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ADVANCES IN
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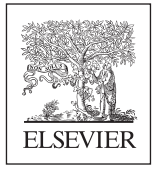

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CHAPTER ONE

Antivirulence Properties of Probiotics in Combating Microbial Pathogenesis

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Contents

Abstract

Probiotics are nonpathogenic microorganisms that confer a health benefit on the host when administered in adequate amounts. Ample evidence is documented to support the potential application of probiotics for the prevention and treatment of infections. Health benefits of probiotics include prevention of diarrhea, including antibiotic-associated diarrhea and traveler's diarrhea, atopic eczema, dental carries, colorectal cancers, and treatment of inflammatory bowel disease. The cumulative body of scientific evidence that demonstrates the beneficial effects of probiotics on health and

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disease prevention has made probiotics increasingly important as a part of human nutrition and led to a surge in the demand for probiotics in clinical applications and as functional foods.

The ability of probiotics to promote health is attributed to the various beneficial effects exerted by these microorganisms on the host. These include lactose metabolism and food digestion, production of antimicrobial peptides and control of enteric infections, anticarcinogenic properties, immunologic enhancement, enhancement of short-chain fatty acid production, antiatherogenic and cholesterol-lowering attributes, regulatory role in allergy, protection against vaginal or urinary tract infections, increased nutritional value, maintenance of epithelial integrity and barrier, stimulation of repair mechanism in cells, and maintenance and reestablishment of well-balanced indigenous intestinal and respiratory microbial communities. Most of these attributes primarily focus on the effect of probiotic supplementation on the host. Hence, in most cases, it can be concluded that the ability of a probiotic to protect the host from infection is an indirect result of promoting overall health and well-being. However, probiotics also exert a direct effect on invading microorganisms.

The direct modes of action resulting in the elimination of pathogens include inhibition of pathogen replication by producing antimicrobial substances like bacteriocins, competition for limiting resources in the host, antitoxin effect, inhibition of virulence, antiadhesive and antiinvasive effects, and competitive exclusion by competition for binding sites or stimulation of epithelial barrier function. Although much has been documented about the ability of probiotics to promote host health, there is limited discussion on the above mentioned effects of probiotics on pathogens. Being in an era of antibiotic resistance, a better understanding of this complex probiotic–pathogen interaction is critical for development of effective strategies to control infections. Therefore, this chapter will focus on the ability of probiotics to directly modulate the infectious nature of pathogens and the underlying mechanisms that mediate these effects.

1. INTRODUCTION

According to the World health Organization, globally 18 million people die annually from infectious diseases (WHO, 2014). Furthermore, infectious diseases pose a significant threat to human health particularly with the increase in the incidence and prevalence of antibiotic resistant bacteria (Fair & Tor, 2014). Infections associated with multidrug resistant pathogens result in increased morbidity and mortality, prolonged hospital stays, and prolonged periods during which individuals are infectious. The annual impact of resistant infections in the United States is estimated to be ~$20 billion in increased health care costs and 8 million additional hospital days (CDC, 2013). Hence, health organizations around the world have identified antibiotic resistance as one of the top health challenges around the globe. The extensive and

unrestricted uses of antibiotics have contributed to the emergence of resistant pathogens (Fair & Tor, 2014). However, an enhanced understanding of pathogen biology has enabled the development of many potential antibiotic-free alternative strategies to treat infectious diseases. In this regard, agents that impede the disease causing ability of pathogens provide a potential alternative that is less likely to induce selective pressure for the development of bacterial resistance than traditional bactericidal or bacteriostatic strategies. Along these lines, probiotic-based approaches have been successfully used for decades in complementary and alternative medicine. Additionally, there exists substantial evidence that demonstrates their efficacy in the treatment of gastrointestinal infections and disorders (Hussain & Quigley, 2006). Probiotics are harmless live microorganisms which when administered in adequate amounts confer both well-being and health benefit on the host (FAO/WHO, 2002). The supplemented beneficial flora can thus act as "prebiotics" and stimulate the growth of part of the gut microbiota.

This chapter will provide a summary of various virulence mechanisms in pathogens and insight into the direct modes of action of probiotics in combating microbial infections. Additionally research demonstrating the mechanistic basis of probiotics' antivirulence property will be included.

2. MECHANISMS OF BACTERIAL PATHOGENICITY

In differentiating between pathogenic and nonpathogenic microbes, early investigators identified certain components that confer upon them the capacity for causing disease. This led to the development of the concept of "virulence." Disease conferring determinants that promote the establishment and maintenance of diseases are called as virulence factors (Casadevall & Pirofski, 1999; 2001; 2009). Pathogens utilize an array of mechanisms to cause disease in human hosts and express several biomolecules to elicit adverse host responses. Therefore, virulence is defined as the relative ability of a pathogen to instigate damage in the host, which can be attenuated or enhanced under different contexts.

The word "virulence" is derived from the Latin word "virulent," which means "full of poison" (Casadevall & Pirofski, 2001; Gauwerky, Borelli, & Korting, 2009). Traditionally, the term virulence has been considered as a pathogenic characteristic rather than as a complex and dynamic phenomenon that is contingent on the availability of susceptible host and microbial factors (Casadevall, Feldmesser, & Pirofski, 2002; Casadevall & Pirofski, 2001;

Thomas & Elkinton, 2004). However, recent advances in postgenomic approaches such as transcriptomics and proteomics have helped to accelerate the recognition of virulence as a multifaceted property of microbes. Primarily, these attributes that confer pathogenicity can be outlined under several categories like those that enable the pathogen to enter a host, evade host defenses, grow in a host environment, counteract host immune responses, or acquire nutrients from the environment and dynamically respond to environmental changes (Casadevall & Pirofski, 2009). However, most virulence factors have a multitude of effects on the host and grouping them within distinct functional categories is difficult. Therefore, to discuss principal virulence factors that are critical for the infection process across all microorganisms, we have very broadly categorized virulence factors based on their primary function.

2.1 Adherence and Colonization Factors

At the initiation of infection, pathogenic bacteria adhere to the host cell epithelium that enables their rapid multiplication and colonization. A common trait of microbial pathogens that enables this attachment is the expression of ligands that bind to host cell receptors, thereby rendering them resistant to mechanical "washing off" (Peterson, 1996). Once adhered to the host cell surface, pathogens then initiate their specific biochemical and physiological processes, including proliferation, toxin secretion, host cell invasion, and activation of host cell signaling cascades resulting in the disease condition (Casadevall & Pirofski, 2001). Therefore, pathogen attachment to host surfaces is a key step in the pathogenesis. Adhesins are such virulence determinants that enable a microbe to attach to host tissues.

Several bacterial cell wall components consisting of proteins or polysaccharides serve as adhesins or ligands. For instance, lectin enables the attachment of *Entamoeba histolytica* to colonic cells, whereas lipoteichoic acids and M protein serve as adhesins for *Streptococcus pyogenes* (Casadevall & Pirofski, 2009). Cellular appendages such as flagella and pili or fimbriae have evolved as potential adhesins in several Gram-negative bacteria, including intestinal and extraintestinal *Escherichia coli*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Aeromonas* and *Neisseria* species (Kirov, Castrisios, & Shaw, 2004; Pratt & Kolter, 1998). On the other hand, a diverse family of proteins called the microbial surface component recognizing adhesive matrix molecules (MSCRAMM) mediate attachment of several Gram-positive pathogens like *Staphylococcus aureus* (Wann, Gurusiddappa, & Höök, 2000) and *Enterococcus faecalis* (Sillanpää, Xu, Nallapareddy, Murray, & Höök, 2004). Similarly, the fungal pathogen *Candida albicans* elaborates a complex system of adhesion

molecules consisting of cell surface glycoproteins of the adhesion-like family, integrin-like proteins, surface proteins, and polysaccharides (Gale et al., 1998; Hoyer, 2001; Staab, Bradway, Fidel, & Sundstrom, 1999).

2.2 Invasion Factors

After adhering to a host surface, pathogens such as *Salmonella*, *Rickettsia*, and *Chlamydia* gain deeper access into the host tissues to perpetuate the infection cycle (Peterson, 1996). Invasion occurs when a microbe actually penetrates the host cell and survives within the intracellular milieu (Wilson et al., 2002). Pathogens like *Chlamydia* spp, *Rickettsia* spp, and *Mycobacterium leprae* have an obligate intracellular lifecycle that mandates host cell invasion for their survival, growth, and replication. Other pathogens such as *Salmonella* and *Yersinia pseudotuberculosis* are facultative intracellular organisms that use this ability to enter and survive within host cells as a means of proliferation and dissemination (Finlay & Falkow, 1997). Advances in bacterial pathogenesis research led to the identification of critical genes that allow pathogens to invade the host nonphagocytic cells. These invasion genes in several pathogens were found to encode a related type III secretion system that injects microbial signaling proteins into host cells. This type of biochemical cross talk identified between the host and pathogen is essential for host cell invasion (Wilson et al., 2002).

However, several pathogens, including group A-β-hemolytic *Streptococcus*, *S. aureus*, and *P. aeruginosa* can break down tissue barriers while remaining outside the host cells. These enzyme virulence factors include, (1) hyaluronidase: cleaves proteoglycans in connective tissue; (2) streptokinase and staphylokinase: break down fibrin clots; (3) lipase: degrades accumulated host lipids; (4) nucleases: digest released RNA and DNA; (5) hemolysin: punch holes on host cells, and (6) elastase: degrades extracellular molecules causing tissue necrosis and keratitis. Through these mechanisms that favor the breakdown of host extracellular matrix, these enzymes contribute to the successful dissemination of the pathogen in the host. This strategy is called extracellular invasion (Wilson et al., 2002). Digestion of the extracellular matrix by the enzymes provides much-needed nutrients for microbial survival and replication in the host extracellular milieu (Casadevall & Pirofski, 2009).

2.2.1 Intracellular Survival

Following the invasion, certain pathogenic microbes also have the ability to survive and replicate within host phagocytic cells. These intracellular pathogens have unique determinants and mechanisms that ensure their survival

within phagosomes (Casadevall & Pirofski, 2009). During the entry of pathogens into host cells, they are initially engulfed by vacuoles. Inside this vacuole, bacteria secrete proteins that enable the lysis of vacuolar membrane and promote replication in the cytosol. For instance, the foodborne pathogen *Listeria monocytogenes* escapes phagosomal killing by producing listeriolysin O and type C phospholipases. Listeriolysin delays the maturation of the phagosome and disrupts the membrane using phospholipases (Ray, Marteyn, Sansonetti, & Tang, 2009). Similarly, other microbes such as *Legionella pneumophila* interfere with phagosome maturation and the soil fungus, *Histoplasma capsulatum* interferes with phagosomal acidification (Casadevall & Pirofski, 2009). However, unlike avoiding phagocytosis, *M. tuberculosis* preferentially targets the macrophage vacuoles. The pathogen can tolerate the low pH and reactive oxygen and nitrogen species in the macrophage and can actively downregulate the normal cellular mechanisms to avoid being killed (Warner & Mizrahi, 2007).

2.3 Bacterial Cell Wall Components

Bacterial cell wall derived constituents can induce a septic shock that often accompanies severe microbial infections. Septic shock is one of the fatal outcomes of a host—microbe interaction mediated by the combined action of cytokines, complement components, and coagulation cascade components (Horn et al., 2000; Wilson et al., 2002). For example, the release of toxic bacterial cell wall components like lipopolysaccharide (LPS) trigger host inflammatory responses that result in septic shock. Bacterial LPS, commonly known as endotoxins are amphiphilic molecules embedded in the outer membrane of Gram-negative bacteria. LPS is often considered to be the principal component responsible for the induction of septic shock in infections caused by Gram-negative microbes such as *E. coli*, *P aeruginosa*, and meningococci (Wilson et al., 2002). Moreover, recent studies identified that secretion of LPS in outer membrane vesicles plays a critical role in bacterial infections leading to sepsis (Vanaja et al., 2016).

Although endotoxin-mediated sepsis is not encountered with Gram-positive infections, bacterial species such as *S. aureus*, *S. epidermidis*, and Streptococci are increasingly recognized as major contributors to nosocomial sepsis. This is due to the presence of peptidoglycan fragments and teichoic acids found in the Gram-positive cell wall, which can trigger similar pathological responses as seen with LPS (Nau & Eiffert, 2002). Irrespective of whether a septic shock is induced by Gram-positive or Gram-negative bacteria, the toxic cell wall components initiate an

inflammatory response through the stimulation of monocytes and macrophages resulting in the subsequent release of proinflammatory cytokines, especially TNF-α and interleukin-1 (Wilson et al., 2002).

In addition to the role of the aforementioned cell wall components, bacterial capsules also play a role in host immune evasion and clearance. In fact, encapsulated strains of bacteria are found to be more virulent and more resistant to phagocytosis and intracellular killing than nonencapsulated strains (Peterson, 1996). Many pathogenic bacteria possess polysaccharide capsules, including *S. pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenza* whereas the capsule of *Bacillus anthracis* is composed of poly-gamma-D-glutamic acid (Kozel et al., 2004). In addition to protecting pathogens from host immune response, capsules exert immunomodulatory effects as well. Therefore, several polysaccharide-based vaccines have been generated against pathogens like *S. pneumoniae* (pneumococcus) and *N. meningitidis* (meningococcus). Additionally, virulent capsules produced by *P. aeruginosa* causes "frustrated phagocytosis" due to the failure of the phagocyte to engulf the microbial target (Peterson, 1996; Wilson et al., 2002; Wu, Wang, & Jennings, 2008).

2.4 Secretion Systems

Bacterial secretion systems export microbial effector proteins that are essential for virulence. Such secretory proteins are known to modify the host cell environment and make it conducive for bacterial survival (Casadevall & Pirofski, 2009). Pathogenic bacteria express several distinct secretion systems (types I-IV) to transport their cytoplasmic proteins into host cells (Table 1). Autotransporters (ATs), are a family of proteins collectively secreted by the type V secretory pathway in certain pathogenic Gram-negative bacteria like *Y. pestis*. This secretory pathway helps transport bacterial effector proteins across the cell envelope into the extracellular environment (Dautin & Bernstein, 2007).

2.5 Exotoxins

Toxins have been recognized as potent virulence factors ever since toxigenic bacteria associated diseases like diphtheria, cholera, and tetanus were described. Exotoxins are another class of secretory proteins that are secreted by viable Gram positive and Gram-negative bacteria and are not required for the viability of the cell. They are delivered to eukaryotic cells by secretion into the surrounding environment or by direct injection into the host

Table 1 Antibacterial effects of probiotics

Antimicrobial substances produced by probiotics	Mechanisms of action	References
Lactic acid and volatile acids	Decrease luminal pH, disruption of cellular metabolism	Maloney (1990)
Hydrogen peroxide, carbon dioxide	Destruction of biomolecules by unwanted oxidation reaction	Mishra and Lambert (1996) and Pridmore et al. (2008)
Diacetyl	Interference of arginine utilization	Jay (1982)
Bacteriocins and microcins	Disruption of cytoplasmic membrane and pore formation. Disrupt the macromolecules synthetic pathway	Cleveland et al. (2001)

cytoplasm via several mechanisms including the type III secretion systems (Casadevall & Pirofski, 2009; Wilson et al., 2002).

Based on their amino acid composition and function, bacterial exotoxins are broadly categorized into (1) A-B toxins, (2) proteolytic toxins, (3) pore-forming toxins, and (4) other toxins like superantigens. Superantigens secreted by *S. aureus* and *S. pyogens* are known to stimulate host cell and lead to extensive inflammatory reactions. Exotoxins are also classified based on their mechanism of action as Type I: cell surface-active (heat-stable enterotoxins); Type II: membrane damaging (RTX toxins, pneumolysin); Type III: intracellular (diphtheria toxin, pertussis toxin, Shiga toxin), Type IV: extracellular matrix damaging (hyaluronidase, collagenase) (Schaechter, 2010).

A-B toxins are produced by several different species of Gram-negative (*P. aeruginosa*, *E. coli*, *V. cholera* and *Bordetella pertussis*) and Gram-positive (*Corynebacterium diphtheria* and *Clostridium tetani*) bacteria. These toxins possess two components: one binds to the host cell receptor and the other enters and damages the cell. Usually, the A subunit possesses the enzymatic activity (proteolytic like tetanus and botulinum toxins or ADP-ribosylating activity like cholera and diphtheria toxins); and the B subunit is responsible

for binding and delivery of the toxin into the host cell (Wilson et al., 2002). Furthermore, *C. botulinum* (botulinum), *C. tetani* (tetanus) and *P. aeruginosa* (elastase and protease) produce proteolytic toxins which break down specific host proteins resulting in the clinical manifestation of the disease (Wilson et al., 2002).

Pore-forming or membrane-disrupting toxins are also associated with several pathogens. These toxins form pores on the host cell membrane leading to cell lysis. Toxins belonging to RTX [containing repeats of arginine (R) threonine (T) X motifs] family such as α hemolysin are pore-forming toxins secreted through type I system or membrane vesicles by pathogens such as uropathogenic *E. coli* (UPEC) and enterohaemorrhagic *E. coli* (EHEC) (Aldick et al., 2009; Linhartova et al., 2010). Other cellulolytic toxins called cytolysins are produced by Gram-positive bacteria like *L. monocytogenes*. Listeriolysin O is a well-characterized cytolysin of *Listeria* which aids in the escape of pathogen from the phagosome (Andrews & Portnoy, 1994).

