [1]. These new approaches will need application of biological solutions, including the manipulation and exploitation of beneficial plant–microbe interactions. Microbes are key players in nutrient cycle, ecosystem functioning and plant's adaptation to biotic and abiotic stresses [2, 3]. Additionally, many other studies illustrate the importance of plant–microbe feedbacks [4, 5]. Both

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rhizosphere and phyloplane are highly dynamic environments which harbor a stunningly diverse collection of microbial species. Fungi and bacteria are the most plentiful species in these habitats. Numerous studies have characterized the diversity, morphology, and functional groupings of abundant taxa in microbial communities. The large diversity of root associated bacteria is found to be dominated by Gram negative bacteria, while that in non-rhizosphere soil is typically dominated by Gram positive bacteria [6]. The solubilization of insoluble phosphates in the rhizosphere is one of the most common modes of action of plant growth promoting microorganisms. Some strains are capable of synthesizing plant hormones such as indole-3-acetic acid (IAA) and gibberellins (GA_3) [7]. Bacterial species also differ in their ability to produce important cellular enzymes such as catalase and nitrate reductase. Siderophores produced by these microorganisms scavenge Fe and form complex with other essential elements (i.e., Mo, Mn, Co, and Ni), making them available to plants [8, 9]. PGPR-mediated chemotaxis, biofilm formation and motility play an important role in the bacterial colonization of plant roots [10, 11]. Thus, accurate characterization of the beneficial plant-associated microbes is an important step in understanding the complex natures of their function in plant–microbe interactions. Moreover, microbial profiling based on the sequencing of the 16S ribosomal RNA (rRNA) and internal transcribed spacer (ITS) region has been the approaches of choice to currently analyze and understand bacteria and fungi, respectively, at the molecular level. These regions reveal a sufficient interspecies variability to track microbial ecology and evolution [12]. Over the past decades, considerable progress has been made in the identification and characterization of beneficial plant microbes. Different methods have already been used to the assay the microbial potential of phosphate solubilization [13], phytohormone (IAA and GA_3) production [7, 14], dinitrogen fixation [15], hydrolytic enzymes (catalase and nitrate reductase) production [16], cell motility [17], biofilm formation [18], and chemotactic response [19] as well as reaction to Gram's staining [20]. Similarly, there are many different protocols available to perform nucleic acid extraction, polymerase chain reaction (PCR), electrophoresis, PCR purification and DNA sequencing [7, 21–23]. These methods vary from very basic manual protocols to more sophisticated methods. However, it would be ideal to select those that perform best in terms of cost-effectiveness and time efficiency. Therefore, the aim of this chapter is to provide highly useful and sensitive, but inexpensive methods of microbial characterization. Our chapter is expected to be helpful to specialists and students from multidisciplinary areas and will serve as a comprehensive laboratory guide for plant–microbe interaction research. Researchers will be able to conduct research independently by following steps given in this chapter.

2 Materials

Sterilize utensils, glassware, and plasticware prior to using them for plating procedures. Prepare all solutions using sterilized deionized or milliQ water and analytical grade reagents. Maintain all laboratory rules and safety precautions. Clean work area with disinfectant to minimize possible contamination.

2.1 Isolation of Microorganisms from the Plant Rhizosphere

1. Plastic bags.
2. Forceps.
3. 5% NaOCl.
4. Mortar and pestle.

2.2 Determination of Phosphate Solubilization Activity in Microorganisms

1. PVK agar medium (for 1 L) (*see* **Note 1**): 10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$, 0.002 g $FeSO_4 \cdot 7H_2O$, and 15 g agar (*see* **Note 2**). Dissolve each chemical by continuous stirring (*see* **Note 3**). Adjust pH to 7.0 (*see* **Note 4**). Dispense into Erlenmeyer flask as desired and cover the flask mouth with a cotton plug and/or aluminum foil (*see* **Note 5**). Autoclave at 121 °C for 20 min.
2. National Botanical Research Institute's phosphate growth medium (NBRIP) (for 1 L): 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 15 g agar. Autoclave at 121 °C for 20 min.
3. NBRIP-bromophenol blue (NBRIP-BP) medium (for 1 L): 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, 0.025 g bromophenol blue, and 15 g agar. Autoclave at 121 °C for 20 min.
4. Test tubes.
5. Sterile glass petri dishes.

2.3 Quantification of Dinitrogen Fixation: Acetylene Reduction Assay (ARA)

1. Nutrient Agar (NA) medium (for 1 L): 3 g beef extract, 5 g peptone, 5 g NaCl, and 15 g agar. Adjust pH to 7.0 ± 0.2. Autoclave at 121 °C for 20 min.
2. Trace element solution (for 1 L): 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.03 g $MnCl_2 \cdot 4H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.01 g $CuCl_2 \cdot 2H_2O$, and 0.02 g $NiCl_2 \cdot 6H_2O$.
3. Nitrogen free semi solid basal medium (for 1 L): 5 g glucose, 5 g mannitol, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 5 mg $Na_2MoO_4 \cdot 2H_2O$, 0.9 g K_2HPO_4, 0.1 g KH_2PO_4, 0.01 g FeSO4·$7H_2O$, 5 g $CaCO_3$, and 5 g agar. Add 1 mL of trace element solution. Adjust pH to 6.8. Autoclave at 121 °C for 20 min.
4. Acetylene and ethylene (*see* **Note 6**).
5. Glass vials (30 and 50 mL) with rubber stopper cap.

6. 1 mL gas tight syringe.
7. 7 mL vacuum tube.
8. Gas chromatograph equipped with a flame ionization detector (FID).