2.6 Quorum Sensing and Biofilm Formation

Quorum sensing (QS) refers to cell to cell communication between members of a bacterial population. By virtue of the QS regulation, bacteria can sense their population density by the production of signaling molecules. Accumulation of these small molecules happens as a function of increasing bacterial population when a threshold has reached (Podbielski & Kreikemeyer, 2004). Such QS related regulatory mechanisms are also closely associated with physiology and virulence in many microbes, including *S. aureus, P. aeruginosa* and *Streptococcus spp*. (Antunes, Ferreira, Buckner, & Finlay, 2010; Cvitkovitch, Li, & Ellen, 2003; Smith & Iglewski, 2003; Yarwood & Schlievert, 2003). QS molecules actively involved in pathogenesis affect the expression of many microbial virulence traits, including biofilm formation and toxin production (Antunes et al., 2010).

As reviewed by Bronner, Monteil, and Prévost (2004), the two-component QS system in *S. aureus* has a complex sensing apparatus which regulates its virulence. Similarly, a two-component system in *B. pertussis* regulates the expression of hemagglutinin, fimbria, toxins and type III secretion proteins which have been implicated in its virulence (Mattoo, Foreman-Wykert, Cotter, & Miller, 2001). Several secreted factors (such as proteases), cell-associated factors (such as LPS and flagella), as well as the ability to form biofilms contribute to virulence in *P. aeruginosa* associated disease. QS in the pathogen thus regulates the production of several

extracellular virulence factors, the expression of antibiotic efflux pumps, and biofilm maturation (Antunes et al., 2010; Lyczak, Cannon, & Pier, 2000). Another bacterial species that uses QS to control virulence gene expression is *E. coli*. Transcriptomic studies in EHEC and enteropathogenic *E. coli* (EPEC) revealed that the global regulator LuxS controls the expression of over 400 genes including type-3 secretion system, the locus of enterocyte effacement (LEE) pathogenicity island and flagellar movement (Antunes et al., 2010; Sperandio, Mellies, Nguyen, Shin, & Kaper, 1999). Additionally, QS has proved important for bacterial virulence in *Salmonella enterica* serovar Typhimurium and *Francisella tularensis* (Rasko et al., 2008). Furthermore, biofilm formation is recognized to be a critical component of pathogenesis in several infectious diseases (Dolan & Costerton, 2002; Parsek & Singh, 2003). In essence, microorganisms possess a varied repertoire of virulence mechanisms that make them successful pathogens. Therefore, elucidation of the different virulence factors, their cellular targets in host cells, the underlying mechanism of action and their intricate regulation are critical to the development of targeted and efficacious therapeutic strategies to control and prevent infectious diseases, particularly in the era of antibiotic resistance.

3. MECHANISM OF ACTION OF PROBIOTICS—INDIRECT EFFECT ON HOST

The human intestinal microbiota is made up of trillions of microorganisms that are considered to be nonpathogenic and beneficial. This inherent microbiota helps in the development of normal intestinal morphology and functions with host defenses to maintain a continued and immunologically balanced inflammatory response (Belizário & Napolitano, 2015). It helps to reinforce the barrier function of the intestinal mucosa and protect against pathogen colonization and invasion. It also contributes to the body's metabolic requirements by acting as a source of essential nutrients and vitamins. Moreover, the beneficial microflora aid in the extraction of energy and nutrients, such as short-chain fatty acids (SCFAs) and amino acids from food (Krajmalnik-Brown, Ilhan, Kang, & DiBaise, 2012). Therefore, there is a huge interest to sustain, improve, or restore the healthy microbiota in diseased individuals through bacteriotherapy (bacteria-based therapy). The supplemented beneficial flora can thus act as "prebiotics" and stimulate the growth of part of the gut microbiota (Ohland & Macnaughton, 2010). Beyond promoting a healthy gut microbiome, supplementation with beneficial flora including probiotics

reinforces intestinal barrier function, thereby preventing the attachment of pathogenic microorganisms and the entry of allergens. This contributes to the enhancement of nonimmunologic gut defense barrier leading to altered gut microecology. Healthy intestinal microbiota thus contributes to the maturation of the immune system and the maintenance of balanced immune response.

Probiotics enhance the epithelial barrier function in numerous and diverse ways thereby promoting gut homeostasis. They limit bacterial movement across the mucous layer by increasing mucin expression and secretion by goblet cells (Ohland & Macnaughton, 2010). Beneficial microbes also enhance β-defensin production and secretion into the intestinal lumen thereby preventing the proliferation of pathogens (Howarth & Wang, 2013). Furthermore, probiotics decrease epithelial permeability to pathogens by enhancing tight junction stability. As indicated by several studies, the enhanced expression of genes involved in tight junction signaling is a possible mechanism to reinforce intestinal barrier integrity (Bermudez-Brito, Plaza-Diaz, Munoz-Quezada, Gomez-Llorente, & Gil, 2012). For instance, lactobacilli modulate the regulation of several genes encoding adherence junction proteins, such as E-cadherin and β-catenin and the abundance of protein kinase C (PKC) isoforms, which positively influence the epithelial barrier function (Anderson et al., 2010; Hummel, Veltman, Cichon, Sonnenborn, & Schmidt, 2012). Additionally, *E. coli* Nissle 1917 (EcN1917) was reported to initiate epithelial barrier repair and enhance mucosal integrity when exposed to EPEC (Zyrek et al., 2007).

Interaction of probiotics with host epithelial cells and the release of soluble factors trigger a signaling cascade leading to host immune modulation. These bacteria can directly interact with epithelial and dendritic cells (DCs), monocytes/macrophages, and lymphocytes (Bermudez-Brito et al., 2012). This immune stimulation results in increased secretion of immunoglobulin-A (IgA), elevated numbers of natural killer cells, and enhanced phagocytic activity of macrophages. The increased secretion of IgA, in turn, may decrease pathogenic organisms in the gut, thus improving the composition of the microflora (Fujimura, Slusher, Cabana, & Lynch, 2010). Probiotics and probiotic-derived products are detected by the intestinal membranous cells (M cells), presented to naive T cells which in turn activate the helper lineage of T cells namely T helper 1 (Th1), T helper 2 (Th2), or the T regulatory (Treg) cells. Activation of such T helper cells leads to the production of IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-13. Treg cells in turn secrete IL-10 and TGF-β to downregulate Th1 and

Th2 cells activities and aid in maintaining immune homeostasis in the intestine (Gill & Prasad, 2008, pp. 423–454). More interestingly, probiotics have also been shown to modulate the innate immunity in addition to the acquired immune responses. They enhance the phagocytic activity of peripheral blood leukocytes (Gill, 2003) and are found to upregulate the expression of phagocytic receptors to activate neutrophils (Gill, Rutherfurd, Cross, & Gopal, 2001; Pelto, Isolauri, Lilius, Nuutila, & Salminen, 1998). Additionally, probiotic bacteria have been shown to elicit nonspecific host resistance to microbial pathogens with a potential to downregulate hypersensitivity reactions (Isolauri, Sutas, Kankaanpaa, Arvilommi, & Salminen, 2001).

Furthermore, the effects of probiotics on digestion, nutrient absorption, and gut motility are documented in farm animals (Fioramonti, Theodorou, & Bueno, 2003). In humans, the vital role of probiotics in digestion is owed to their compensation for lactase insufficiency. The probiotic bacteria known to promote lactose digestion in the small intestine could alleviate the symptoms of undigested lactose in the gut (de Vrese et al., 2001). The de novo synthesis and supply of B and K vitamins can be stimulated by probiotic supplementation as well (Macfarlane & Cummings, 1999). Studies have also shown the ability of probiotics to increase intestinal fermentation thereby enhancing colonic peristalsis and high amplitude propagated contractions. The increased production of SCFA, a major end-product of bacterial fermentation, thus stimulate ileal propulsive contractions and release neuroendocrine factors and lower the intracolonic pH (Choi & Chang, 2015). Furthermore, these SCFAs have also been demonstrated to exert antimicrobial, antiinflammatory and anticancerous effects in the gut.

4. DIRECT EFFECTS OR ANTIVIRULENCE ATTRIBUTES OF PROBIOTICS ON PATHOGENS

In addition to the aforementioned protective effects in hosts, probiotics protect the host from infection by exerting direct antimicrobial effects on the pathogen. In general, the antimicrobial effect of probiotics is achieved through the production of antimicrobial substances such as bacteriocins, organic acids, and hydrogen peroxide. Furthermore, probiotics also modulate the virulence attributes of pathogens through several mechanisms, including colonization resistance, toxin neutralization, and disruption of QS. Although much is known about the ability of probiotics to promote host health, there is limited literature available on the aforementioned effect

of probiotics on pathogens. The following section of chapter will therefore focus on the ability of probiotics to directly modulate the infectious nature of pathogens and the underlying molecular mechanisms (Fig. 1).

4.1 Antibiosis Effects of Probiotics

Among the different desirable characteristics, the ability to produce antimicrobial products provides the probiotics with a competitive advantage over other pathobionts. The major groups of inhibitory products elaborated by beneficial flora are (1) organic acids such as lactic acid (2) metabolites like hydrogen peroxide and diacetyl, and (3) bacteriocins and microcins (Mishra & Lambert, 1996). Each of these compounds and particularly a combination of them can effectively kill pathogen both in the host and in food systems. The mechanisms of antibiosis of probiotics are listed in Table 1.

Lactic acid and acetic acids are major antibacterial organic acids produced by probiotics depending on the nutrient substrate and their metabolic profile. These acids mainly assert their bactericidal activity by lowering the pH. Low pH retards the growth of unfavorable microbes, reduces intracellular pH, and disturbs the transmembrane potential resulting in impaired cellular metabolism (Maloney, 1990). Hydrogen peroxide produced by lactobacilli is known to inhibit Gram-negative *Pseudomonas* and Gram-positive *S. aureus* (Mishra & Lambert, 1996). Hydrogen peroxide inactivates essential biomolecules by superoxide anion chain reaction and by oxidizing thiocyanate to release detrimental toxic oxidation products (Fernandes, Shahani, &

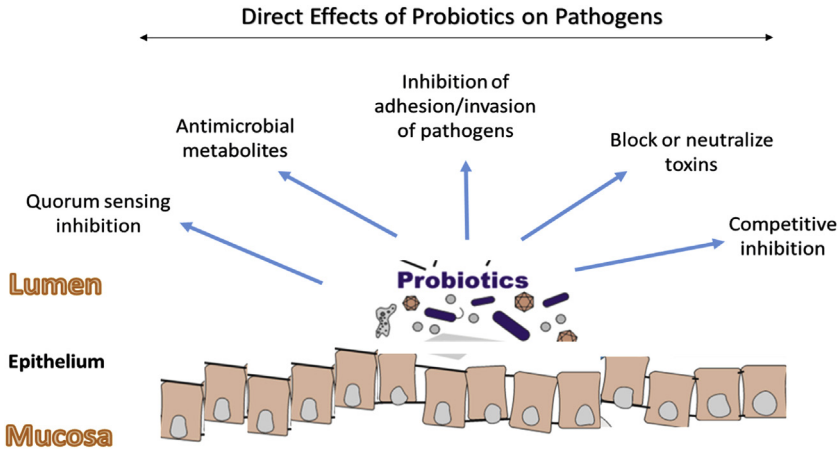

Figure 1 Anti-virulence property of probiotics.

Amer, 1987; Mishra & Lambert, 1996). For instance, the anti-*Salmonella* activity of *Lactobacillus johnsonii* and *Lactobacillus gasseri* was found to be due to their ability to produce hydrogen peroxide in the presence of oxygen (Pridmore, Pittet, Praplan, & Cavadini, 2008). Similarly, diacetyl produced by certain lactobacilli has been reported to interfere with arginine utilization, particularly in Gram-negative bacteria by binding to arginine binding proteins (Jay, 1982).

Bacteriocins and microcins are bioactive proteinaceous compounds identified as antimicrobial peptides that selectively kill and inhibit the growth of competing bacteria (Liévin-Le Moal & Servin, 2006). Bacteriocins are peptides produced by Gram-positive bacteria while Gram-negative bacteria elaborate microcins. They are ribosomally synthesized secondary metabolites with strain-specific molecular composition and narrow spectrum of activity (Cleveland, Montville, Nes, & Chikindas, 2001) (Table 2). Based on their structure and function, Klaenhammer (1993) broadly divided bacteriocins into different groups (Table 3).

Generally, bacteriocins permeabilize the cytoplasmic membrane of bacteria leading to the disruption of cell wall synthesis and formation of pores eventually leading to cell death (Duquesne, Destoumieux-Garzón, Peduzzi, & Rebuffat, 2007). For instance, bacteriocin ABP-118 produced by *Lactobacillus salivarius* strain UCC118 has been shown to inhibit the growth of *Bacillus*, *Staphylococcus*, and *Enterococcus* species. It has also been shown to protect mice against infection with the foodborne pathogen *L. monocytogenes* (Corr et al., 2007; Flynn et al., 2002). Similarly, a heat-resistant bacteriocin has been observed to inhibit the growth of *S. thermophilus* (Van de Guchte, Ehrlich, & Maguin, 2001). Certain lipophilic molecules produced by *Bifidobacterium* strains have also been shown to inhibit the viability of *E. coli*, *Klebsiella pneumonia*, *Y. pseudotuberculosis*, *S. aureus*, and *S.* Typhimurium (Lievin et al., 2000). Nisin is another heat stable, cationic lantibiotic exhibiting significant antimicrobial activity against a wide array of Gram-positive pathogens (Punyauppa-path, Phumkhachorn, & Rattanachaikunsopon, 2015).

Likewise, microcins from Gram-negative flora target the enzymes that are involved in biomolecule structure and synthesis, including DNA, RNA, and proteins (Duquesne et al., 2007). Microcins are bacteriocins of less than 10 kDa produced by *E. coli* and related enterobacteria through the ribosomal pathway (Rebuffat, 2012). Microcins J25 is a

Table 2 Classification of bacteriocins

Classification	Characteristics	Bacteriocins (class representatives)	Producing strain	References
Class I	Lantibiotics, small <5 kDa. Peptides containing lanthionine and β-methyl lanthionine	Labrynthopeptin	*Lactococcus*, *Streptococcus*, *Bacillus*, *Actinomadura*	Field et al. (2012), Meindl et al. (2010), and Yang, Lin, Sung, and Fang (2014)
Ia	Flexible molecules	Nisin		
Ib	Globular peptides	Mersacidin		
Class II	Heat stable, unmodified, nonlanthionine containing small peptides		*Pediococcus*, *Lactobacillus*, *Lactococcus*, *Carnobacterium*	Yang et al. (2014) and Tulini et al. (2014)
IIa	Pediocin (PA-1) like bacteriocin, synthesized in precursor form	Pediocin		
IIb	Two component systems: two different peptides required to form an active poration complex	Lactacin F		
IIc	Circular heat stable peptide	Carnocyclin A		
IId	Linear, nonpediocin like single peptide	Lactococcin A		
Class III	Large, heat unstable proteins	Helveticin J, Casecisin 80	*Lactobacillus helveticus*, *Lactobacillus caesei*	Joerger and Klaenhammer (1990)
Class IV	Complex bacteriocins containing lipid or carbohydrate moieties	Sublancin	*Bacillus subtilis* 168	Oman, Boettcher, Wang, Okalibe, and van der Donk (2011)

Table 3 Classification of microcins

Classification	Characteristics	Bacteriocins (class representatives)	Producing strain	References
Class I	Low molecular weight peptides (<5 kDa), post translationally modified	J25, B17, D93	*Escherichia coli*	Collin et al. (2013) and Yang et al. (2014)
Class II	Larger (5–10 kDa) peptides, with or without post translational modifications		*E. coli Klebsiella*	Corsini et al. (2010) and Vassiliadis, Destoumieux-Garzón, Lombard, Rebuffat, and Peduzzi (2010)
IIa	Require more than one gene to synthesize and assemble peptides	N/24		
IIb	Linear peptides with post translational modifications	E492		

classic example of microcins produced by several strains of *E. coli*. This antimicrobial peptide provides an efficient and subtle weapon that provides a competitive edge for probiotic *E. coli* to thrive within bacterial communities. It is a cytoplasmic membrane degrading peptide elaborated by probiotic *E. coli* known to kill several closely related bacteria (Rintoul, de Arcuri, Salomon, Farias, & Morero, 2001; Sable, Pons, Gendron-Gaillard, & Cottenceau, 2000).

4.2 Competition for Limiting Nutrients

The human gut is a prolific source of nutrients not only for the host but also for the millions of microorganisms that cohabit the gut. Hence, competition for nutrients plays an important role in shaping the gut microbial community. Studies interestingly suggest the requirement of only one nutrient can be limiting for outcompeting the invading bacterium. For instance,

competition for monomeric glucose, N-acetyl-glucosamine and sialic acid in the colon favors the elimination of pathogenic *Clostridium difficile* from the gut of healthy individuals (Fuller, 1991; Wilson & Perini, 1988). Similarly, host inflammation alters iron availability in the gut and *S.* Typhimurium thrives in an inflamed gut by efficiently scavenging for iron through the production of siderophores. However, administration of *E. coli* strain Nissle 1917 in a mouse model led to competition for iron between the probiotic and pathogen, thereby successfully reducing *S.* Typhimurium colonization (Deriu et al., 2013). Different lactobacilli are also able to bind ferric hydroxide at their cell surface rendering it unavailable to colonizing pathogens (Elli, Zink, Rytz, Reniero, & Morelli, 2000). *L. acidophilus* and *L. delbrueckii* are examples of such ferric oxide binding lactobacilli making iron unavailable to pathogens. The bound iron in turn plays a role in the pyrimidine and purine metabolism of lactobacilli (Elli et al., 2000).