2.4 Colorimetric Assay for Indole-3-Acetic Acid Production

1. Jensen's broth (for 1 L): 20 g sucrose, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.1 g $FeSO_4$, 0.005 g $NaMoO_4$, 2 g $CaCO_3$, and 2 mg/mL L-tryptophan. Dispense into Erlenmeyer flasks as desired and cover the flask mouth with cotton plug and/or aluminum foil. Autoclave at 121 °C for 20 min. Dispense 10 mL broth into sterile screw-cap test tubes.
2. Salkowski reagent: Prepare 10 mL of 0.5 M $FeCl_3$ solution by dissolving 0.8 g of $FeCl_3$ in distilled water and store it until use. Prepare 50 mL of 35% perchloric acid solution (*see* **Note 7**). Add 1 mL of 0.5 M $FeCl_3$ solution in 50 mL of 35% perchloric acid. Mix it well and store it in a bottle protected from light.
3. Purified commercial IAA (*see* **Note 8**).
4. Spectrophotometer.

2.5 Colorimetric Assay for Gibberellic Acid (GA_3) Production

1. Potato dextrose broth: Boil 200 g of sliced peeled potatoes in 1 L of distilled water for 30 min. Filter through cheesecloth, saving effluent and dissolve 20 g dextrose. Make the volume up to 1 L with water. Dispense 200 mL portions into 500 mL Erlenmeyer flasks. Cover the flask mouth with cotton plug/aluminum foil. Autoclave at 121 °C for 20 min.
2. Nutrient broth (for 1 L): 3 g beef extract/yeast extract, 5 g peptone and 5 g NaCl. Adjust the pH to 7.4 ± 0.2. Dispense 100 mL medium into 250 mL Erlenmeyer flasks and cover the flask mouth with aluminum foil. Autoclave at 121 °C for 20 min.
3. HCl (3.75 and 0.1 M).
4. Ethyl acetate.
5. Absolute ethanol.
6. Commercial purified GA_3.
7. 0.5 M phosphate buffer: Mix 802 mL of 0.5 M K_2HPO_4 with 198 mL of 0.5 M KH_2PO_4. Adjust final pH to 7.0 with 5 N HCl. Autoclave at 121 °C for 20 min. Store at 4 °C.
8. Separating funnel.
9. Spectrophotometer.

2.6 Catalase Test

1. Catalase reagent: 3% hydrogen peroxide.
2. Trypticase soy agar (TSA) (for 1 L): 15 g casein peptone, 5 g soya peptone, 5 g NaCl, and 15 g agar. Adjust final pH to 7.0 ± 0.2. Autoclave at 121 °C for 20 min. Store prepared media below 8 °C protected from direct light.

3. Microscope slides.
4. Pasteur pipettes.

2.7 Nitrate Test

1. NA medium: Prepare as in Subheading 2.3.
2. Nitrate broth (for 1 L): 5 g peptone, 3 g beef extract, and 1 g KNO_3. Adjust final pH to 7.0 ± 0.2. Dispense 5 mL of medium into test tubes and autoclave at 121 °C for 20 min.
3. Sulfanilic acid solution (Reagent A): Dissolve 8 g of sulfanilic acid in 1 L of 5 N acetic acid (*see* **Note 7**). Store Reagent A protected from direct light at room temperature for up to 3 months.
4. Naphthylamine solution (Reagent B): Dissolve 6 g of *N*,*N*-dimethyl-1-naphthylamine in 1 L of 5 N acetic acid (*see* **Note 7**). Store Reagent B protected from direct light at 2–8 °C for up to 3 months.
5. Zinc powder.

2.8 Assay for Siderophore Production

1. NA medium: Prepare as in Subheading 2.3.
2. Modified M9 liquid medium (for 1 L): 64 g $Na_2HPO_4{\cdot}7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 0.25 g $MgSO_4$, 0.011 g $CaCl_2$, and 20 g glucose. Dispense in 200 mL flasks. Autoclave at 121 °C for 20 min.
3. 2 mM Chrome-azurole S (CAS) solution.
4. 1 mM $FeCl_3{\cdot}6H_2O$ solution: Dissolve 0.027 g of $FeCl_3{\cdot}6H_2O$ in 100 mL of 100 mM HCl. Heat with constant stirring to completely dissolve the $FeCl_3$.
5. CAS-Fe-(HDTMA) dye: Dissolve 21.9 mg of hexadecyl-trimethyl-ammonium bromide (HDTMA) in 25 mL of water with low heating and constant stirring. In a separate container, mix 5 mL of 1 mM $FeCl_3{\cdot}6H_2O$ with 7.5 mL of 2 mM CAS. Add this solution to the HDTMA solution while stirring.
6. MES buffer: Dissolve 9.76 g MES buffer (2-[*N*-morpholino] ethane sulfonic acid) in 50 mL of water. Adjust the pH to 5.6 with 50% KOH.
7. CAS Assay solution: Add the MES buffer solution to the volumetric flask containing the CAS-Fe-(HDTMA) dye solution. Add water to make the volume up to 100 mL.
8. Spectrophotometer.

2.9 Gram Staining

1. NA medium: Prepare as in Subheading 2.3.
2. Crystal violet: Dissolve 2 g of crystal violet in 20 mL of 95% ethyl alcohol. Dissolve 0.8 g of ammonium oxalate monohydrate in 80 mL of water. Mix the crystal violet and ammonium oxalate monohydrate solutions. Store the solution up to 24 h.
3. Gram's iodine: Weigh 1 g of iodine and 2 g of KI and grind in a mortar with pestle while slowly add water with continued

grinding until all the iodine has completely dissolved. Make the volume up to 300 mL with water. Store the solution protected from direct light.

4. 95% ethanol.
5. Safranin: Dissolve 2.5 g of safranin in 10 mL of 95% ethanol. Make the volume up to 100 mL with water.
6. Clean glass slides.
7. Microscope.
8. Test tubes.
9. Blotter paper.
10. Immersion oil.

2.10 Biofilm Formation Assay

1. Nutrient broth: Prepare the broth medium as described in Subheading 2.5.
2. 96-well microtiter plates and microtiter plate reader.
3. 100% methanol.
4. 95% ethanol.
5. 0.1% crystal violet.

2.11 Motility Test

1. Nutrient broth: Prepare as in Subheading 2.5.
2. Motility test medium (for 1 L): 3 g beef extract, 10 g casein, 5 g NaCl, and 4 g agar. Adjust pH to 7.0 ± 0.2. Add 5 mL of 1% triphenyl tetrazolium chloride (TTC) solution. Dispense in 10 mL aliquots into test tubes and plug with silicon cap. Autoclave at 121 °C for 20 min.

2.12 Microbial Chemotaxis

1. Nutrient broth: Prepare as in Subheading 2.5.
2. 0.1 M ethylenediaminetetraacetic acid (EDTA).
3. 0.5 M potassium phosphate buffer: Mix 802 mL of 0.5 M K_2HPO_4 with 198 mL of 0.5 M KH_2PO_4. Adjust final pH to 7.0 with 5 N HCl. Autoclave at 121 °C for 20 min. Store at 4 °C.
4. Chemotaxis buffer: Add 20 mL 0.5 M potassium phosphate buffer and 20 μL 0.1 M EDTA and complete to 100 mL with water.
5. 1% Hydroxypropyl methyl cellulose: Heat approximately 30 mL of water to at least 90 °C. Add 1 g of hydroxypropyl methyl cellulose to the heated water and stir the mixture until the particles are thoroughly wetted and evenly dispersed. Add 70 mL of cold water to lower the temperature and agitate for at least 30 min.
6. Root exudates or individual organic acids.
7. Standard 1 μL capillary.
8. Syringe.

2.13 DNA Extraction from Fungi

1. Potato dextrose broth: Prepare as in Subheading 2.5.
2. 10% sodium dodecyl sulfate (SDS).
3. Skim milk: Dissolve 2 g of skim milk in 10 mL of water by heating at 65 °C. Autoclave at 115 °C and 2 atm pressure for 10 min. Store at −20 °C.
4. Extraction buffer: 1 mL 1 M Tris–HCl, 20 mL 0.5 M EDTA, 0.2 mL 5 M LiCl, and 3.8 mL water. Store at 4 °C.
5. 3 M sodium acetate: Dissolve 40.82 g of sodium acetate trihydrate in 80 mL of glacial acetic acid. Adjust final pH to 5.2. Add water to complete the volume to 100 mL.
6. Benzyl chloride.
7. RNAase.
8. Isopropanol at −20 °C.
9. 70% ethanol at −20 °C.
10. TE buffer: 1 mL 1 M Tris–HCl, 0.2 mL 0.5 M EDTA, 98.8 mL water.
11. Mortar and pestle.
12. Liquid nitrogen.

2.14 DNA Extraction from Bacteria

1. Nutrient broth: Prepare as in Subheading 2.5.
2. 10% SDS.
3. Minimal salts solution:
4. Phenol saturated with 1 M Tris–HCl (pH 7.5): Liquefy phenol at 65 °C in a water bath for 1 h (*see* **Note 9**). Pour 100 mL of liquid phenol in a graduated cylinder and transfer to a sterilized amber bottle. Add 99 mL of Tris–HCl (pH 7.5) and stir continuously for 1 h. Allow the solution to stand for separation of the two layers. Remove the upper Tris–HCl layer carefully with a glass pipette and pour in a measuring cylinder. Take 50 mL of water in a sterilized conical flask and pour a few mL of water into the bottle containing the phenol. Stir the bottle again for 30 min and transfer the upper Tris–HCl layer again to the graduated cylinder and pour a few mL of water into the bottle. Repeat this procedure to replace 50 mL of water. In another sterilized conical flask, add 100 mL of water, 1 mL of 1 M Tris–HCl and 200 μL of 0.5 M EDTA. Transfer the solution to an amber bottle containing phenol. Add a pinch of 8-hydroxylquinoline. Stir continuously overnight on a magnetic stirrer. Store at 4 °C.
5. Bacterial cell lysis buffer: 1 mL 1 M Tris–HCl, 20 mL 0.5 M EDTA, 0.2 mL 5 M NaCl, and 73.8 mL water. Store at 4 °C.
6. 7.5 M ammonium acetate.
7. TE buffer: Prepare as in Subheading 2.13.
8. Mortar and pestle.
9. Absolute ethanol.

2.15 Amplification of a Target Gene by PCR

1. 10× Taq reaction buffer (without $MgCl_2$).
2. 25 mM $MgCl_2$.
3. 100 mM dNTP.
4. Taq DNA Polymerase (5 units/μL).
5. 20 μM forward and reverse primers: Select primers as reported [7, 21–23] (*see* **Note 10**).
6. PCR tubes.
7. Thermo cycler.