4.3 Antiadhesive Effects (Colonization Resistance)

Adhesion to intestinal mucosa is regarded as a prerequisite for colonization in the case of both probiotic strains and pathogens. Competitive colonization of the host intestinal cells by probiotics is equally important for active modulation of the immune system and antagonism against pathogens (Bermudez-Brito et al., 2012). The induction of mucus-binding proteins and the competition for host cell surface receptors contributes mainly to the antiadhesive effects of the beneficial bacteria against pathogens. For instance, upregulation of MUC2 mucin protein and specific cytokines by selected lactobacilli was reported to inhibit the attachment of *E. coli* O157:H7 to intestinal epithelial cells (Kim, Kim, Whang, Kim, & Oh, 2008). On the other hand, Walsham et al. (2016) reported that exposure of human intestinal epithelial cells to mucin binding proteins (MUB) of *Lactobacillus reuteri* significantly inhibited adhesion of EPEC. MUB produced by *L. reuteri* increased the binding of the probiotic to intestinal cell lines, thereby competitively excluding EPEC adherence. Likewise, Collado, Grześkowiak, and Salminen (2007) showed that presence of *Bifidobacterium lactis* Bb12 and/or *Lactobacillus rhamnosus* LGG reduced the adhesion of pathogenic *Salmonella*, *Clostridium*, and *E. coli* strains to the intestinal mucus of swine. Similar studies conducted by Bernet, Brassart, Neeser, and Servin (1994) demonstrated that exposure of cultured human intestinal cell lines to four different *L. acidophilus* strains inhibited cell attachment and cell invasion by several enterovirulent bacteria (enterotoxigenic, diffusely adhering and

EPEC *Salmonella* Typhimurium, and *Y. pseudotuberculosis*). However, the strong antiadhesion activity of *Lactobacillus crispatus* JCM 8779 against *E. faecalis* observed was due to the combined effect of the antibacterial action and competition for the adhesion receptor (Todoriki, Mukai, Sato, & Toba, 2001). Other *lactobacillus* strains have been shown to compete with enterohemorrhagic *E. coli* O157:H7 for attachment to HT-29 cells and that with EPEC for attachment to mucus in pig ileum (Davidson & Hirsh, 1976; Mack, Michail, Wei, McDougall, & Hollingsworth, 1999). Interestingly, in vitro studies have also shown the potential of yeast probiotics like *Saccharomyces boulardii* in inhibiting the attachment of *E. histolytica* trophozoites to erythrocytes (Rigothier, Maccario, & Gayral, 1994).

Furthermore, degradation of carbohydrate receptors by secreted proteins, production of receptor analogs, and the induction of biosurfactants are other mechanisms by which probiotics prevent pathogen adherence. Antiadhesive biosurfactants are produced by several members of the genus *Lactobacillus*, for example, *L. acidophilus* ATCC 4356. Recently this biosurfactant in a coincubation study was found to interfere with the adhesion and biofilm formation of different *Serratia marcescens* strains (Shokouhfard, Kermanshahi, Shahandashti, Feizabadi, & Teimourian, 2015). Thus colonization resistance by competitive receptor binding can inhibit pathogen adhesion to host intestinal cells. This ability of probiotics to exert colonization resistance and competitively exclude pathogens is being utilized in the development of designer probiotics for controlling infections.

4.4 Antiinvasion Effects

Invasion of host epithelial cells is an important prerequisite for a pathogen to establish a successful infection. In addition to the production of various antibacterial substances that kill intracellular bacteria probiotics are also able to interfere directly with bacterial invasion of host tissue. For instance, the administration of *E. coli* Nissle (EcN) strain with *S.* Typhimurium was found to reduce the invasion efficiency of the pathogen by 70% (Altenhoefer et al., 2004). In the same study, the authors also reported the ability of EcN to inhibit invasion of several gut pathogens, including *Y. enterocolitica*, *Shigella flexneri*, *L. pneumophila*, and *L. monocytogenes* without affecting the viability of the pathogens. The interference with bacterial invasion by EcN was identified to be mediated by a secreted component produced by the probiotic. In another study, Schierack et al. (2011) investigated the inhibitory effects of EcN on *S.* Typhimurium invasion of porcine intestinal epithelial cells. They observed that EcN inhibited *Salmonella* invasion by modulating

Salmonella virulence gene regulation and *Salmonella* SiiE-mediated adhesion. Similar secreted factors released from *Lactobacilli* and *B. bifidum* were also reported to interfere with the invasion of host epithelial cells by *S.* Typhimurium (Botes, Loos, van Reenen, & Dicks, 2008). Likewise Moroni, Kheadr, Boutin, Lacroix, and Fliss (2006) identified bifidobacterial strains with increased potential for preventing listeriosis in humans.

4.5 Antitoxin Effects

Bacterial toxins are important virulence factors that mediate microbial pathogenesis. The antidiarrheal effect of probiotics in most cases is based on their ability to protect the host against such toxins. In vitro and in vivo experiments conducted by Asahara et al. (2004) demonstrated the ability of *Bifidobacterium* species to inhibit verotoxin production in EHEC. Interestingly, the high amount of acetic acid produced by Yakult strain was found to be responsible for this inhibition of toxin expression in vitro. Later in 2008, Carey et al. tested 15 different probiotics strains against EHEC toxin gene expression and observed that the SCFAs produced by the test strains downregulated Shiga toxin 2A gene expression. On the other hand, the probiotic yeast *S. boulardii* induces an antitoxin A IgA immune response and secretes a protease, which can destroy *C. difficile* toxin A within the host. This protective effect was confirmed in cell culture models and rat ileal loop assays (Castagliuolo, LaMont, Nikulasson, & Pothoulakis, 1996; Pothoulakis et al., 1993). Valdés-Varela, Alonso-Guervos, García-Suárez, Gueimonde, and Ruas-Madiedo (2016) identified the protective effect of *Bifidobacterium* and lactobacilli on the cytotoxic effect of *C. difficile* on human intestinal epithelial cells in vitro. Similarly, Trejo, Pérez, and De Antoni (2010) identified an antagonistic effect on the cell cytotoxicity induced by *C. difficile* when cocultured with beneficial probiotics. Furthermore, recently the ability of a protease secreted by the probiotic *Bacillus clausii* strain O/C was shown to counteract the cytotoxic effects induced by *C. difficile* and *Bacillus cereus* toxins (Ripert et al., 2016).

Inactivation of fungal toxins is also an antivirulence property exhibited by beneficial flora. The addition of dead or live *L. rhamnosus* GG to mycotoxin contaminated cereals was found to reduce the incidence of gastroenteritis. It was found that probiotics in the feed effectively bound to the mycotoxin and reduced its bioavailability (Turner et al., 2008). Further, in vivo experiments with *Lactobacillus* strains resulted in the increased fecal excretion of aflatoxin thereby alleviating liver injury. This was found to be due to the ability of the lactobacilli to bind aflatoxin, thereby reducing

their effective concentration in the blood and liver (Gratz, 2007). Furthermore, probiotics such as *S. boulardii* have the ability to modify toxin receptors in host cells, thereby reducing toxin binding and tissue cytotoxicity (Castagliuolo et al., 1996).

4.6 Inhibitors of Quorum Sensing

Bacteria rely on chemical communication or QS to coordinate cellular activities necessary for their survival and biofilm formation. Moreover, the entire coordinated expression of many virulence determinants during the infectious process is regulated by the QS system. Subsequently, several compounds with inhibitory activity against the QS systems have been identified and such compounds are termed as QS inhibitors. In this regard, organic acids secreted by probiotics are identified as QS inhibitors against various multidrug resistant *P. aeruginosa* and *S. aureus* strains (Ioana Cotar, Ionescu, et al., 2013; Ioana Cotar, Saviuc, et al., 2013). A real-time qPCR study conducted by Cotar et al. (2010) showed that in *P. aeruginosa* strains grown in the presence of sterile probiotic culture filtrates, the expression of QS genes was reduced compared to those in the control samples.

The antibiofilm properties of some probiotics against biofilm forming enteropathogens have also been evaluated with respect to their aforementioned antivirulence properties. Initially, Collado, Jalonen, Meriluoto, and Salminen (2006) observed that specific probiotic combinations were able to enhance the inhibition of pathogen adherence to intestinal mucus when compared to single probiotic strains. Similar studies by Kim and Kim (2009) demonstrated that exopolysaccharides released from *L. acidophilus* A4 drastically decreased enterohemorrhagic *E. coli* biofilm formation on polystyrene (87%) and polyvinyl chloride surfaces (94%) by downregulating genes critical to biofilm formation. Similarly, *L. acidophilus* La-5 was reported to secrete molecules that could acts as a QS signal inhibitor controlling the transcription of EHEC O157 genes critical for colonization (Medellin-Pena, Wang, Johnson, Anand, & Griffiths, 2007).

The antibiofilm effect of *L. pentosus* and *L. plantarum* cell-free supernatants against a foodborne pathogen (*B. cereus*) and plant pathogen (*P. aeruginosa*) was described by Khiralla, Mohamed, Farag, and Elhariry (2015). Similarly, Teanpaisan, Piwat, and Dahlen (2011) and Teanpaisan and Piwat (2014) as well as Vuotto, Barbanti, Mastrantonio, and Donelli (2014) found that Lactoba*cillus* SD1−SD6 and *L. brevis* exhibited an inhibitory effect

against biofilms formed by dental pathogens, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Prevotella melaninogenica*, respectively. Furthermore, the antiadhesive and antibiofilm properties of biosurfactants isolated from *L. jensenii and L. rhamnosus* were observed against clinical multidrug resistant strains of *Acinetobacter baumannii*, *E. coli*, and *S. aureus* by Sambanthamoorthy, Feng, Patel, Patel, and Paranavitana (2014). Thus, in addition to the beneficial effects exerted by probiotics on the host, direct inhibition of pathogen survival and virulence is critical for effectively controlling infections (Table 4).

Table 4 Examples of probiotic strains with antivirulence property

Probiotic strains	Inhibited pathogen	Activity	References
1 *Escherichia coli strain Nissle* 1917	*Salmonella Typhimurium*	Competition for iron, reduce the invasion efficiency of the pathogen	Deriu et al. (2013) and Altenhoefer et al. (2004)
2 *Lactobacillus reuteri*	*E. coli* O157:H7	Mucin binding proteins inhibited the adhesion	Kim et al. (2008)
3 *Bifidobacterium lactis* Bb12	*Salmonella*, *Clostridium*	Reduced the adhesion to intestinal cells	Bernet et al. (1994)
4 *Saccharomyces boulardii*	*Clostridium difficile*	Induces an antitoxin immune response	Pothoulakis et al. (1993), Castagliuolo et al. (1996), and Chen et al. (2006)
5 *Bifidobacterium*	*E. coli* O157:H7	Inhibits toxin gene expression	Carey, Kostrzynska, Ojha, and Thompson (2008)
6 *Lactobacillus acidophilus* A4	*E. coli* O157:H7	Inhibit biofilm formation	Kim and Kim (2009)
7 *Lactobacillus* SD1−SD6	*Porphyromonas gingivalis*	Reduces periodontal infections	Teanpaisan et al. (2011) and Teanpaisan and Piwat (2014)

5. CONCLUSION

Although beneficial microbes have been used for centuries in the manufacture of fermented foods, advances in research has greatly enhanced our understanding of their health benefits. Furthermore as we enter the postantibiotic era, there is an increasing trend for the use of probiotics as complementary and alternative medicine. An increasing body of documented evidence currently supports the potential application of probiotics in the prevention and treatment of infections. However, to fully exploit this alternative approach, a better understanding of probiotic biology and their interactions with the pathogen, and the host is warranted. In this regard, the availability of next generation technologies, refinement in techniques, and user-friendly data mining tools will help provide science-based evidence for the development of novel and effective probiotic-based biotherapeutics for the treatment and prevention of infectious diseases.

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CHAPTER TWO

Iron and Fungal Physiology: A Review of Biotechnological Opportunities

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Contents

Abstract

Iron is an essential inorganic micronutrient. Because of its low toxicity only a few studies have dealt with the importance of iron in fungal physiology. Most of the studies published so far focus on iron sequestration by animal fungal pathogens, iron uptake by mycorrhizal fungi, or iron redox activities by fungal wood degraders. However, a general overview on the relationship between fungal physiology and iron is still lacking.

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In this review we present a summary of the types of physiological activities that participate in iron homeostasis in fungi and how these activities can be used for the development of original biotechnological applications in relationship to iron-containing matrices. Concrete examples of biotechnological applications involving iron and fungi are also discussed. In the last part, a specific research project in biotechnology focusing on the use of fungi for the conservation of archaeological objects in iron is described in detail. This project aims at developing a new conservation-restoration method to preserve archaeological iron artifacts exploiting the ability of fungi to transform and uptake iron. Preliminary results obtained in this project regarding iron-reduction, iron uptake, and biogenic formation of iron minerals are presented and discussed.

1. INTRODUCTION

Nowadays, biotechnology has become a field of increasing interest. Researchers are developing sustainable and environmentally friendly technologies exploiting the physiological capabilities of plants and microbes to decrease the footprint of humans on earth. In this field fungi have a remarkable potential, as many of them exhibit a filamentous and modular structure called a mycelium in which they are able to translocate, accumulate, volatilize, and transform substances. Ultimately some compounds can also be transferred into macroscopic structures such as the carpophores formed in higher fungi. Due to these metabolic abilities, fungi are employed in several domains such as agriculture, pharmaceutical and food industries, bioremediation, and also more recently in conservation-restoration science (Broadbent, 1966; Feng, Poprawski, & Khachatourians, 1994; Joseph et al., 2011; Zouboulis, Matis, Loukidou, & Šebesta, 2003). For example, in the case of agriculture, *Beauveria bassiana* is currently used as a biological alternative to chemical insecticides (Feng et al., 1994). Besides this, arbuscular mycorrhizal fungi are reported as being able to improve the growth of plants in saline soils by increasing their tolerance to osmotic stress induced by salts (Feng et al., 2002). In addition, fungi can control the development of phytopathogens. For example, fungal endophytes isolated from the plant *Artemisia annua* are reported to have antifungal activities against phytopathogens such as *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum*, *Fusarium graminearum*, *Gerlachia nivalis*, and *Phytophthora capsici* (Liu, Zou, Lu, & Tan, 2001). In the pharmaceutical industry, since the discovery of penicillin, great attention has been given to the discovery of fungal antibacterial substances. In addition to those, research into the function of fungal extracellular polymeric substances (EPS) is starting to

emerge. In fact, these fungal metabolites are considered as high-value biomacromolecules with multiple applications (Mahapatra & Banerjee, 2013). For instance, experimental evidence demonstrates that EPS from *Aureobasidium pullulans* can be used as thickener in the production of biodegradable and edible plastics (Mahapatra & Banerjee, 2013; Paul, Morin, & Monsan, 1986). Moreover, fungal β-glucans are an EPS class studied in human health promotion for its ability to confer a protective behavior against mutation, microbial infections, and diabetes (Chen & Seviour, 2007; Mantovani et al., 2008). In addition, EPS are also employed as biosorbent matrixes in wastewater treatment because of their ability to chelate toxic metals like lead, zinc, and copper (Moon, Park, Kim, & Park, 2006; Yin, Hu, & Xiong, 2011). Finally, another fungal ability exploited to treat polluted water or soil is the biosorption of metals into fungal biomass (Gadd, 1993; Mali, Pandit, & Majumder, 2014; Singh, 2006, chap. 11). More recently, the production of biogenic oxalate minerals by the entomopathogenic fungus *B. bassiana* has been explored to develop a sustainable conservation-restoration method for copper-based alloyed artifacts (Joseph et al., 2011, 2013; Joseph, Simon, Mazzeo, Job, & Wörle, 2012).

Iron is an essential trace element in the microbial metabolism. It is an important cofactor of many enzymes (Howard, 1999) and it is essential during DNA synthesis (ribonucleotide synthetase) and cleavage (endonuclease III) (Matzanke, 1994). This metal is also crucial for some wood decay fungi since it is involved in the nonenzymatic degradation of lignocellulosic polysaccharides and lignin. In fact, Fe^{2+} is necessary to start the Fenton-based reactions leading to the production of oxygen-free radicals that cause the nonenzymatic degradation of wood components (Arantes, Milagres, Filley, & Goodell, 2011). Despite its ubiquitous involvement in physiology, microorganisms face a conundrum to obtain iron; the main reservoirs of this metal in nature are not bioavailable, and microbes, and in particular fungi, have developed several strategies to overcome this. Two of those strategies are the production of siderophores (Neilands, 1995; Philpott, 2006) and organic acid overexcretion (Fomina et al., 2005; Gadd, 2007). The multitude of roles of iron and its low environmental bioavailability explain the importance of this element in fungal physiology. Therefore, iron is often considered as a limiting factor for fungal growth and development.

Even if iron is an essential element for fungal physiology, at present the general knowledge of possible fungal reactions involving iron is incomplete. For instance, the role of filamentous fungi in the geochemical cycling of iron is still poorly documented (e.g., Kosman, 2003). To

develop new biotechnological approaches related to iron, the first step is to understand fungal abilities regarding this metal. Therefore, the aim of this review is to present an overview of the known fungal physiological capabilities in relationship to iron cycling and their possible application in biotechnology.

2. FUNGAL METABOLIC ACTIVITIES REGARDING IRON

2.1 Redox-Active Reactions

Fungal iron oxidation is a recurring process in soil due to fungal metabolic activity and the oxidative conditions present in this type of environment. Indeed, fungal dissolution of minerals produces free Fe^{3+} and Fe^{2+}: these ions are subsequently exposed to oxidative conditions due to the presence of H_2O and O_2, but also to the protons and oxidative exoenzymes produced by fungi (Guillén, Martínez, Gutiérrez, & Del Rio, 2005). This leads to a rapid oxidation of iron in soil near fungal biomass.