2.16 Visualization of Amplicons by Agarose or Polyacrylamide Gel Electrophoresis

1. Agarose.
2. 10× Tris borate EDTA (TBE) (for 2 L): 218 g Tris base, 110 g boric acid and 9.3 g EDTA. Adjust the pH to 8.3 with NaOH.
3. 6× loading dye: Dissolve 25 mg of bromophenol blue or xylene cyanol and 4 g of sucrose in 10 mL of water.
4. Ethidium bromide staining solution: Add 15 μL ethidium bromide to 300 mL of water and store at 4 °C (*see* **Note 11**).
5. Electrophoresis unit and power supply.
6. UV transilluminator.
7. 1.5 M Tris–HCl (pH 8.8): Dissolve 90.825 g of Tris base in 400 mL water. Adjust pH to 8.8 with 5 N HCl. Complete the volume to 500 mL with water.
8. 0.5 M Tris–HCl (pH 6.8): Dissolve 30.275 g of Tris base in 400 mL of water. Adjust pH to 6.8 with 5 N HCl (*see* **Note 12**). Complete the volume to 500 mL with water.
9. 30% acrylamide stock solution: Dissolve 150 g of acrylamide monomer and 4 g of *N*,*N*′-methylene bisacrylamide in 500 mL of water and filter through a 0.45 μm membrane filter. Store at 4 °C (*see* **Note 13**).
10. 10% APS (ammonium peroxodisulfate).
11. TEMED (*N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine).
12. 10× Tris-glycine buffer (for 1 L): 30.3 g Tris base and 1444.1 g (*see* **Note 14**).
13. Hypodermic syringe.
14. Filter paper.

2.17 Removal of dNTP and Unused Primer

1. 30% (w/v) polyethylene glycol 6000 (PEG) + 1.6 M NaCl solution (for 50 mL): 15 g polyethylene glycol 6000, 4.68 g NaCl in 40 mL of water. Shake and let PEG go into solution. Put it into a shaking incubator set to 37 °C until everything is dissolved. Complete the volume to 50 mL with water.
2. 70% ethanol at −20 °C.
3. NanoDrop or similar.

2.18 Sequence Reaction Using Sequencing Kit

1. Sequencing Kit (Ready reaction mix and 5× sequencing buffer). Store the kit at −15 to −25 °C. Sequencing buffer may be stored at 4 °C.
2. 1.6 μM primers (forward and reverse).
3. PCR product.
4. 95% and 70% ethanol.
5. 3 M sodium acetate.
6. Formamide.
7. Sanger-sequencing genetic analyzer.

3 Methods

Carry out all procedures stepwise at room temperature unless otherwise specified.

3.1 Isolation of Microorganisms from the Plant Rhizosphere

1. Collect 2–5 g of fresh roots and the soil adhered to the roots in sterilized plastic bags. Separate the soil by gentle tapping using sterilized forceps. For endophytes, wash the roots under running tap water, surface-sterilize in 5% NaOCl for 1 min and wash three times with water.
2. Grind 1 g of roots in a small amount of water with a sterilized mortar and pestle and dilute the root or soil suspension in 9 mL of sterilized water.
3. Prepare a dilution series from 10^{-1} to 10^{-6}. Set out six test tubes, each containing 9 mL of water. Pipette 1 mL of the sample suspension into tube 1 and thoroughly mix. Then transfer 1 mL of the suspension from tube 1 to tube 2 and mix thoroughly again. Repeat this procedure until the dilution series is completed. Plate the dilutions to isolate colonies.

3.2 Determination of Phosphate Solubilization Activity in Microorganisms

1. Pipette 100 μL of the sample culture onto the appropriate phosphate solubilization agar plate and spread with the spreader evenly all over the surface (*see* **Note 15**).
2. Incubate the plates for 7 days. Mark colonies with a clear halo positive for phosphate solubilization.

3.3 Quantification of Dinitrogen Fixation: Acetylene Reduction Assay (ARA)

1. Incubate the studied bacteria in NA medium for 24 h.
2. Dispense 10 mL aliquots of nitrogen-free semisolid medium into sterile 30 mL vials (prepare four replicates for each strain).
3. Inoculate a single colony from the NA plate in the semisolid medium of the vials (*see* **Note 16**).
4. Seal the vial with an airtight rubber stopper and incubate for 48 h.
5. Check for pellicle formation over the medium surface.

6. Following pellicle formation, remove 2 mL of air with a gas-tight syringe. Then inject the vials with an equivalent volume of acetylene gas to create a 10% (v/v) acetylene atmosphere (*see* **Note 17**).
7. Incubate the vials for 24 h.
8. To determine in planta nitrogenase activity, take plants inoculated with isolated strains at 48 days after inoculation. Separate root samples from seedlings and carefully remove any adhered soil.
9. Place the plant samples in 50 mL glass bottles and seal with a rubber septum. After removing an equivalent volume of air, inject acetylene into these bottles to give a final concentration of 10% and incubate for 24 h.
10. Withdraw 1 mL gas samples with a gas-tight syringe and transfer to a 7 mL vacuum tube.
11. In all cases, analyze the presence of ethylene by gas chromatography and operate under the following conditions: carrier gas: hydrogen and oxygen with a flow rate of 4 kg/cm^2, detector temperature: 165 °C; pressure: 4.0 psi.
12. Prepare a standard curve using standard ethylene in concentrations ranging from 1 to 1000 μmol.
13. Calculate the ethylene concentrations from the calibration based on the standard curve and peak-area percentage: μmol C_2H_4 $mL^{-1} = a + bx$ (where x is the peak area from the gas chromatograph, a and b are derived from the calibration curve).
14. Calculate net ethylene accumulation after deducting the ethylene values for a blank treatment without samples.

3.4 Colorimetric Assay of Indole-3-Acetic Acid Production

1. Inoculate bacteria or fungi from pure colonies into Jensen's broth.
2. Incubate the culture at room temperature with continuous shaking at 125 rpm for 48 h.
3. Centrifuge 1 mL of culture solution at 15,000 rpm for 1 min in a tabletop centrifuge.
4. Add 1 mL of the supernatant to 2 mL of Salkowski's reagent and gently vortex.
5. Incubate the mixture for 20 min in darkness at room temperature.
6. Prepare an IAA standard curve with pure IAA solutions at different concentrations (0, 10, 20, 30, 40, 50, and 60 μg/mL) in acetone or ethanol (*see* **Note 8**). Take 1 mL of IAA standard solutions and mix with Salkowski's reagent as previously mentioned. Measure the absorbance at a wavelength of

540 nm in a spectrophotometer. Plot an IAA standard curve of absorbance versus IAA concentration.

7. Measure the absorbance of the studied samples at a wavelength of 540 nm, and plot the absorbance value of the sample solution on the IAA standard curve and calculate the IAA content of the sample solution.

3.5 Colorimetric Assay for Gibberellic Acid (GA_3) Production

1. Grow fungus on potato dextrose broth for 7 days, and bacteria in nutrient broth for 2 days at constant shaking.
2. Centrifuge to remove biomass and collect the supernatant.
3. Adjust the pH of the supernatant between 1 and 2 with 0.1 M HCl.
4. Transfer 8 mL of the supernatant to a 100 mL separating funnel. Add water to make the volume up to 10 mL.
5. Add 20 mL of ethyl acetate and shake vigorously for 1 min.
6. Transfer the aqueous phase to a second separating funnel.
7. Add 20 mL of ethyl acetate to the second funnel and shake vigorously for 1 min.
8. Discard the aqueous phase.
9. Transfer the organic phase to the first separating funnel.
10. Add 20, 15, and 10 mL phosphate buffer (pH 7.4), successively. Shake each time for 1 min and combine each extraction in a 50 mL volumetric flask.
11. Complete the volume to 50 mL with phosphate buffer.
12. Mix 1 mL of the sample extraction with 1 mL of absolute ethanol in a 10 mL flask.
13. Add 3.75 M HCl to the flask and complete the volume to 10 mL. Mix vigorously for 10 s.
14. Prepare a GA_3 standard curve: Dissolve 0.08 g pure GA_3 in 100 mL absolute ethanol in a flask. Dilute and prepare GA_3 solutions at different concentrations (0.6, 0.4, 0.2, and 0.1 g/L) with absolute ethanol. Read the absorbance at a wavelength of 254 nm and plot the absorbance versus GA_3 concentration.
15. Measure the absorbance of the solution at 254 nm and determine the concentration of GA_3 from standard curve.

3.6 Catalase Test

1. Inoculate bacteria from pure colonies in TSA medium.
2. Incubate plates for 24 h.
3. Smear the growth from the plate on a microscope slide and place a drop of 3% hydrogen peroxide on the smear (*see* **Note 18**).
4. Copious bubbles liberated in the hydrogen peroxide indicate presence of catalase.

3.7 Nitrate Test

1. Incubate bacteria in NA medium for 24 h.
2. Inoculate the nitrate broths in a tube and seal the tube with Parafilm.
3. Incubate the tubes for 24–48 h.
4. Put five drops of reagent A and five drops of reagent B into the tube containing the culture. Shake the tube well to mix reagents with the medium.
5. A distinct red color, which should develop within a few minutes, indicates that nitrate was reduced to nitrite.
6. If the suspension is colorless after the addition of reagents A and B, add a small amount (sharp knife point) of zinc powder to the medium. Shake the tube vigorously and allow it to stand at room temperature for 10–15 min. If the medium remains colorless after the addition of Zn powder, the test result is positive. If the medium turns red-pink after the addition of Zn powder, the result is negative (*see* **Note 19**).
7. The negative control should also be tested. There should be no red-pink color formation after adding reagent A and B and if zinc powder is added the color should change to red-pink (*see* **Note 20**).

3.8 Assay for Siderophore Production

1. Incubate bacteria overnight in NA medium.
2. Transfer bacterial cells to modified M9 liquid medium containing no added Fe and incubate for 2 days with continuous shaking.
3. Take 10 mL of the culture and centrifuge at 15,000 rpm for 5 min in a tabletop centrifuge.
4. Collect the supernatant and filter through a 0.45 μm membrane filter.
5. Mix 0.5 mL of the filtrate with 0.5 mL CAS assay solution and incubate for 3–4 h.
6. The change in color of the blue dye to orange indicates the presence of siderophores.
7. Prepare a reference solution with a mixture of 0.5 mL CAS assay solution and 0.5 mL of uninoculated sterile M9 medium.
8. Measure the absorbance at 630 nm and calculate the percentage of siderophore units as $[Ar - As/Ar] \times 100$, where As = absorbance of samples and As = absorbance of the reference solution.

3.9 Gram Staining

1. Incubate bacteria in NA medium for 48 h.
2. Add 1 mL of water into a test tube using a disposable pipette.
3. Take a colony with an inoculating loop, transfer to the test tube containing water and gently shake for 5–10 s.

4. Transfer a small amount of the bacterial suspension from the test tube to a clear dry glass slide. Tilt the slide to spread the drop out slightly to create a thin smear.
5. Air-dry and fix by gentle heat by passing the slide over a flame several times (*see* **Note 21**).
6. Flood the fixed smear with crystal violet solution and incubate for 1 min.
7. Wash with water.
8. Flood the slide with iodine solution and incubate for 1 min.
9. Rinse off the iodine solution with water.
10. Flood the slide with ethanol until the blue dye no longer flows from the smear.
11. Wash with water.
12. Flood the slide with safranin and incubate for 30–60 s.
13. Wash with water.
14. Air-dry or blot-dry by gently passing the paper down for few times.
15. Add a drop of immersion oil on the stained sample and examine the slide under a 100× objective. Observe several fields on the slide for bacterial organisms. Gram-positive bacteria stain deep violet to blue and Gram-negative bacteria stain pink to red.