Little is known about fungal iron reduction activity. Some research, focusing on yeast and human pathogens, demonstrated the presence of an iron reductase and oxidase coupled with a permease that allows iron to penetrate into fungal cells (Kosman, 2003). However, reduced iron is a not only relevant during animal infection, but also in several other fungal metabolisms, such as wood degradation. The production of free oxygen radicals that allows the degradation of lignin and cellulose wood compounds is caused by the interaction between Fe^{2+} and H_2O_2. Even if the process is still not completely elucidated, a Fenton reaction-like model has been widely accepted. To start this reaction (Eq. 1), Fe^{2+} is required. Nevertheless, in soils and wood, the main fraction of available iron is in its trivalent oxidation state (Fe^{3+}). Therefore, to start the Fenton reaction, wood decay fungi have to be able to produce Fe^{3+}-reductive enzymes or low molecular weight Fe^{3+}-reducing compounds able to convert Fe^{3+} into Fe^{2+} (Shah et al., 2015).

$$Fe^{2+} + H_2O \rightarrow Fe^{3+} + \bullet OH + {}^{-}OH \quad (1)$$

Fungi can produce several compounds for this purpose, and a general enzyme or Fe^{3+}-reductant used by the majority of wood decay fungi does not exist. In most cases, these compounds are organic acids, phenol, or alcohols, and each fungus possesses its own set of Fe^{3+}-reductants (Arantes et al., 2011). Recently, this type of reactive molecule has also

been described in mycorrhizal fungi. In fact, Shah et al. (2015) demonstrated that the ectomycorrhizal fungus *Paxillus involutus* produces involutin, a molecule able to reduce Fe^{3+} during Fenton-based decomposition of organic matter.

Although free Fe^{2+} is required for the Fenton reaction and wood degradation, an excessive quantity of this ion can be detrimental. Indeed, excessive quantities of toxic oxygen-free radicals produced as result of the Fenton reaction can lead to degradation of the fungal biomass. To overcome this issue, fungi have developed several strategies to maintain the needed ionic equilibrium. Precipitation, complexation, adsorption (binding to cell walls), and active uptake are the most frequently described mechanisms (Gadd, 1993).

2.2 Uptake

As previously described, iron is an essential element for fungal growth, and the ability to acquire and accumulate this ion in their biomass allows fungi to survive under iron-limiting conditions. Fungi have developed different strategies to facilitate their access to insoluble iron. Two of the most relevant strategies are the production of low molecular weight organic acids (LMWOA) and the production of specific chelating compounds. Siderophores are an important family of the latter type of molecules able to chelate metals, which is produced under iron-limiting conditions (Neilands, 1995). Either of these mechanisms enhances fungal iron uptake, allowing fungi to compete more efficiently for this important limiting growth factor. However, once inside the cell, free intracellular iron may become extremely cytotoxic, and to avoid cellular damage, efficient immobilization and storage mechanisms are required. The mechanisms of iron immobilization involve adsorption in the cell wall and active uptake followed by intracellular iron storage. Those mechanisms will be presented in the following section.

2.2.1 Adsorption

The first site of direct interaction between fungi and metals is the cell wall. The fungal cell wall is a multilayered structure composed of chitin, β-1,3/1,6-glucan, and an external layer of mannoproteins that offers the fungal cell both a mechanical and a chemical protection (Eisenman & Casadevall, 2012). Under stress conditions, some fungi produce also melanin, which can be synthetized within the cell wall (over the layer of mannoproteins) or produced extracellularly (Gadd, 1993; Philpott, 2006). Due to its complex composition, the cell wall can act as a protective barrier against

toxic metals. Indeed, it is widely recognized that melanin, mannoproteins, and chitin have metal-binding properties (Gadd, 1993; Philpott, 2006) that allow fungi to adsorb not only toxic metals, but also beneficial ones such as iron.

Melanin is a fungal pigment, synthetized in response to different stress conditions such as ultraviolet radiation (Eisenman & Casadevall, 2012). Furthermore, it has been shown that the presence of copper in the culture medium of *Gaeumannomyces graminis* is associated with an enhancement of the melanin content in the cell wall (Caesar-Tonthat, Van Ommen, Geesey, & Henson, 1995). In another study, it was demonstrated that the melanin from *A. pullulans* has the ability to bind significant amounts of copper as well as iron (Gadd & de Rome, 1988; Senesi, Sposito, & Martin, 1987). Chitin and chitosan are also considered to be important metal biosorbent molecules, binding several metals on their amine nitrogen groups (Gadd, 1993). Experiments conducted by Franco et al. (2004) demonstrate that chitin has also the ability to chelate iron.

The diversity of molecules involved in metal sorption in the fungal cell wall has a clear biotechnological interest. Metal biosorption abilities of fungi have been studied to decontaminate water and soil from toxic metals. Experimental evidence demonstrates that both living and dead biomass can be exploited as bio-sorbent and the efficacy of this approach has been proven also for a mixed solution of several toxic metals (Bishnoi & Garima, 2005; Mali et al., 2014; Paknikar, Puranik, Agate, & Naik, 1998). Despite the fact that toxic metals have received more attention, cell wall compounds are also reported to be able to bind iron (Murugesan, Sathishkumar, & Swaminathan, 2006). Therefore these adsorption mechanisms have to be considered when studying fungal interactions with iron not only in vitro but also in the environment (e.g., in soils).

2.2.2 Active Uptake

While the mechanisms described previously are based on the passive adsorption of metals in the fungal cell wall, fungi also display active iron uptake, which is metabolism dependent and occurs only in living cells (Bishnoi & Garima, 2005; Kosman, 2003; Philpott, 2006). In biosorption by living biomass, various parameters such as pH of the culture, temperature, composition of the growth medium, and especially culture age, all influence metal uptake. Experimental evidence suggests the active uptake of Cu, Cd, Ni, Zn, Co, Mn, Sr, Mg, and Ca. Active uptake of iron has been widely studied in yeast and pathogenic fungi, as this is an important virulence factor of

human pathogens (Ruddat et al., 1991). *Saccharomyces cerevisiae* has been reported as being able to retain a substantial amount of iron in the cell wall and in the periplastic environment (Philpott, 2006). Experimental evidence suggests that under iron-depletion conditions this yeast overexpresses genes for the synthesis of mannoproteins responsible for the retention of siderophore-iron complexes in the cell wall, called facilitator of iron transport proteins (Philpott, 2006; Protchenko et al., 2001). Less information is available in the case of filamentous fungi. The mechanisms described until now are (1) extracellular reduction of Fe^{3+} and uptake via a ferrous permease; (2) extracellular oxidation of Fe^{2+} and uptake via a ferric permease; (3) uptake of Fe^{3+} and Fe^{2+} through a ferric and ferrous permease respectively; and (4) uptake of Fe^{3+}-siderophore complexes via a siderophore permease (Kosman, 2003; Philpott, 2006) (Fig. 1).

Due to its high reactivity, iron is rarely found free inside the cell. Fungi have developed several iron storage molecules for intracellular storage similar

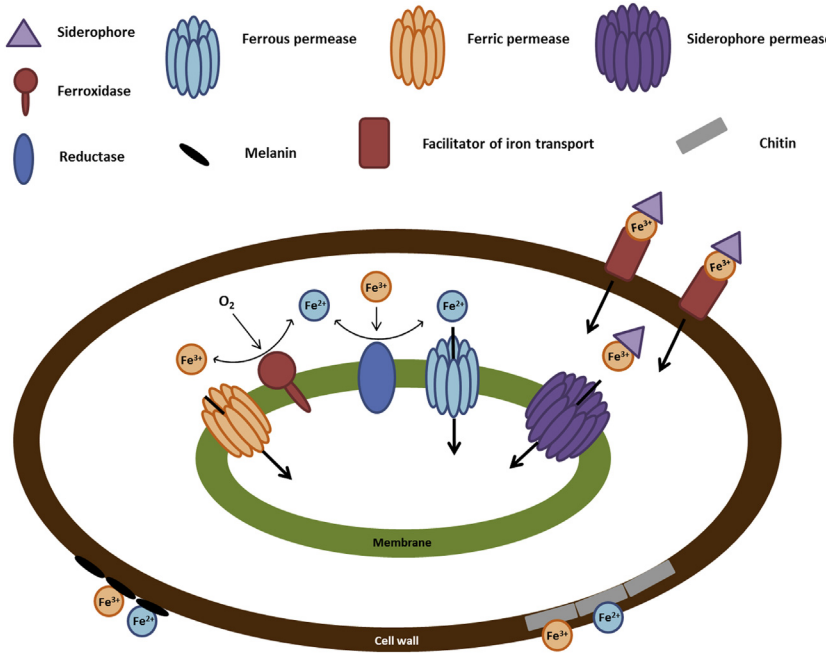


Figure 1 Schematic representation of possible mechanisms of iron uptake in fungi. *Adapted from Kosman, D. J. (2003). Molecular mechanisms of iron uptake in fungi.* Molecular Microbiology, 47, *1185–1197 and Philpott, C. C. (2006). Iron uptake in fungi: a system for every source.* Biochimica et Biophysica Acta, 1763, *636–645.*

to siderophores, as well as proteins such as ferritins and metallothioneins, and also polyphosphates (Howard, 1999; Matzanke, 1994).

2.2.2.1 Siderophores

Siderophores are low molecular weight Fe^{3+} chelating molecules produced under iron-limiting conditions (Neilands, 1995). With a few exceptions fungal siderophores identified so far are of the hydroxamate-type and can be classified into three structural families: fusarinines, coprogenes, and ferrichromes (Renshaw et al., 2002). Iron acquisition by siderophores is an energy-dependent mechanism that can occur via four different ways: (1) shuttle transport mediated by penetration of the iron—siderophore complex in the fungal cell and cleavage of these two compounds by an intracellular reductase; (2) taxicab transport mediated by the production of a siderophore that chelates iron and remains outside the membrane, while iron is transferred through ligand exchange to an internal siderophore pool (Renshaw et al., 2002; Van der Helm & Winkelmann, 1994); (3) hydraulic acquisition mediated by the penetration of the iron—siderophore complex into the fungal cell and subsequent cleavage of the complex by intracellular reductive and degradative reactions (Renshaw et al., 2002; Van der Helm & Winkelmann, 1994); and (4) reductive acquisition mediated by the production of a siderophore that chelates iron and remains outside the fungal cell, followed by iron reduction near the membrane and uptake of Fe^{2+} (Renshaw et al., 2002; Van der Helm & Winkelmann, 1994).

For some species, siderophore production is an essential virulence factor that regulates pathogenicity. In fact, mutants of the plant pathogen *Microbotryum violaceum* producing a reduced amount of siderophores showed lower or no pathogenicity (Ruddat et al., 1991). Interestingly, it has been reported that fungi unable of producing siderophores have the ability to use these molecules when secreted by other microbes (Howard, 1999). The use of heterologous siderophores by nonpathogenic fungi can then suppress the growth of phytopathogenic fungi, and fungi able to do this are then reported to be beneficial for plant health (Campbell, Renwick, & Coe, 1986; Renshaw et al., 2002). There is also evidence that plants can exploit microbial siderophores to obtain iron (Crowley, Reid, & Szaniszlo, 1988).

The metal chelating capabilities of siderophores are studied and exploited in many biotechnological fields such as medicine, reprocessing of nuclear fuel, remediation of metal contaminated fields, and treatment of industrial waste compounds. These possible applications are described exhaustively (Renshaw et al., 2002). Even if siderophores are considered to be one of

the most important mechanisms developed to acquire iron under limiting conditions, they also play an important role in intracellular iron storage in fungal cytoplasm and spores (Winkelmann, 1992).

2.2.2.2 Proteins

Ferritin-like molecules are a broad superfamily of iron-rich proteins used for iron storage in different organisms. Three types are reported in the literature: (1) ferritin; present in animals (Carrano, Böhnke, & Matzanke, 1996), (2) phytoferritin; present in plants, and (3) bacterioferritin found in bacteria. These types of iron-rich proteins are reported to be present only in Zygomycetes. Three types of ferritin-like molecules have been discovered in fungi: (1) mycoferritin, similar to ferritin present in animals; (2) zygoferritin, found only in Zygomycetes; and (3) bacterioferritin, discovered in *Absidia spinosa* (Carrano et al., 1996). Other proteins able to bind metals in the fungal cytosol are metallothioneins and phytochelatins, two families of low-molecular weight cysteine-rich proteins containing sulfur-based clusters able to bind metals (Bellion, Courbot, Jacob, Blaudez, & Chalot, 2006; Gadd, 1993; Kameo, Iwahashi, Kojima, & Satoh, 2000).

2.2.2.3 Other Molecules

Polyphosphates are molecules present in many microorganisms. Their role is still not clear but there is some evidence suggesting that they could play a role in the storage of Fe^{2+}. Experimental evidence reveals accumulation of other divalent cations bound to polyphosphates in microbes. For instance, Ca^{2+} in yeast (Ashford, Vesk, Orlovich, Markovina, & Allaway, 1999), Mn^{2+} in the bacterium *Lactobacillus plantarum* (Archibald & Fridovich, 1982), and Al^{2+} in the cyanobacterium *Anabaena cylindrica* (Pettersson, Kunst, Bergman, & Roomans, 1985). Böhnke and Matzanke (1995) suggest that polyphosphates could play the role of Fe^{2+} storage molecules in *Escherichia coli*. It can then be supposed that polyphosphates may be used as iron storage molecules in the fungal vacuole as well.

2.3 Translocation

Fungal translocation is a phenomenon frequently discussed in relationship to fungal nutrient cycling. Indeed, it is widely recognized that fungi have the ability to transport carbon sources from the substratum to another distal location through their mycelial network. Through the use of radioactive isotopic tracers several studies have demonstrated the ability of fungi to translocate phosphorus (Lindahl, Finlay, & Olsson, 2001) or amino acids

(Tlalka, Watkinson, Darrah, & Fricker, 2002). This ability allows fungi to colonize environments with low resource availability. Translocation has been exhaustively described by Lindahl and Olsson (2004). Translocation and accumulation of toxic metals from polluted soils into the fruiting body of edible fungi is a well-known mechanism that can cause serious human health issues (Brown & Hall, 1990; Gadd, 1993). Even if metal translocation through the mycelial network has rarely been proved in vitro, the accumulation of heavy metals in the fruiting body demonstrates that fungi have the ability to translocate these. Studies by Furuno et al. (2012) and Schamfuss et al. (2013) proved the translocation of toxic polycyclic aromatic hydrocarbons (PAH) in the fungal-like mycelial network of the oomycete *Pythium ultimum*. These researchers suggested that translocation of PAH pollutants could be an interesting ability to improve degradation of toxic substances by increasing the dispersal and the bioavailability of the pollutant into the soil matrix to enhance bacterial degradation.

Further work is still required to elucidate the mechanisms and the molecules involved in the translocation of iron. Nonetheless, fungal iron translocation can be used to provide trace elements to plants. For example, a microcosm experiment has demonstrated that the arbuscular mycorrhizal fungus *Glomus mosseae* is able to mobilize, uptake, and translocate iron from soil to the shoots of sorghum plants (Caris, Hördt, Hawkins, Römheld, & George, 1998). That study shows that fungi are also able to translocate beneficial trace elements such as iron and not only amino acids or toxic metals.

2.4 Solubilization/Weathering

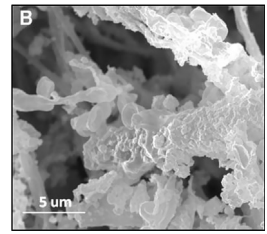
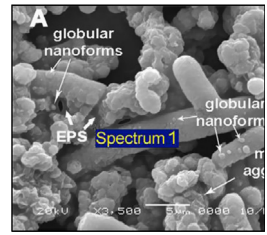
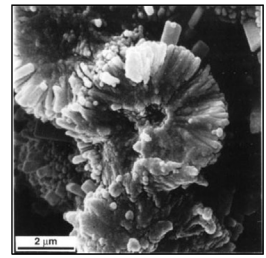
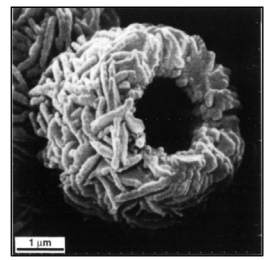
In natural environments, iron bioavailability is poor. To overcome this issue, fungi have developed different strategies to acquire this element from insoluble minerals. The first is the enhancement of mineral solubilization via acidification of the environment near their mycelial network. This occurs either by proton secretion or by producing extracellular LMWOA (Ehrlich, 2006; Fomina et al., 2005; Gadd, 1993, 1999). LMWOA are important extracellular metabolites that promote the solubilization of unavailable ions from mineral substrata by decreasing the pH of the microenvironment, as well as by chelating the ions released. This is one of the reasons why fungi are considered important geological weathering agents (Sterflinger, 2000). Several types of fungal organic acids have been reported: citric acid, gluconic acid, itaconic acid, L-lactic acid, fumaric acid, L-malic acid, and succinic acid. However, the most representative is

oxalic acid (Magnuson & Lasure, 2004). This compound is also considered as an important virulence factor, since it is responsible for host tissue acidification and sequestration of important ions like Ca^{2+}, Mn^{2+}, Mg^{2+}, and $Fe^{2+/3+}$ (Godoy, Steadman, Dickman, & Dam, 1990). Finally, citric and oxalic acid have also the property to chelate divalent and trivalent metals (Fomina et al., 2005; Gadd, 1993, 1999). The ability of several fungi to produce organic acids with chelating properties is considered an important extracellular resistance mechanism against heavy metal toxicity. Indeed, as a result of the interaction between oxalic acid and cations, crystals of metal oxalates will be produced. This important property allows fungi to decrease the concentration of toxic metals in solution producing secondary biogenic minerals, like metal-oxalates (Gadd, 1993).