3.10 Biofilm Formation Assay

1. Incubate bacteria in nutrient broth for 48 h with continuous shaking.
2. Adjust the OD_{600} of the cultures to 0.5.
3. Add 200 μL of cell suspension in 96-well microtiter plates and incubate for 48 h.
4. Discard the growth medium from the microtiter plate wells.
5. Wash the wells twice, each with 200 μL of water and pipette out slowly.
6. Air-dry for 30 min.
7. Add 200 μL of 100% methanol per well for fixation.
8. Incubate at 37 °C for 15 min and pipette out the fixative.
9. Air-dry for 15 min.
10. Add 200 μL of 0.1% (v/v) crystal violet solution to each well and incubate for 25 min at room temperature.
11. Pour out the excess dye.
12. Wash three times with water and tap the plate on paper towels to get all the water out.
13. Air-dry for 30 min.
14. Add 200 μL of 95% (v/v) ethanol to dissolve crystal violet.
15. Measure the OD at 578 nm in a microtiter plate reader.

3.11 Motility Test of Bacteria

1. Incubate bacteria in nutrient broth for 36 h with continuous shaking.
2. Stab 2/3 of the semisolid motility agar medium with an inoculation needle containing the microorganism of interest. Withdraw the needle as vertically as possible to avoid spreading the inoculum beyond the original stab line.
3. Incubate the tubes for 48 h.
4. A positive motility test is indicated by a red turbid area extending away from the line of inoculation. A negative test is indicated by red growth along the inoculation line but no further.

3.12 Microbial Chemotaxis

3.12.1 Plate Method

1. Incubate bacteria in nutrient broth for 48 h with continuous shaking.
2. Collect cells grown by centrifugation and resuspend in 12 mL of chemotaxis buffer.
3. Add 4 mL of 1% hydroxypropylmethyl cellulose solution to the cell suspension.
4. Pour the cell suspension to a 60 mm-diameter petri dish.
5. Add 10 μL concentrated root exudate or individual organic acids (0.1 M) to the center of each petri dish.
6. Incubate for 10–15 min at room temperature.
7. Check whether a ring of turbidity near the center of each petri dish appears. This is the indicator of chemotactic response triggered by bacterial cells.

3.12.2 Capillary Assay

1. Grow bacteria in NB medium and collect by centrifugation as described as above.
2. Resuspend bacteria twice in 10 mL chemotaxis buffer and collect by centrifugation.
3. Resuspend the cells in 10 mL of the same buffer and pour in a 60 mm-diameter petri dish.
4. Load the standard 1 μL capillaries with the concentrated root exudates or individual organic acids at different concentrations (10, 25, 50, and 100 μM, respectively) and immerse in the cell suspension.
5. Incubate for 30 min at room temperature.
6. Transfer the liquid in the capillary into a sterilized eppendorf tube via syringe.
7. Dilute the suspension to 10^{-3}, 10^{-4}, and 10^{-5}, and plate 100 μL of the suspension on NA plates.
8. Incubate the plates for 24 h and count the colony forming units (cfu). Compare the cfu between treatments.

3.13 DNA Extraction from Fungi

1. Grow fungi in PDB for 7 days.
2. Harvest the mycelial mat by filtration and store at −80 °C until use.
3. Clean mortar, pestle, and spoon with 70% ethanol.
4. Grind fungal sample in liquid nitrogen with mortar and pestle.
5. Transfer ground sample to a 1.5 mL tube.
6. Add 250 μL of extraction buffer, 50 μL of 10% SDS, 10 μL of skim milk and 5 μL of RNAase to each tube.
7. Vortex for 1 min.
8. Add 150 μL of benzyl chloride.
9. Vortex for 2 min.
10. Incubate at 50 °C for 60 min.
11. After incubation, add 150 μL 3 M sodium acetate (*see* **Note 22**) and vortex briefly.
12. Incubate on ice for 15 min.
13. Centrifuge for 10 min at 15,000 rpm at 4 °C in a tabletop centrifuge.
14. Transfer the upper phase to a new tube.
15. Centrifuge again for 10 min at 15,000 rpm at 4 °C in a tabletop centrifuge.
16. Transfer the upper phase to a new tube.
17. Add 500 μL cold isopropanol and mix.
18. Centrifuge for 20 min at 15,000 rpm at 4 °C in a tabletop centrifuge.
19. Discard the liquid phase.
20. Add 500 μL of cold 70% ethanol to each tube and rinse the wall by rolling the tube.
21. Centrifuge for 5 min at 15,000 rpm at 4 °C in a tabletop centrifuge.
22. Discard the liquid phase.
23. Vacuum-dry for 10 min and add 50 μL of TE buffer (*see* **Note 23**).

3.14 DNA Extraction from Bacteria

1. Incubate bacteria in nutrient broth for 24–48 h with shaking.
2. Transfer 1.5 mL of the culture to a 2 mL eppendorf tube and centrifuge at 10,000 rpm at 4 °C for 10 min in a tabletop centrifuge.
3. Wash the cell pellet with 500 μL of ice-cold minimal salts solution, mix by vortexing, centrifuge as described in **step 2** and discard the supernatant.

4. Suspend the cell pellet in 500 μL of ice-cold water, mix by vortexing, centrifuge as described in **step 2** and discard the supernatant.
5. Suspend the cell pellet in 500 μL of bacterial cell lysis buffer, mix by gentle inverting and place on ice for 5 min.
6. Add 500 μL of phenol saturated with Tris–HCl, mix by gentle inverting and centrifuge for 10 min at 15,000 rpm at 4 °C in a tabletop centrifuge.
7. Transfer the aqueous phase to new 1.5 mL eppendorf tube.
8. Add 500 μL of phenol saturated with Tris–HCl, mix by gentle inverting and centrifuge for 10 min at 15,000 rpm at 4 °C in a tabletop centrifuge.
9. Transfer the aqueous phase to a new 1.5 mL eppendorf tube.
10. Add 150 μL of 7.5 M ammonium acetate and 1 mL of absolute ethanol and store at −20 °C for at least 2 h (*see* **Note 24**).
11. Centrifuge for 15 min at 15,000 rpm at 4 °C in a tabletop centrifuge.
12. Discard the supernatant by pipetting. Invert the tube over some paper towels to drain off the residual liquid.
13. Resuspend the DNA in 50 μL of TE buffer.

3.15 Amplification of Target Gene by PCR

1. Prepare a PCR master mixture: 2.5 μL 10× Buffer, 1.25 μL $MgCl_2$, 2 μL dNTP 10 mM, 0.625 μL primer F (20 μM), 0.625 μL primer R (20 μM), 0.2 μL rTaq polymerase, and 12.8 μL water.
2. Multiply the volume of each reagent by the number of samples and add 10% extra to account for pipetting error.
3. Mix the master mix thoroughly, and then add 5 μL of template DNA.
4. Mix by flicking tube and spin down.
5. Run the program as initial step at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 1.5 min, which is followed by a final extension at 72 °C for 10 min.

3.16 Visualization of Amplicons by Agarose or Polyacrylamide Gel Electrophoresis

3.16.1 Agarose Gel Electrophoresis

1. Weigh the appropriate mass of agarose (1.5–3%) into an Erlenmeyer flask.
2. Add the appropriate volume of running buffer (TAE/TBE) to the agarose-containing flask, depending on the size of the gel, and swirl to mix.
3. Melt the mixture by heating in a microwave. At 30 s intervals, remove the flask and swirl to mix well. Repeat until the agarose has completely dissolved.

4. Place the gel tray into the casting apparatus, pour the molten agarose into the gel mold, and place an appropriate comb into the gel mold. Allow the gel to set completely (30–35 min at room temperature).
5. Pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb.
6. Place the gel in the gel box and add just enough electrophoresis buffer to cover the gel.
7. Mix 5 μL of PCR product with 1 μL of 6× gel-loading dye and slowly load the mixture into the slots of the submerged gel using a micropipette. Load size marker into slots on both the right and left sides of the gel.
8. Run the gel at a desired voltage until the dye has migrated to an appropriate distance.
9. Remove gel from the gel box, place it in ethidium bromide staining solution and wash it after some minutes.
10. Place the gel to UV light.

3.16.2 Polyacrylamide Electrophoresis

1. Pour 8.9 mL of 30% acrylamide solution, 3.3 mL of water, 4.2 mL of 1.5 M Tris–HCl, and 63.8 μL of 10% APS in a conical flask and shake continuously. Add 15.8 μL of TEMED and mix gently.
2. Assemble the glass plates with silicon packing and clips and pour the running gel solution between the glass plates.
3. Immediately spread a thin layer of water over the gel solution.
4. Leave the gel to polymerize. While waiting for the gel to polymerize you can start preparing the stacking gel.
5. Prepare a stacking gel in a small flask or falcon tube using 0.6 mL of 30% acrylamide solution, 2.04 mL of water, 0.96 mL of 0.5 M Tris–HCl, 30 μL of 10% APS, and 4 μL of TEMED.
6. When the polymerization of resolving gel is complete, decant the layer of water (*see* **Note 25**). Dry excess water using blotter paper.
7. Pour the stacking gel solution between the glass plates. Insert the comb carefully avoiding air bubbles. Leave to polymerize until gel gets firm.
8. Once the gel has polymerized, slowly remove the comb under running water.
9. Wash the wells with water using a hypodermic syringe.
10. Remove the silicon packing and clips.
11. Set up the electrophoresis unit and pour Tris glycine buffer into the unit.

12. Mix PCR products with loading dye and load into the wells.
13. Run the electrophoresis (*see* **Note 26**).
14. Stop the electrophoresis 0–30 min after the loading dye has run off the gel.
15. Take the gel out of the glass plates.
16. Stain the gel with EtBr solution for 10 min.
17. Visualize DNA bands under a UV transilluminator.

3.17 Removal of dNTP and Unused Primer

1. Add 0.6 volumes of PEG + NaCl solution to a PCR product and mix well by pipetting.
2. Incubate the PCR + PEG + NaCl at 37 °C for 10 min.
3. Transfer the product to a 1.5 mL tube.
4. Centrifuge at 15,000 rpm for 10 min at room temperature in a tabletop centrifuge.
5. Add 1 mL of cold 70% EtOH to the tube.
6. Centrifuge at 15,000 rpm for 1 min at room temperature in a tabletop centrifuge.
7. Discard the aqueous phase completely.
8. Vacuum-dry for 5 min.
9. Suspend the pellet in 20 μL water.
10. Measure the PCR product concentration in a NanoDrop or similar.
11. Adjust the final concentration to 50 ng/μL with water.