2.5 Biogenic Mineral Production

A direct consequence of fungal solubilization mechanisms such as organic acid production and their interaction with the environment (soil, rock, wood, animal, or vegetal tissues) is the precipitation of secondary biogenic minerals. Bacterial iron mineral production is a domain studied in detail. Since bacteria are able to grow under both aerobic and anaerobic conditions they are able not only to produce Fe^{3+} minerals, such as hematite and goethite (Frankel & Bazylinski, 2003) but also, as a consequence of iron reduction, Fe^{2+} minerals like magnetite, siderite, pyrite, and vivianite, among others (Frankel & Bazylinski, 2003). Biogenic production of iron minerals by fungi is less studied probably because of their supposed inability to grow under anaerobic conditions. In fact, iron chemistry in an aerobic system is circumscribed (Kosman, 2003). Fe^{2+} rapidly oxidizes to its trivalent state in contact with the oxygen contained in the atmosphere. This Fe^{3+} further hydrolyzes, producing insoluble ferric hydroxide species. However, fungi usually induce drastic changes in the microenvironment near their hyphal network (Robson et al., 1996). For instance, a modification of pH is an essential environmental factor influencing the redox state and the stability of iron ions, as well as its solubility. In fact Fe^{2+}, which is stable under anaerobic conditions, is also stable under alkaline conditions (Selwyn, 2004). Nevertheless, few studies have focused on fungal biogenic formation of iron minerals. However, experimental studies have provided evidence for the production of iron oxalate (FeC_2O_4) (Joseph et al., 2011), iron oxi-hydroxides such as hematite (Fe_2O_2) and goethite ($\propto$-FeO(OH)) (Feldmann, Neher, Jung, & Graf, 1997) and sulfates like jarosite ($KFe_3(OH)_6(SO_4)_2$) (Oggerin et al., 2013) (Table 1).

Table 1 Type and scanning electron microscopy image of biogenic iron minerals produced by several fungi in different environments

Fungal species	Biogenic mineral	Electronic Microscopy	References
Beauveria bassiana	Iron oxalate $FeC_2O_4 \cdot 2\,H_2O$		Joseph et al. (2011)
Purpureocillium lilacinum	Jarosite $KFe_3^{3+}(SO_4)_2(OH)_6$		Oggerin et al. (2013)
Unidentified basidiomycete	Goethite (α-FeO(OH))		Feldmann et al. (1997)
Unidentified basidiomycete	Hematite (Fe_2O_3)		Feldmann et al. (1997)

2.5.1 Iron Oxalates

The most studied secondary weathering products are metal oxalates. The interaction between oxalate anions and divalent and trivalent cations leads to the formation of metal oxalates. Fungi are reported to be able to produce

oxalate salts mainly with calcium, copper, magnesium, and strontium. However, many other oxalate minerals exist (Fomina et al., 2005; Gadd, 2007). For instance, iron oxalate crystals have been found on sheeted hyphae of the entomopathogenic fungus *B. bassiana* (Joseph et al., 2011) (Table 1). However, the stability of these crystals is not completely understood. Indeed, Eckhardt (1985) and Varadachari, Barman, and Ghosh (1994) reported that oxalic acid produces insoluble salts only reacting with divalent cations such as Ca^{2+} or Cu^{2+}, while oxalates from the reaction with trivalent cations result in soluble salts. Experimentally, it is suggested that only Fe^{3+} is able to react with oxalic acid (Eick, Grossl, Golden, Sparks, & Ming, 1996), and this could be the reason why fungal iron oxalate is rarely found in nature, even though fungi produce high amounts of oxalic acid and iron is the most abundant metal on Earth. Further research is required to better understand fungal iron oxalate production and the stability of the resulting minerals.

2.5.2 Iron Oxides and Oxyhydroxides

Poorly ordered iron oxides and oxyhydroxydes are frequently reported in association with lichens and are the result of the primary oxidation of iron-bearing rocks induced by the production of organic acids by fungi. The subsequent release and hydrolysis of Fe^{2+} and Fe^{3+} leads to the precipitation of secondary biogenic minerals on rock surfaces (Eick et al., 1996; Siever & Woodford, 1979). An example of this mechanism has been described by Adamo, Colombo, and Violante (1997). These authors show the presence of poorly crystallized ferrihydrite in the interaction zone between a volcanic rock and the thallus of *Stereocaulon vesiuvianum*. Fungal erosion of a quartzite deposit in a Swiss Alpine environment has been studied by Feldmann et al. (1997). Their work revealed the presence of crystalline goethite and hematite spherulites around fungal hyphae produced by an unidentified basidiomycete (Table 1).

2.5.3 Iron Sulfates—Jarosite

Recently, Oggerin et al. (2013) were able to isolate a strain of the fungus *Purpureocillium lilacinum* from the acidic environment of Río Tinto. An indepth study of its biomineralization abilities revealed that this fungus was able to produce biogenic jarosite (Table 1). Well-crystallized jarosite was produced by living and dead fungal biomass. The biomineralization process started in both cases in the cell wall, at the outer part of the fungal cell. However, living and dead fungal biomass performed differently in terms

of biomineralization efficiency. Indeed, only 0.5 g/L of jarosite was produced in cultures inoculated with dead cells compared to 2 g/L in living cultures. The authors explain this difference by the presence of EPS associated with living fungal biomass. They conclude that jarosite formation is a process independent from active metabolism and that requires fungal biomass and EPS as nucleation sites. The biogenic mineral formation starts in the fungal cell wall and EPS probably enhances the cohesion of newly formed jarosite crystals, allowing the formation of larger slime-like aggregates (Oggerin, Rodríguez, del Moral, & Amils, 2014; Oggerin et al., 2013).

3. IRON AND BIOTECHNOLOGY

During the last three decades, increasing attention has been dedicated to the development of fungal biotechnologies. The production of nanoparticles of silver, gold, nickel, and other metals has caught the attention of scientists (Pantidos & Horsfall, 2014). A large quantity of secondary fungal metabolites with interesting properties is produced and studied for their pharmaceutical relevance such as antibiotics, photoprotectors, and antioxidants (Leung, Zhao, Ho, & Wu, 2009; Moliné et al., 2010). Purified fungal compounds are employed as colorants and flavors in the food and textile industries (Mapari et al., 2005; Raisanen, 2009). In this section known or potential biotechnological applications of fungal metabolic activities regarding iron will be discussed.

3.1 Biosorption

Even though iron is not considered a conventional toxic metal, high amounts can lead to a reduction of soil fertility and human health issues. In fact, the maximum concentration of iron recommended by the Food and Agriculture Organization of the United Nations (FAO) in irrigation water is 5 mg/L. A higher amount of iron is not toxic to plants in aerated soils, but can contribute to soil acidification and a loss of availability of essential nutrients such as phosphorus and molybdenum (Board, 1972, p. 244). For drinking water 0.3 mg/L is the limit recommended by the FAO to avoid staining of laundry and sanitary ware. Indeed, studies revealed that an excessive accumulation of iron in the body could cause hemorrhagic necrosis and sloughing of areas of the mucosa in the stomach. The lethal dose for humans is 200–250 mg/kg of body weight, but death has occurred following the ingestion of doses as low as 40 mg/kg of body weight (World Health Organization, 2004). Sources of iron contamination of water are mainly in industry, metallurgic manufacturing, mining, and in some cases

the iron-based flocculants used in waste water treatment plants. Often in these cases, iron is not the only metal pollutant present in water, and studies have focused on the use of dead fungal biomass to remove metals in a nonselective way (Ahluwalia & Goyal, 2007; Mali et al., 2014; Zouboulis et al., 2003). For instance, experimental evidence demonstrated arsenic and iron removal abilities of autoclaved fungal biomass from contaminated groundwater. 100% of As(III), As(V), and Fe(II) was removed by dead fungal biomass after 30 min of contact (Murugesan et al., 2006).

3.2 Bioremediation of Asbestos Using Fungi

Asbestos is a group of minerals composed of fibrous silicates producing hazardous airborne particles. Reaching the respiratory system they can cause pulmonary fibrosis, pleural or peritoneal mesothelioma, and lung carcinoma. The toxicity of these fibers relies on the iron associated with asbestos that produces free hydroxyl radicals and consequently DNA damage (Daghino et al., 2006; Fubini, 1997; Hardy & Aust, 1995; Weitzman & Graceffa, 1984). Due to this, the use of asbestos has been banned in several countries, but this compound is still present in contaminated soils or near abandoned mines. Remediation strategies involving soil removal are not suitable in this case because of the production of airborne fibers of asbestos. Therefore in situ treatments involving fungi have been studied to bioremediate these contaminated environments. Experimental evidence shows that iron removal with fungal organic chelators and siderophores decreases the toxicity of these compounds (Chao & Aust, 1994; Martino et al., 2003; Weitzman & Weitberg, 1985). A study by Daghino et al. (2006) demonstrated that *Verticillium* sp. and *Paecilomyces* sp. were able to remove iron from asbestos fibers. They demonstrated through in vitro tests of DNA damage, that fungal-treated asbestos causes less DNA damage effects. Their study concludes that fungi can be appropriate candidates to develop bioremediation strategies for asbestos-contaminated environments by reducing the amount of iron present in this compound.

4. CASE STUDY: FUNGI TO THE RESCUE OF ARCHAEOLOGICAL IRON

During the last decade, fungal biotechnology has been successfully applied in the field of conservation-restoration of metallic artifacts (Joseph et al., 2011, 2012, 2013). In this section, a specific project aiming to develop sustainable and eco-friendly conservation-restoration method for archaeological iron artifacts with fungi will be presented.

Iron artifacts are among the most recurring items found in archaeological findings. Swords, utensils, and armor are testimony to ancient human activity and allow us to understand the habits of a distant past. Archaeological iron objects during their burial period develop a corrosion layer according to the composition of their surrounding environment. Different parameters can then influence the composition of the corrosion layer, for instance, pH, water flow, and the chemical composition of soil (Neff, Reguer, Bellot-Gurlet, Dillmann, & Bertholon, 2004). After excavation the conditions leading to the formation of the oxidation layer change and the corrosion compounds are no longer stable. Therefore, archaeological iron artifacts are extremely fragile and need to be rapidly treated after their discovery. Indeed, severe corrosion would, without intervention of conservation-restoration, lead to a complete loss of shape resulting in impossible interpretation and recognition. The main problematic compound for archaeological iron objects is chlorine. In fact, chlorine can cause chemical degradation though the production of hydrochloric acid (HCl) (Selwyn, 2004). Moreover, acidic Fe^{2+}-containing chlorides exposed to air oxidize producing oxyhydroxide species. These corrosion products are three times more voluminous than the original compounds and create mechanical pressure, thus producing cracks and finally the breakdown of the artifact. Therefore, archaeological iron objects need to be rapidly treated after their excavation to remove chlorine and stabilize the corrosion layer.

Traditional conservation-restoration methods employed to stabilize archaeological iron objects are principally three, and they focus on a passive removal of chlorine by increasing the porosity of the corrosion layer (Selwyn, 2004). The first desalination method consists of immersion of the object in anoxic aqueous solutions of alkaline sulfide to dissolve out the free chloride ions (Scott & Eggert, 2009, chap. 12; Selwyn, 2004). During this treatment part of the iron oxyhydroxides is converted to magnetite, increasing the porosity of the corrosion layer and allowing a faster diffusion of the chlorine from the objects to the alkaline solution (Selwyn, 2004). However, the alkaline sulfide solution needs to be changed regularly and at the end of the treatment, the used solution requires specific waste neutralization. Moreover, the effective chlorine removal is difficult to assess without destructive analysis (Rimmer, Wang, Watkinson, Eggert, & Schmutzler, 2010; Selwyn, 2004). Another desalination method is electrolytic reduction that consists of immersion of the artifacts (cathode) in an alkaline electrolyte containing an electrode (anode). The voltage that is then applied causes the reduction of the Fe^{3+} oxyhydroxides into magnetite

or Fe^{2+}-containing compounds, increasing the porosity of the corrosion layer and enhancing chlorine diffusion (Selwyn, 2004). The main disadvantage of this treatment is that it has to be carefully monitored because it causes the production of extremely explosive H_2 by the electrolysis of water. Moreover, the production of bubbles of H_2 can also lead to uncontrolled surface damage (Selwyn, 2004). Therefore, this method is exploitable only for large marine findings (Scott & Eggert, 2009, chap. 12). Finally, reactive hydrogen plasma is also used to reduce the corrosion compounds to a lower oxidation state. However, to prevent the alteration of the metallurgical structure, the temperature is maintained below 400°C during the treatment. Due to the fact that experimental evidence proved that chlorine removal is not effective at this low temperature (Schmidt-Ott, 1997), plasma treatment is usually employed only as a pretreatment before alkaline sulfide desalination since it causes cracks and fissures improving the diffusion of chloride ions (Scott & Eggert, 2009, chap. 12). Considering the caveats of the current methods of conservation-restoration of archaeological iron objects, there is a pressing need to develop novel methodologies that could improve chlorine removal from the objects either indirectly by reducing the corrosion layer or directly by active chlorine removal.

4.1 Biopassivation on Iron Objects

To develop an eco-friendly conservation-restoration method for archeological iron artifacts, Joseph et al. (2011) studied the biogenic mineral formation abilities of the fungus *B. bassiana*. That study aimed at producing a protective layer of iron oxalate on the surface of the objects through the conversion by the fungus of the existing corrosion products into less soluble and more stable compounds. In solid media culture amended with different iron sources, no crystal formation was observed. However, in liquid cultures, hyphae on the surface of immerged objects were completely encrusted with biogenic oxalate crystals as detected by optical microscopy and Raman spectroscopy. It was concluded that *B. bassiana* could be considered a good candidate for the development of new conservation-restoration methods for archaeological iron artifacts.

4.2 Microbes for Archaeological Iron Artifacts: the MAIA Project

A related study with the same goal was initiated in 2013. The project called MAIA (Microbes for Archaeological Iron Artifacts) aims at developing innovative and sustainable conservation-restoration methods to preserve

archaeological iron artifacts with fungi and bacteria. As illustrated in Fig. 2 this project proposed two different strategies. The first, designated direct chlorine extraction, focuses on direct removal of chlorine using fungi (Fig. 2A). The second strategy, indicated as indirect chlorine extraction, focuses on the conversion of chlorinated iron corrosion products into more stable compounds and the consequent osmotic diffusion of chlorine out of the objects (Fig. 2B). Promising fungal interactions with iron were then studied and the following paragraphs give an overview of the results. The main structure and the principal tasks of the indirect chlorine extraction are presented in Fig. 2C.

Iron, in the presence of oxygen and water, is exposed to severe oxidation. However optimal fungal growth generally requires oxygen and high relative humidity. Hence, as iron corrosion can be avoided by using an alkaline solution (Selwyn, 2004), a new fungal treatment has to be conducted under alkaline pH. To meet this criterion, the first step was to identify fungal strains that are able to grow under alkaline conditions. In addition, the second requirement for an organism used for the development of a new restoration method is halotolerance, as the removal of iron leads to a concomitant release of chlorine with a consequent increase of the chlorine concentration in the treatment solution. A screening of selected strains with diverse ecologies (Table 2) was performed on solid malt extract (1.2%) agar (1.5%) (MA) medium buffered at pH 9.2 with ($NaHCO_3$ 0.765% and $NaCO_3$ 0.106%). Additional experiments were carried out on solid MA medium amended with 50 mM of NaCl. This concentration was selected according to literature suggesting that archaeological iron objects contain an average of 50 mM of chlorine (Rimmer, Watkinson, & Wang, 2012).

Nine fungal strains were able to grow under alkaline conditions and to tolerate 50 mM NaCl (Table 3). In these nine strains, iron reduction was studied in 1.2% malt extract liquid cultures amended with 10 mM $FeCl_3$ and buffered at pH 9.2. Fe(II) concentration was measured with the Ferrozine reagent (Bell, Mills, & Herman, 1987). *Agrocybe* sp. had the ability to reduce iron (Table 3), which was probably due to the production of extracellular reductive enzymes or Fe^{3+} reductant compounds. Indeed, this fungus is able to degrade lignocellulose through the Fenton reaction mechanism, which involves such iron reducing molecules (Arantes et al., 2011). Once isolated, these substances could be used for proposing a biological treatment that does not involve living microorganisms. This could avoid the sometimes fastidious supply of all the factors affecting fungal growth such as a carbon source, moisture, and pH. Reduction of the chlorinated iron

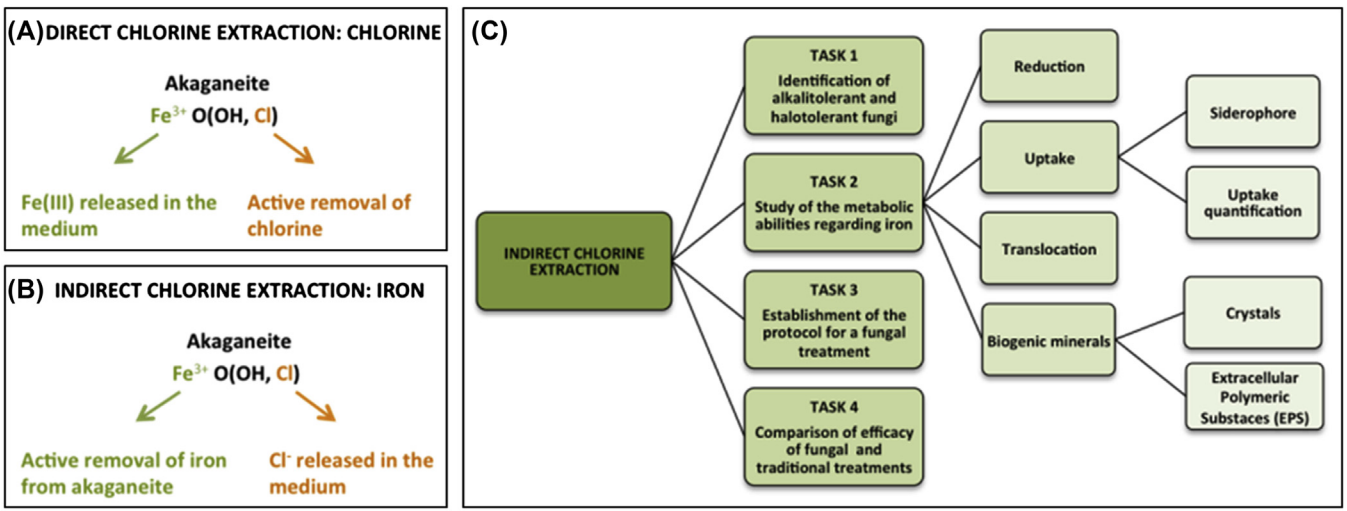


Figure 2 Schematic representation of the Microbes for Archaeological Iron Artifacts project: (A) direct chlorine extraction, (B) indirect chlorine extraction, (C) experimental design of the indirect chlorine extraction.