3.18 Sequence Reaction Using BigDye Terminator V3.1 Cycle Sequencing Kit

1. Completely thaw the contents of the BigDye Terminator v3.1 Cycle Sequencing Kit and your primers and store on ice.
2. Vortex the tubes for 2–3 s, then centrifuge for 2–3 s to collect contents at the bottom of the tubes.
3. Label micro tubes "forward" and "reverse" and add components as indicated: 2 μL 1.6 μM primer, 2 μL 2.5× Ready Reaction Mix, 4 μL 5× Sequencing Buffer, 90 ng PCR product and complete to 20 μL with water.
4. Add 50 μL of 95% ethanol and 2 μL of 3 M sodium acetate.
5. Incubate on ice for 10 min.
6. Transfer to a 1.5 mL Eppendorf tube.
7. Centrifuge at 15,000 rpm for 20 min at room temperature in a tabletop centrifuge.
8. Discard the aqueous phase.
9. Add 250 μL of 70% ethanol.
10. Centrifuge at 15,000 rpm for 20 min at room temperature in a tabletop centrifuge.

11. Discard the aqueous phase.
12. Vacuum-dry for 10 min.
13. Add 10 μL formamide and vortex for 10 s.
14. Load the samples to a 96-well plate for Sanger sequencing reactions.
15. Get the sequence data on the following day.

4 Notes

1. In plate assays, phosphate solubilizing activity of the microorganisms studied could be qualitatively evaluated by growing them in any of the PVK, NBRIP and NBRIP-BP media. However, NBRIP medium with bromophenol blue (NBRIP-BP) has higher clarity and visibility of the yellow-colored halo than other media used in our studies.
2. To avoid precipitation, use tri-calcium phosphate instead of calcium phosphate while preparing phosphate solubilizing media.
3. When stirring the broth solution, take special note in beginning the stir scale at a low setting and adding sequentially more speed.
4. The pH of the prepared medium should be tested when the medium is in its final form at room temperature (25 °C). Agar products will, therefore, be in solid form and ideally require the use of a flat-bottomed pH probe in order to facilitate measurement. The use of automatic temperature adjusting pH meter is not recommended. Our laboratory suggests an electrode such as the BDH double-junction flat-tipped combination electrode.
5. Erlenmeyer flasks should not be filled more than half so that the agar does not boil over.
6. Acetylene is a dangerous, explosive gas, and great care must be taken with its use. It should not be mixed with oxygen and never be used in confined (unventilated) rooms or near sources of ignition. Acetylene is readily obtainable as a compressed gas in steel cylinders from commercial suppliers, or it can be produced by adding water to calcium carbide. Industrial grade acetylene is usually adequate. An incubation atmosphere of 10% acetylene (100 mL/L) is sufficient to assess maximum nitrogenase activity.
7. Prepare the solution in the fume hood. Avoid contact with eyes, skin, and clothing. Avoid ingestion and inhalation. Change gloves frequently. Wear chemical goggles to protect eyes. Always wash hands thoroughly after handling, even if gloves are used.

8. IAA is not soluble in water but it is soluble in acetone and ethanol.
9. Whenever possible, work with phenol in a chemical fume hood, especially when heating it. Never heat or melt phenol in an incubator, microwave, drying oven, or similar appliance.
10. The accuracy of ITS and 16S rRNA amplicon analyses depends strongly on the choice of primers. Using inappropriate primers consequently leads to questionable biological conclusions. Therefore, select primer pairs with the best overall coverage and phylum spectrum for specific applications.
11. Ethidium bromide is carcinogenic. Use of less harmful dye such as GelRedis recommended.
12. Decreasing the pH is much stronger than increasing the pH, so be extra careful when reducing the pH.
13. Wear a mask when weighing acrylamide. Unpolymerized acrylamide is a neurotoxin and care should be exercised to avoid skin contact.
14. Running buffer could be used multiple times (twice or thrice) of electrophoresis. However, multiple use of running buffer prolongs the duration of electrophoresis.
15. Run the laminar airflow and clean the bench surface with 70% ethanol. Leave the bench under UV for 30 min before starting the work.
16. Flaming the mouths of vials, test tubes and flasks during opening and replacing cap will prevent contamination.
17. Note that this concentration may not be sufficient for bacteria such as those belonging to the genera *Beijerinckia* or *Derxia*. These bacteria were reported to require a very high partial pressure of CH_2 [24].
18. Do not introduce a metallic loop into the drop of catalase reagent, because this often causes a false-positive reaction. Bacteria grown on media with low levels or no glucose may yield conflicting results from pseudocatalase, a non-iron enzyme. The pseudocatalase reaction can be prevented by using media with 1% glucose.
19. The addition of zinc reduces the nitrate to nitrite, and the nitrite in the medium forms nitrous acid, which reacts with sulfanilic acid. The diazotized sulfanilic acid that is thereby produced reacts with the α-naphthylamine to create the red complex.
20. Addition of too much zinc powder can result in false-negative reaction.
21. During Gram staining, the slide should be passed very quickly through the flame and not be heated excessively. Don't place

the slide directly in the flame so that the bacteria will not be burned.

22. At this point it is possible to stop the extraction and store the mixture for 1 day at 4 °C.
23. The volume of the TE buffer could be changed depending on the size of the pellet.
24. At this point it is possible to stop the extraction and store the mixture overnight at −20 °C.
25. A sharp line between water layer and the gel, and formation of dimples of irregular shapes near the edges of the gel indicate completion of polymerization.
26. Perform electrophoresis at 300 V for approximately 5–6 h. An overnight electrophoresis at a lower voltage (50–60 V) is also possible. For overnight electrophoresis, first run the electrophoresis at 300 V for 15 min, then set to 50–60 V in the night and change to 300 V in the morning to complete.

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