Table 2 List of the 30 strains selected for the screening of alkalitolerance and halotolerance abilities

Strains tested	Ecology
Acremonium sp.	Saprophyte
Agaricus lignicola AAM42	Saprophyte
Alternaria alternata	Phytopathogen
Armillaria mellea	Saprophyte, phytopathogen
Aspergillus niger F75	Saprophyte
Aureobasidium sp.	Saprophyte, endophyte
Beauveria bassiana	Entomopathogen
Beauveria caledonica	Entomopathogen
Botritis cinerea	Phytopathogen
Alternaria sp.	Phytopathogen
Clitocybe geotropa	Saprophyte
Coprinus comatus	Saprophyte
Cordiceps sinensis	Entomopathogen
Fusarium culmorum NEU F376	Phytopathogen
Fusarium graminearum	Phytopathogen
Fusarium sp.	Phytopathogen
Ganoderma lucidum	Saprophyte
Laetiporus sulphureus	Saprophyte
Lepista nuda	Saprophyte
Morchella sp.	Saprophyte, ectomycorrhiza
Phanerochaete chrysosporium	Saprophyte
Pleurotus ostreatus	Saprophyte
Pycnoporus cinnabarinus 1	Saprophyte
Pycnoporus cinnabarinus 2	Saprophyte
Pycnoporus cinnabarinus 3	Saprophyte
Trametes versicolor	Saprophyte
Trichoderma sp.	Saprophyte, mycoparasite
Ulocladium sp.	Saprophyte
Verticillium dahliae	Phytopathogen

corrosion compounds present in the archaeological object will then allow a decrease in the porosity of the corrosion layer and consequently dissolve out the chlorine from the object.

Spectophotometric analyses with the Ferrozine were conducted to assess iron uptake by the selected fungal strains. Iron uptake in *Alternaria* sp., *Fusarium culmorum*, and *F. graminaerum* was demonstrated (Table 3). After 2 days of incubation the iron concentration decreased from 4514 μM to only 627 μM in the culture medium of *Alternaria* sp (Fig. 3), while the two strains of *Fusarium* were less efficient.

Table 3 Summary of the results obtained through the study of fungal metabolic activities regarding iron of selected alkalitolerant and halotolerant fungi

Alkalitolerant and halotolerant fungal strains	Reduction	Uptake	Siderophore	Crystals	Extracellular polymeric substances
Acremonium sp.	−	−	−	−	−
Agaricus lignicola	+	−	+	−	−
Beauveria bassiana	−	−	+	+	+
Alternaria sp.	−	+	−	−	−
Coprinus comatus	No growth	No growth	+	−	−
Cordiceps sinensis	−	−	−	−	−
Fusarium culmorum	−	+	+	−	−
Fusarium graminearum	−	+	+	−	−
Morchella esculenta	No growth	No growth	+	−	−

Siderophore production was also studied in the nine selected fungal strains using chrome azurol S (CAS)-amended media (Daghino et al., 2006). Interestingly, *F. culmorum* and *F. graminearum* were able to produce siderophores, whereas this capacity was not observed in *Alternaria* sp (Table 3). This suggests that in the latter iron uptake is not directly related to siderophore production but to another mechanism. It can be supposed that different strategies are employed and that since *Alternaria* sp. is able to melanize its cell wall, this fungus could exploit cell wall adsorption for iron mobilization and further uptake. This capacity could then be used to develop a biological cleaning of the object, removing the external part of the corrosion layer without damaging the original surface. A biological removal of a part of the corrosion layer could, for example, replace electrolytic reduction that has to be monitored carefully due to the production of H_2.

Optical microscopy investigations allowed observation of biogenic particles only for *B. bassiana*. The production of biogenic iron minerals was assessed using solid MA medium amended with different iron sources (soluble iron citrate and insoluble FeO(OH)). Brown rosette-like aggregations similar to copper oxalate were detected after 1 month of incubation in cultures amended with 10 mM iron citrate (Fig. 4A and B), as well as 100 mM and 150 mM FeO(OH) (Fig. 4C−F). However, the low amount of crystals formed did not allow their identification with Raman Spectroscopy. Further investigation is needed to better understand the mechanism of crystal formation and to increase the amount of biogenic crystals produced by *B. bassiana*.

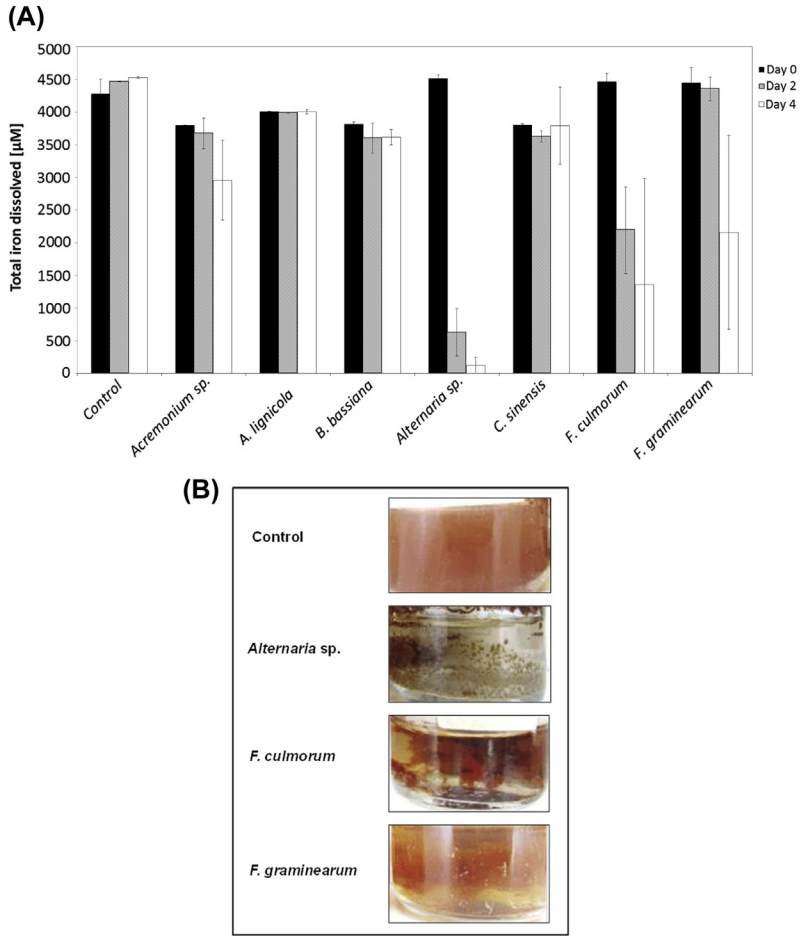


Figure 3 (A) Total dissolved iron measured after 0, 2, and 4 days incubation of nine alkalitolerant and halotolerant fungal strains, in liquid malt extract medium amended with 10 mM $FeCl_3$. (B) Control and cultures of *Alternaria* sp., *F. culmorum*, and *F. graminearum* after 4 days incubation.

The production of EPS and its ability to chelate iron was also evaluated in *B. bassiana* by incubating this fungus in 1.2% malt extract liquid medium amended with 10 mM iron citrate. Scanning electron microscopy coupled with Energy dispersive X-ray spectroscopy (SEM-EDX) observations were carried out to study the size, shape, and composition of the particles newly formed in the presence of iron in liquid cultures. SEM observations showed the presence of aggregates on the hyphae. Iron, carbon, and oxygen

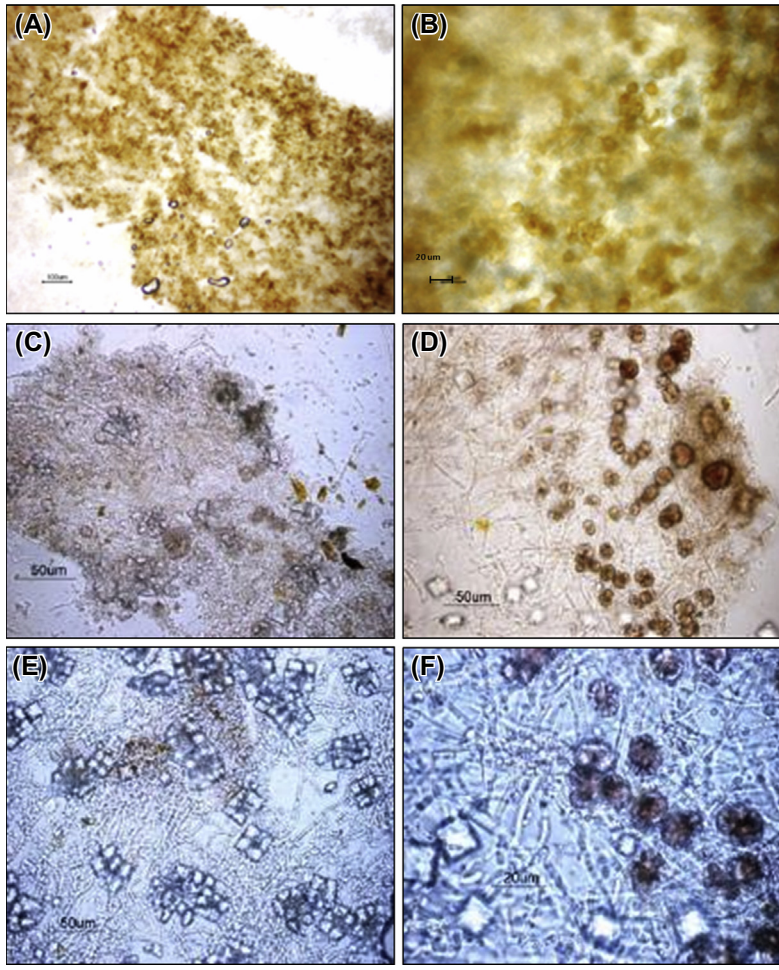


Figure 4 Biogenic minerals produced after 1 month of incubation of *Beauveria bassiana* with (A and B) iron citrate 10 mM, (C—F) FeO(OH).

were present in these particles, which were smaller than 1 μm (Fig. 5). Either the formation of biogenic iron crystals or iron-containing EPS could be exploited to convert a part of the corrosion layer into more stable minerals. Additionally, fungal biogenic production of iron particles is an interesting ability that can be employed to form low molar volume compounds and hence increase the porosity within the corrosion layers of archaeological artifacts.

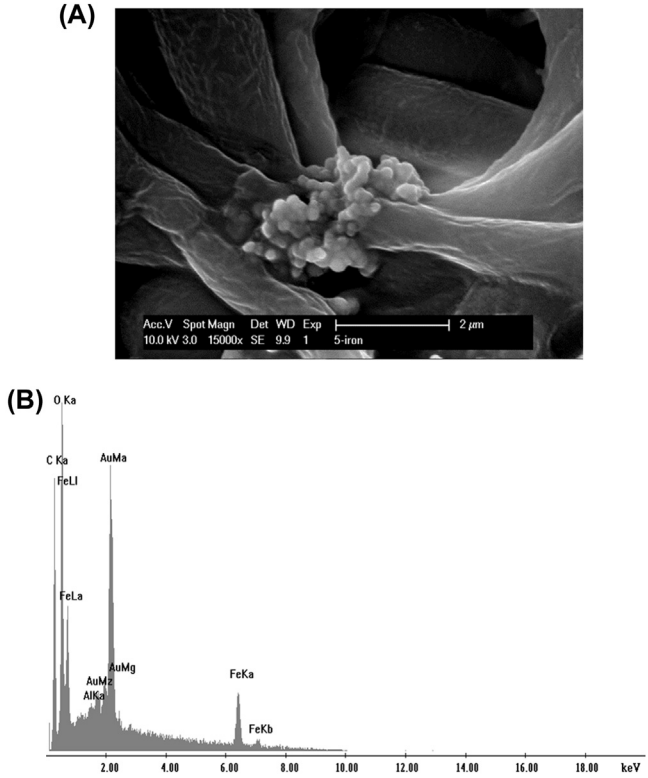


Figure 5 (A) Secondary electron micrograph showing the aggregates on *Beauveria bassiana* hyphae and (B) corresponding energy dispersive spectroscopy spectrum.

5. CONCLUSIONS

Fungal metabolic activities regarding iron are a particularly exciting research field but are still underexplored. Fragmentary information is available with focus on specific topics such as fungal pathogens, yeast, mycorrhizal fungi, and wood-degrading fungi. However, an overall background regarding the interactions between fungi and iron does not yet exist. In addition to this, most of the interest is dedicated today to the study of the interactions between fungi and toxic metals and, although in some cases also iron is included, it can only be supposed that information available for toxic divalent and trivalent metals could be applied for iron. Because iron is a key element in soil dynamics, and generally in the environment, and as fungi are significant weathering agents, it would be important to put more effort into the study of the metabolic abilities of fungi regarding iron. As a

consequence, a better understanding of this domain would allow exploitation of the metabolic abilities of fungi for the development of biotechnological applications in fields where iron needs to be preserved or removed. Such an example is the MAIA project, in which these fungal abilities are studied to develop an eco-friendly and sustainable conservation-restoration method for archaeological iron objects. Further investigation is still required, but the isolation of one or several iron reductive molecules could permit developing a conservation-restoration method based on the use of enzymes for the reduction of the iron compounds present in the corrosion layer. Additionally, rapid iron uptake abilities were shown for *Alternaria* sp., *F. culmorum* and *F. graminearum*. This capacity could be exploited to develop a biological treatment aiming to remove the corrosion layers in a selective way without damaging the original surface, replacing, for example, electrolytic reduction methods. Biogenic iron minerals were produced by *B. bassiana* in solid media amended with several iron sources and might in the future allow production of a passivation layer on the surface of iron objects, indirectly removing also the chlorine present that instigates further corrosion. The production of low molar volume compounds, such as iron oxalates or EPS chelating metal, will allow increasing the porosity of the corrosion layer and enhancing then the removal of chlorine. In conclusion, fungi possess a great variety of metabolic abilities in relationship to iron. The further study of this fascinating domain will allow us not only to better understand these microorganisms and their role in ecosystems, but also to exploit their abilities to develop novel sustainable and eco-friendly biotechnologies for the recovery and the protection of iron-containing matrices.

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CHAPTER THREE

Contamination Control for Scientific Drilling Operations

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Contents

Abstract

Drilling is an integral part of subsurface exploration. Because almost all drilling operations require the use of a drill fluid, contamination by infiltration of drill fluid into the recovered core material cannot be avoided. Because it is impossible to maintain sterile conditions during drilling the drill fluid will contain surface microbes and other contaminants. As contamination cannot be avoided, it has to be tracked to identify those parts of the drill core that were not infiltrated by the drill fluid. This is done by the addition of tracer compounds. A great variety of tracers is available, and the choice depends on many factors. This review will first explain the basic principles of drilling before presenting the most common tracers and discussing their strengths and weaknesses. The final part of this review presents a number of key questions that have to be addressed in order to find the right tracer for a particular drilling operation.

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1. INTRODUCTION

Exploration of deep subsurface environments relies on drilling. Many different drilling techniques are being used, the choice is mainly based on the type of sediment or rock to be penetrated and the maximum depth that has to be reached. Almost all drilling operations require the use of a drill fluid for cooling the bit, transporting cuttings out of the borehole and stabilizing the well (Kallmeyer, Mangelsdorf, Cragg, Parkes, & Horsfield, 2006). The most simple drill fluid is water, but in many cases this is not sufficient and additional compounds have to be added, e.g., clay minerals to increase specific gravity of the fluid and/or thickeners of variable composition (natural compounds such as cellulose or guar gum or synthetic polymers).

1.1 Sterile Drilling—An Impossibility

As the drilling fluid travels from the surface down to the drill bit, it comes in contact with many surfaces (holding tank, pump, pipes, etc.) and will inevitably transport some surface material down into the borehole. From a geomicrobiological or biogeochemical perspective drilling is a very dirty business, even in relatively small drilling operations, it is impossible to maintain sterile conditions or avoid contamination of the drill core with foreign compounds such as hydrocarbons. A drill core is never completely pristine but will always have at least some contamination on the outside. A drilling rig and the associated drill rods very quickly add up to a total weight of many tons. The weight of the drill string varies significantly between different diameters and wall thicknesses, as a rule of thumb a 6 m piece of drill string for a deep drilling operation weighs between 100 and 150 kg, so a kilometer of string weighs 18–25 tons. Such huge amounts of equipment are much too large and heavy to be sterilized. Even if it were possible to sterilize an entire drill string of up to several kilometers length, it would be a futile exercise because as soon as the drill string enters the drill hole it will be immediately contaminated with microbes from the surrounding sediment or rock. Due to the fact that the drilling fluid is usually an opaque mixture of water and suspended particles that are larger than microbial cells, the fluid can neither be filter-sterilized nor UV treated. In cases where only small volumes of water without any additives can be used as a drill fluid, then presterilization is indeed an option. However, this option is limited to rather small

operations. In normal sized drilling operations the massive volumes of drill fluid of up to hundreds of cubic meters and flow rates of hundreds of liters per minute preclude any sterilization. At best the drilling equipment is thoroughly cleaned before use to avoid contamination with foreign hydrocarbons from the pipe grease or other chemicals, and the drill mud is prepared with clean tap water instead of well or river water. There are several ways to keep the drilling operation as clean as possible, for example, employing very strict cleaning protocols and carefully designing the operations around the drill rig with contamination avoidance in mind (Russell, Phelps, Griffin, & Sargent, 1992).

So even under the best possible conditions, drilling inevitably causes infiltration of nonsterile drilling fluid into the core, not just along cracks and fissures but also into the pore space of even undisturbed fine-grained sediments (McKinley & Colwell, 1996; Smith et al., 2000a, 2000b). While drill fluid contamination is problematic for many analyses, it poses particular challenges for geomicrobiological studies.

Compared to the surface, microbial cell abundances in the subsurface are several orders of magnitude lower. As an example for typical cell abundances, a coastal shallow marine or lacustrine sediment contains between 10^6 and 10^8 cells/cm^3. In very organic rich sediments from upwelling areas or other eutrophic systems, cell abundances are in the 10^9 cells/cm^3 range but can reach, or in rare cases even exceed, 10^{10} cells/cm^3 (Andrén, Barker Jørgensen, Cotterill, Green, & the IODP expedition 347 scientific party, 2015). At the other end of the spectrum are deep subsurface sediments, which normally only have cell densities around 10^3–10^4 cells/cm^3 (e.g., D'Hondt et al., 2015; Kallmeyer, Pockalny, Adhikari, Smith, & D'Hondt, 2012) or even less (Inagaki et al., 2015). So there are several orders of magnitude difference in cell abundance between the shallow and the deep subsurface. Thus even the slightest infiltration of drilling fluid into a deep subsurface sample (in the order of nanoliters per cubic centimeter sediment) renders the sample unsuitable for microbiological and also certain geochemical investigations (Yanagawa et al., 2013). One could argue that it should be possible to avoid any contact of the drill fluid with surface sediments, use clean but not necessarily sterile equipment, and employ strict contamination control. All these are possible and have been done in the past with various degrees of success. Still, preparation of the drill fluid is a key issue for minimizing contamination. Due to the large volumes of water that are required and the often remote location of the drill site, it is often impossible to use relatively clean tap water; instead water has to be sourced

locally from wells, springs, rivers, or lakes instead. In ocean drilling the drilling liquid of choice is normally surface ocean water. However, even in the most extreme oligotrophic parts of the world's ocean cell abundance at the surface is still around 10^5 cells/mL (D'Hondt et al., 2011). In coastal waters or lakes, cell abundances are in the 10^6 cells/mL range or higher (e.g., Daley & Hobbie, 1975; Noble & Fuhrman, 1998). So even under the best possible conditions the drill fluid will have a cell concentration that is orders of magnitude higher than a deep subsurface sample, and it will inevitably infiltrate at least into the outer layers of the drill core.

2. DRILLING TECHNIQUES

Drilling is an integral part of deep earth exploration. The review of Wilkins et al. (2014) provides an excellent overview of terrestrial scientific drilling operations. A recent book (Stein, Blackman, Inagaki, & Larsen, 2014) describes the state of the art and future challenges of deep life exploration in the marine realm.

Most scientists who had to deal with a large-scale drilling operation will agree that a very close collaboration between the science and drilling team a project from the earliest possible date a project is vital for its success. Scientists should not try to tell the drillers how to drill, but should be able to formulate their specific needs with regard to drilling operations. In my experience, even the most unusual requirements can be accommodated with relative ease, if they are communicated early enough. It also helps tremendously when scientists and drillers both speak the same language in terms of technical terms and definitions. Therefore before I start with a description of the different drilling techniques, I would like to introduce the most common terms and definitions as they are often mixed up or used incorrectly, leading to confusion and misunderstandings.

2.1 Glossary of Common Terms

Drilling is a technique by which a hole is created, normally by a rotating drill bit, sometimes also by hammering. Drilling almost always includes the use of a drilling fluid. For scientific purposes, drilling almost always includes the recovery of a core, but there are cases where no core is recovered and only the hole itself is of interest. Also, there are cases where only specific intervals are cored, the rest is just drilled, not cored. In industry, coring is

rarely done as it is very time-consuming and therefore expensive. A key feature of drilling is the use of a so-called drill string, which is a set of steel pipes that connect the drill bit at the bottom of the hole with the drill rig at the surface. The drill string is extended by addition of more pieces of drill pipe. The drill fluid is pumped through the drill string, exits through the drill bit, and travels upward through the drill hole, carrying the drill cuttings to the surface.

Coring is a term that is used to describe the retrieval of a core, irrespective whether drilling was used or not. Mainly in limnology and oceanography, cores of unconsolidated sediment are taken without drilling but with a gravity or piston corer (see below). Even in operations with continuous coring over hundreds of meters a core is not taken continuously but in sections of variable lengths, usually between 3 and 10 m. Between the individual cores, there is usually a small gap caused by mechanical disturbances from the drill bit or the core catcher. In cases where an absolute continuous record without gaps is required, e.g., for stratigraphy or paleoclimate research, multiple holes are being drilled. Coring at different holes will be arranged with an overlap to cover the gap in one hole with a continuous core in the other. So if a single core run will be 10 m long, then the first core of the second hole will be drilled only to 5 m depth to have sufficient overlap.

Core tube or core barrel: The central piece of any corer is the core tube or barrel, as it will retrieve and hold the core. The core barrel is usually made from metal, as it has to withstand the mechanical forces during coring and protect the inner plastic liner (see below) from breaking. Depending on the type of coring operation the core barrels have to be very sturdy and thick walled to withstand the forces that occur when pushing into the sediment, especially in piston coring. These core barrels can weigh up to hundreds of kilograms, depending on the total length of the corer. For example, the long piston corer from Woods Hole Oceanographic Institution has steel core barrels with wall thicknesses of 1.25″; each barrel is 20′ long and weighs 1500 lbs (Fig. 1).

Liner is a tube (normally plastic) that sits inside the core barrel and collects the core. After bringing the core barrel to the surface the liner with the core inside is pulled out of the barrel (Fig. 2A) and laid down horizontally and usually cut into sections of 1 or 1.5 m length to allow for easier handling (Fig. 2B). To prevent the core from falling apart, it is left in the liner. Depending on the type of analysis the horizontally placed core, including the liner, is either split open horizontally to see the internal structure of

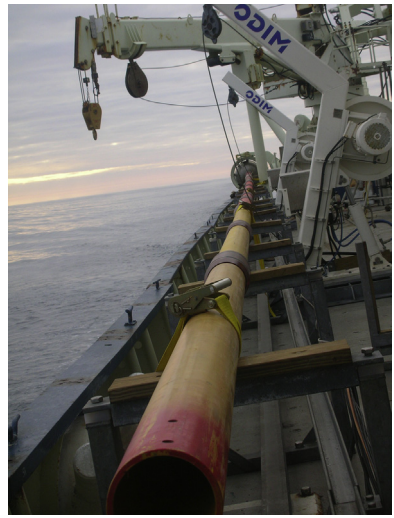

Figure 1 The long piston corer of Woods Hole Oceanographical Institution. The thick-walled steel core barrels are held together by joints. The core shoe and the core catcher are removed to allow inserting a liner. The large weight set is in the back. *Photo: Kallmeyer, GFZ Potsdam.*

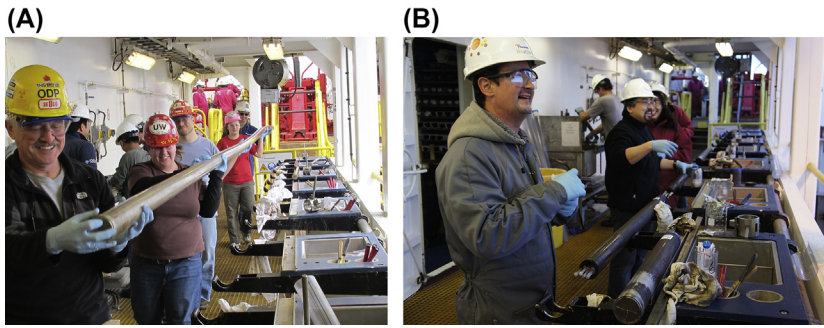

Figure 2 (A) A team of happy technicians pulls a 9 m-long liner out of the core barrel and brings it to the science area, the so-called catwalk, on board the Integrated Ocean Discovery Program drill ship JOIDES Resolution. At the cat walk the core will be sectioned and sampled of time-sensitive parameters such as perfluorocarbon tracer. (B) A team of even happier scientists cuts the core into 1.5-long sections and takes subsamples. Note the sets of syringes at the freshly cut ends. This way samples for perfluorocarbon tracer are taken. *Photo: Kallmeyer, GFZ Potsdam.*

the cored material or cut vertically into small intact core pieces, the so-called whole round cores (WRC). The latter is commonly used geomicrobiological or biogeochemical research because the exposure to oxygen is reduced.

For taking short cores (usually <1 m) from very soft sediment, small gravity corers are being used that do not have a separate core barrel and liner but only a single tube of either steel or plastic in which the core is collected. The recovered core is then usually pushed out of the tube, immediately sampled, and the tube reused.

Core catcher: Core loss normally occurs once the corer is lifted up and the core slides down. To help prevent this loss of core, there is usually a check valve at the top of the core that lets the water out that is displaced by the drill core but closes and creates suction to keep the core inside the tube as soon as the corer is pulled upward.

This way a vacuum is created that keeps the sediment inside the tube. However, the longer the core the more likely suction alone will not hold the core in place and some loss will occur. To improve core recovery a core catcher is installed at the bottom of the core tube (Fig. 3).

Different mechanisms are being used, but the most common one is a circle of plastic or metal lamellae, forming a circular flexible barrier. The core pushes the lamellae to the side and glides past them when entering, but when the core tries to fall back out the lamellae close and keep the core in place. However, the lamellae can disturb softer sediments as

Figure 3 A selection of drill bits and core catchers used on the Integrated Ocean Discovery Program drill ship JOIDES Resolution. *Photo: Anna Ling, University of Miami.*

they enter. This is more the case with metal core catchers than with softer plastic ones. By choosing a core catcher with the right stiffness a compromise between optimal sample integrity and minimal core loss can be found.

2.2 Drilling/Coring Techniques

Industry has developed many different drilling and coring techniques for almost any rock type or environment imaginable. However, only a few play a role in scientific drilling, but even those few offer many options and might confuse a scientist at first.

In rare cases, airlift drilling has been used for scientific purposes (Colwell, 1989; Colwell et al., 1992; McKinley & Colwell, 1996), where air or an inert gas like argon was used to lift the cuttings out of the hole. Given the rarity of airlift operations in scientific drilling, it will not be further discussed in this paper. The study of Colwell et al. (1992) is recommended for a good overview of the air-drill technique.

The next few paragraphs will describe those techniques that are most often used in scientific drilling.

Piston and gravity coring: Both techniques do not require the use of any drilling liquids and thereby reduce the chance of contamination with foreign material. However, they only work in soft, unconsolidated sediments, not in hard rocks. Quite often gravity or piston coring operations are called drilling, which is not correct; the correct term is gravity or piston coring. There is however an exception to this rule and that is hydraulic piston coring (see below), which is in fact a drilling technique. While the difference between coring and drilling seems rather semantic, it does make a major difference in terms of contamination control. While for piston or gravity coring operations, contamination control is of minor concern and usually not employed due to lack of drilling fluid, it is absolutely crucial for drilling operations. So having a core sample from a drilling or coring operation does make a difference for many analyses.

In its simplest form a gravity corer consists of a core barrel that is closed by a flap valve at the top and a set of weights that allows it to penetrate the soft sediment. It hangs vertically on a rope or cable on which it is lowered toward the sea or lake floor. While a gravity core is lowered rather slowly from the vessel into the lake or seafloor, a piston corer is only lowered to a few meters above the bottom and then dropped in free fall by releasing a loop of additional cable. In a gravity corer the cable is connected to the head of the corer, whereas in a piston corer it is

connected to a piston that sits at the bottom of the barrel. The length of the free fall is calculated so that when the corer touches the seafloor the cable stops the piston right above the sediment surface while the corer continues its movement and penetrates the sediment. The vacuum created by the immobile piston on the sediment surface prevents the sediment from compaction caused by the downward moving tube. This way the sediment enters the tube comparatively undisturbed and allows for longer and heavier coring systems compared to gravity cores. Both gravity and piston corers have proven to provide largely undisturbed cores, but the short free fall of the piston corer allows for deeper penetration at the cost of operating a more complicated and failure-prone system. The maximum depth limit for gravity coring is usually in the 10 m range. Piston corers allow for deeper penetration. The largest systems (Calypso corer of French research vessel Marion Dufresne and Woods Hole Long Core system, Fig. 1) reach maximum depths around 50 m.

Hydraulic piston coring (HPC): This is the most common technique for recovering non- to semiconsolidated sediment from greater depths beyond the limits of gravity or piston coring. In ocean drilling operations, this technique is also called Advanced Piston Coring (APC). Depending on the sediment properties, cores in excess of 400 m length can be recovered by this technique (Pälike et al., 2010). For HPC operations a core tube with a liner inside, a flap valve on top, and a core shoe (Figs. 3 and 4) with a core catcher at its bottom forms a close seal at the bottom end of the drill string, so the drill fluid inside the string can be pressurized. Eventually the pressure

Figure 4 A cutting shoe of a hydraulic piston corer. The front edge is bent and damaged from hitting harder material. *Photo: Kallmeyer, GFZ Potsdam.*

exceeds the breaking strength of the shear pins that hold the core tube in place. By selecting the right number and type of shear pins the drillers can adjust to varying lithologies. After breaking of the shear pins the core tube shoots forward and pushes into the sediment. The core shoe has to cut through the sediment and might get damaged when hitting harder layers or pieces of gravel (Fig. 4). When the core tube has come to a stop a circular drill bit will drill around the core tube and extend the drill hole. During this operation the drill string will move downward and push the core tube back inside the string. After extending the hole to the maximum depth that was reached by the core tube the core tube will be pulled upward for retrieval of the drill core. HPC is usually used in combination with wireline coring (see below).

Extended Core Barrel (XCB): In case the sediment becomes too stiff for HPC but is still too soft for rotary drilling, other tools are being used, the most common one is XCB, which features a short (<50 cm) core barrel that extends forward from the actual drill bit, hence the name extended core barrel (Fig. 3). As the drill bit moves downward the core barrel pushes into the sediment ahead of the bit. There are different versions of this tool, some have a nonrotating barrel, others have a rotating one. In some cases the barrel is spring loaded, others are fixed. There are different names for these tools: extended nose, extended shoe, extended core bit, etc. As different as these systems are, they all have in common that the quality of the recovered cores is usually not as good as the HPC cores. Very often they are mechanically disturbed and contaminated by drilling fluid and therefore unsuitable for geomicrobiological analyses. Despite many years of development, there is still no suitable technique available that delivers high quality cores from sediments that can neither be cored by HPC or rotary drilling (RCB).

Rotary drilling (RCB): For real hard lithologies, rotary drilling is being used. If the sediment is still too soft, then it will be fractured into small pieces and more or less destroyed. In RCB operations a core bit equipped with either three or four rotating cones (Fig. 3) or a ring-shaped crown cuts the rock.

Wireline Coring: Wireline coring is used in almost every deep drilling operation where coring is an important component. It can be used in both rotary coring and hydraulic piston coring operations. Its main advantage is that it is not necessary to pull the entire drill string out of the hole to retrieve the core and replace the liner but to retrieve many consecutive cores through the drill string. In shallower terrestrial drilling operations (up to a few tens to hundred meters) with stable boreholes, it is not much of a

problem to pull out the entire drill string for every few meters of core. In cases where stability of the borehole is an issue, e.g., in sandy aquifers, pulling the string out of the hole should be avoided as this will cause additional disturbance that might lead to a caving or even collapse of the hole.

Pulling the drill string out of the hole becomes a huge problem for deeper drilling operations or for operations from drill ships or swimming platforms. It takes several hours to move one or more kilometers of string in and out of the hole. Although a reentry into a drill hole on the sea or lake floor is possible, it is technically challenging and requires specially equipped ships or drilling platforms with highly accurate dynamic positioning systems. Also a large reentry cone has to be installed on the sea floor to help reentering the drill string into the hole. Given the technical difficulties and required additional equipment and time for a reentry, it might be cheaper to drill a new hole instead of reentering an existing one.

For wireline coring a unit consisting of a core tube with a liner inside, a flap vale, and a core-retrieval mechanism (the so-called spearhead or fishneck, Fig. 5) at the top and a core catcher at the bottom is sent through the drill string to the bottom of the hole, where it attaches itself to the bottom segment of the string, the so-called bottom hole assembly (BHA). After coring has commenced a cable with a catching mechanism, the so-called overshot assembly is lowered through the string, connects itself to the core tube, and releases it from the BHA. Then the core tube is pulled up. Upon retrieval the core tube is laid horizontally, the core catcher is removed,

Figure 5 A wireline corer is about to be deployed onboard JOIDES Resolution. The spearhead sits on top of the corer, and the overshot assembly is hanging on chains at the left. A piece of drill string can be seen in the foreground on the right. *Photo: Kallmeyer, GFZ Potsdam.*

and the liner with hopefully a core inside is pulled out of the core tube. Then a new liner is loaded, the core catcher is fixed, and the unit is ready for another trip down the drill string.

3. CONTAMINATION TRACERS

Because contamination cannot be avoided, at least not completely, it is essential to trace contamination of the drill core to identify uncontaminated samples. To assess the degree of infiltration a tracer is added to the fluid. To attribute the detected tracer to the infiltration of drilling fluid into the sample, it is necessary that tracers (1) have no natural source, (2) are easy to detect even at extremely low concentrations, and (3) are chemically inert.

Several techniques have been used in past drilling operations to assess microbial contamination, including fluorescent dyes (Pellizzari et al., 2013; Phelps, Fliermans, Garland, Pfiffner, & White, 1989; Russell et al., 1992), perfluorocarbon tracers (PFTs) (Colwell et al., 1992; House, Cragg, Teske, & Party, 2003; Lever et al., 2006; Russell et al., 1992; Smith et al., 2000a, 2000b), microsphere tracers (Colwell et al., 1992; Kallmeyer et al., 2006; Smith et al., 2000b; Yanagawa et al., 2013), dissolved salts such as lithium bromide (Haldeman, Amy, Russell, & Jacobson, 1995), potassium bromide (Phelps et al., 1989), and barium (Chapelle & Lovley, 1990), sulfonic acids (Hirtz, Kunzman, Broaddus, & Barbitta, 2001), and foreign microbes such as cyanobacteria (Colwell, Pryfogle, Lee, & Bishop, 1994) or fluorescent proteins (Juck et al., 2005). Other studies used molecular biological techniques to differentiate between the microbial community of the drill fluid and the sample (Chandler, Brockman, & Fredrickson, 1997; Gronstal et al., 2009).

Gronstal et al. (2009) give a good overview of the different scientific drilling operations and their respective methods for contamination control.

3.1 Fluorescent Dyes

Fluorescent dyes like fluorescein or rhodamine are inexpensive, easy to handle, and allow sensitive detection of contamination (Russell et al., 1992). Another major advantage is the fact that fluorescein is nontoxic and has been used as a groundwater tracer for a long time. In areas with strong legal constraints on drilling, e.g., close to drinking water wells, fluorescein might be the only tracer for which a permit can be obtained as the authorities already have experiences with it. The detection limit for fluorescein in an aqueous solution without any interfering compounds is in the

order of 0.05 ppb (Gunderson, Parini, & Sirad-Azwar, 2002), however in actual geologic samples the detection limit is more in the range of 1 ppb (Pellizzari et al., 2013), other dyes are in a similar range. Detection and quantification is easy, the only required equipment is a fluorometer. Pellizzari et al. (2013) provide a good description of the protocol to extract the dye, in this case fluorescein, from the drill core sample and measure a large number of samples at once with a plate reader.

In most cases the dye concentration in drill mud is in the ppm range (mg/L), resulting in a detection range of c. three orders of magnitude. Although a detection limit in the ppb range sounds impressive, it also means that a drill fluid contamination in a concentration around 0.5 μL/cm^3 would not be detected, assuming a dye concentration of 1 ppm. Using normal lake or seawater with a microbial cell concentration of c. 10^6 cells/cm^3 as a drill fluid, then the sample contains up to 500 foreign cells per cubic centimeter. For a drill core from shallow depths or from an organic rich deposit with concomitantly high cell counts (i.e., above 10^5 cells/cm^3), these 500 cells/cm^3 only represent less than 0.5% of the community. Depending on the planned analysis, this might not be much of an issue. However, even in shallow subsurface samples with a large indigenous microbial population, introduction of 500 foreign cells from the surface renders the sample unusable for cultivation approaches, because subsurface microbes normally have much lower metabolic activity and growth rates than surface microbes (Hoehler & Jorgensen, 2013; Jørgensen, 2011) and can therefore be outcompeted by the introduced microbes.

For deep subsurface environments with only a few 100 or 1000 cells per cm^3 the situation is much worse, as even such a small (and in case of a dye tracer undetectable) contamination would massively change the microbial community composition. Considering the fact that a contamination of 0.5 μL drill fluid per cm^3 of sample would fall below the minimum detection limit, a more sensitive contamination control method would be required.

Also, fluorescent dyes have other potentially problematic features. (Diehl & Horchak-Morris, 1987) showed that fluorescein is sensitive to light degradation. Normally the drill fluid or drill mud is stored in large holding tanks that are open at the top. So while it is easy to mix the tracer with the drill fluid in the holding tanks, it might decay to a certain degree and thereby change its initial concentration and lower the minimum detection limit.

Another aspect that has to be taken into account when using fluorescent dyes is their sensitivity to low pH values. In a detailed study about the

fluorescence intensity of fluorescein and several other compounds that are being used as tracers, (Zhu et al., 2005) showed that intensity remains largely stable in the alkaline range up to pH 10.5 but decreases in the acidic range, this trend seems to be more pronounced at higher concentrations.

Sorption of fluorescent dyes onto clays is another important aspect that has to be taken into account (Magal, Weisbrod, Yakirevich, & Yechieli, 2008). Clays are a common drill mud additive; they are used to increase density to improve the capability of the mud to carry cuttings out of the hole and stabilize the walls of the drill hole. The sorption characteristics depend on the type of mineral and dye, so no general recommendations can be given, only the strong advice to carefully test the tracer before deciding on its use in a drilling operation. Also, in a detailed study about factors that influence fluorescence intensity of fluorescein (Weidner, Naurath, Rüde, & Banning, 2011) showed that dissolved Fe^{2+} and Mn^{2+} can significantly decrease the signal. While this might not be of general concern, it should be taken into account when drilling through metal-rich formations or through aquifers with iron-rich waters.

When drilling through organic-rich deposits such as peat or coal the drill fluid can extract substantial amounts of humic substances, which react with the fluorescent dyes and cause quenching, thereby decreasing the fluorescence signal (Hafuka, Ding, Yamamura, Yamada, & Satoh, 2015). Moreover, fluorescent dyes will stain the entire drilling fluid in a bright color (Fig. 6), which might cause problems for disposal of the mud after drilling.

Figure 6 Drill fluid stained with fluorescein in a holding tank. *Photo: Alawi, GFZ Potsdam.*

Although fluorescent dyes are the tracer that is most easy to obtain and use, there are several limitations that have to be taken into account as they limit the applicability of fluorescent dyes in deep drilling campaigns.

3.2 Perfluorocarbon Tracers

PFTs are fluorinated hydrocarbons that have no known natural source. These tracers have been used extensively in drilling operations on land and at sea (Lever et al., 2006; McKinley & Colwell, 1996; Russell et al., 1992; Smith et al., 2000a). A common PFT is Perfluoromethylcyclohexane, which has boiling point of 76°C, its solubility is ~1 mg/L in water and 10 g/L in methanol (Colwell et al., 1992).

Its low solubility in water combined with a low boiling point facilitates gas phase partitioning through heating of the sample followed by quantitative headspace analysis via electron capture gas chromatography (GC-ECD). This is by far the most sensitive detection method for any tracer, reaching down to levels of c. 2×10^{-12} g PFT, which translates to a minimum detection limit for drill mud infiltration in the range of 4–5 nL/cm^3 (Lever et al., 2006).

Because PFT has such a low solubility in water and a low boiling point, it cannot be premixed into the drill mud but have to be fed constantly into the mud immediately before being pumped down the drill hole. The normal rate at which the PFT is introduced into the mud stream is 1 mg/L. For feeding the PFT into the mud stream, high pressure liquid chromatography pumps have proven to be a good choice. Another PFT that has been used in scientific drilling is gaseous Halon 1211 (Gronstal et al., 2009). During drilling operations at the Cheaspeake Bay impact structure, a gas mixture of 1% Halon in N_2 was added into the mud stream to reach a final concentration of 1 ppm Halon. Due to the pressure in the borehole the gas was completely dissolved into the drill mud.

Core samples for contamination control have to be taken as quickly as possible after retrieval of the core to avoid any losses due to evaporation (Gronstal et al., 2009). Samples have to be placed immediately in gas-tight (usually glass) vials and sealed with a septum. After heating the vial to facilitate partitioning of PFT into the headspace a small (0.5–5 mL) gas sample is taken from the headspace and analyzed via GC-ECD. Smith et al. (2000a, 2000b) note that the glass syringe used for transferring the sample from the vial to the GC should also be heated to 70°C to avoid adsorption of PFT onto the glass walls. Due to its high volatility and extremely low detection limit, they recommend that all handling of PFT should be carried out in

a well-ventilated area and away from the drilling operations or sample handling areas to avoid false positives.

Given the high sensitivity of detection, PFT should be the method of choice for any biogeochemical or geomicrobiological coring operation. However, there are good reasons for not using it. The first one is the required technology for delivering the tracer into the drill mud. Because it cannot be premixed a small pump has to be installed and the tracer fed into the mud stream at a rate that is proportional to the flow rate of the mud pump. Depending on the type of drill rig and the willingness of the drilling team to cooperate, it might be technically and strategically challenging or impossible to install a delivery system for PFT. The second reason for not using it under all circumstances is its high volatility. If a contamination control sample is not taken shortly after retrieval of the core, an unknown fraction of PFT will have evaporated from the sample. So under conditions where the cores cannot be subsampled immediately after retrieval, e.g., in lake drilling operations, where only a very small drilling barge is being used that does not provide any space for detailed subsampling, the PFT will be gone by the time the cores reach the laboratory. And even if an initial subsampling immediately after retrieval is possible the high volatility of PFT still precludes detailed contamination control at a later date. In many cases highly specialized and laborious, time-consuming analyses are not carried out at the drill site, but later in the home lab. Usually each group that plans detailed analyses in the home lab receives a single larger WRC from a sampling interval instead of subsampling immediately for each measurement. This way the processing time at the drill site can be minimized, which is often necessary to keep up with the core flow. All further subsampling is then carried out right before the analyses. To make sure that every individual sample is in fact not contaminated, it would be highly desirable to measure the level of contamination on every individual sample prior to analysis. When using PFT, this would not be possible because the PFT has long since evaporated.

Another issue is the detection of PFT, which requires a GC with an electron capture detector (ECD). An ECD is mainly used for pesticide detection and in pharmaceutical research, it is not a common detector in biogeochemistry and therefore not readily available in most geomicrobiology and biogeochemistry laboratories. So unless a suitable system can be borrowed, it has to be included in the budget. For a one-off operation, it might be too expensive. In cases where largely unknown lithologies are drilled, it is extremely helpful to have at least a few contamination control results right

at the drill site to adjust the drilling strategy. This however requires a GC-ECD on site, including a supply of a suitable high-purity carrier gas. Depending on the location of the drilling operation, this might be a very challenging task. PFT is the standard contamination control tracer on both drill ships of the Integrated Ocean Discovery Program , but they have all required equipment permanently installed and all necessary protocols in place, optimized over decades. In other operations, e.g., smaller drilling operations at remote locations such as on a tropical lake in the rainforest of Sulawesi (Russell et al., 2016), in the desert (Cohen et al., 2016), or in the high arctic (Dallimore et al., 2005), it might be a considerable challenge to organize the logistics to have such a system up and running at the drill site.

3.3 Microspheres and Other Particulate Tracers

Other very popular tracers are microspheres, sometimes also called microbeads. These are microbe-sized fluorescent plastic particles. Microspheres are available from several suppliers in many different colors and size ranges. The rationale for using microspheres is that particles that have the same size as microbes will, when mixed into the drill mud, penetrate the sample in a similar fashion as microbes and therefore mimic their distribution in a sample. Detection and quantification of microspheres by fluorescence microscopy is relatively easy and fast (Fig. 7). For contamination control, often green fluorescent microspheres with 0.5 μm diameter are used, as this is the average size of a subsurface microbe (Kallmeyer et al., 2012), and detection is

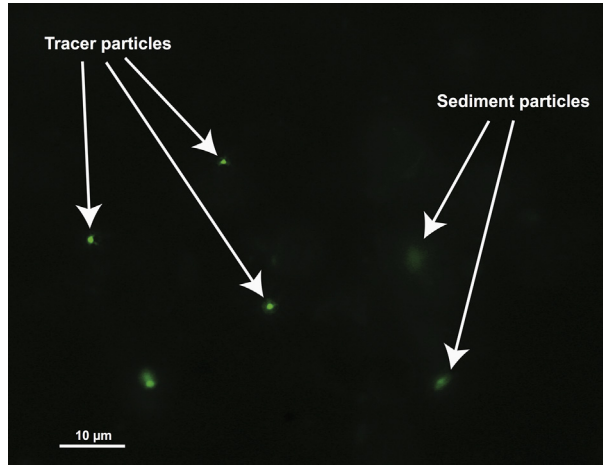

Figure 7 Microphotograph of fluorescent particles in a contaminated sediment sample. *Photo: Friese, GFZ Potsdam.*

possible with the same optical filter set as for the common stains Acridine Orange or SYBR Green I. The minimum detection limit is between 10^5 and 10^6 particles/cm^3 (Kallmeyer et al., 2006), similar to subsurface cell counting without cell separation (Fry, 1988; Kallmeyer, 2011; Morono, Terada, Masui, & Inagaki, 2009). However, separating the microspheres from the sample by density separation can lower the detection limit by at least one order of magnitude (Kallmeyer et al., 2006). Using the equation of (Kallmeyer, Smith, D'Hondt, & Spivack, 2008) and assuming that the drill fluid has a microbial cell concentration of 10^6 cells mL and a microsphere concentration in the range of 10^9 particles/mL, (Friese et al., 2016) calculated the minimum detectable concentration of drill mud infiltration to be 117 nL/cm^3 or 117 foreign cells/cm^3. This is better than fluorescent dyes but not as sensitive as PFT.

Microspheres have many advantages over fluorescent dyes and PFT. Except for temperatures >100°C that can be encountered in drilling operations in geothermal systems (Yanagawa et al., 2013), they are inert under most physical and chemical conditions, and they do not evaporate. However, they do have one major drawback, and that is their price. Microspheres are sold as aqueous suspensions with a concentration around 10^{12} particles/mL. When aiming for a concentration of 10^9 particles/mL, then 1 mL of microsphere suspension will be sufficient for 1 L of drill mud. Considering the price of tens of dollars per milliliter of microsphere solution and the usual volumes of drill mud in the range of thousands of liters, the use of microspheres very quickly reaches financial limits. Only in very rare instances (Kallmeyer et al., 2006) microspheres were directly mixed into the drilling mud. In most operations the tracer is packed into small plastic bags and taped to the core catcher at the bottom of the core where it bursts open once the sediment enters the liner (Fig. 8). This way the core is bathed in a high concentration of microspheres without the need to add them to the entire volume of drill mud (e.g., Lever et al., 2006; Russell et al., 1992; Smith et al., 2000a). However, this method has a drawback, which is the uneven delivery of microspheres (House et al., 2003; Yanagawa et al., 2013). If the drill fluid does not form a homogenous suspension with the microspheres, then chances are high that infiltration of drill fluid into the core will go unnoticed. Still, this technique has been used in many drilling operations with satisfactory results. Of course there were attempts to overcome these limitations. Juck et al. (2005) coated the inside of a liner with a microsphere suspension, so as soon as the core hits the liner, it will be in contact with the tracer. They used this approach only in rather

Figure 8 A small plastic bag filled with microsphere tracer solution taped at the bottom of a piston corer. The bag will burst open when the corer hits the sediment and release the microspheres that will bathe the core in a highly concentrated tracer solution. *Photo: Kallmeyer, GFZ Potsdam.*

small-scale operations, and it remains to be seen whether this approach can be expanded to larger operations.

The ultimate tracer to detect infiltration of microbes into a core would be a microbe that does not occur in this environment naturally and is very easy to detect. Several studies made such attempts. In a laboratory study, (Colwell et al., 1994) used the Cyanobacterium *Aphanocapsa delicatissima* to measure infiltration of water into basalt cores. Two detection methods were used to quantify infiltration of cyanobacteria in the core, cultivation, and spectrophotometric chl-a measurements. Although the method proved successful for studying flow through the pore space of the basalt cores, its suitability for larger drilling operations remains questionable, as this would require larger volumes of cyanobacteria. Also, chl-a concentrations decreased by 20% over 30 days. Although the decrease is much slower as PFT, cyanobacteria might still not be the best choice when long-term stability for tracing contamination is an issue. Colwell et al. (1994) also added microspheres as an inorganic tracer and found that the cyanobacteria move faster than the microspheres, despite having a similar size.

Juck et al. (2005) used the strain *Pseudomonas Cam*1-*gfp*2, expressing a green fluorescent protein (GFP), as a contamination tracer for drilling in permafrost and ground ice. They painted the culture on the inside walls of the liner. Like Colwell et al. (1994), they used two detection methods,

cultivation of the organism and much more sensitive PCR of the GFP-gene. Both approaches indicated good transfer of microbes from the painted walls of the liner into the core. Like in the approach discussed previously, these techniques might work very well in small-scale operations, but it remains questionable whether such approaches can be scaled up for deep drilling. Also, the use of genetically modified organisms in a drilling operation might also cause some major problems for permitting.

Very recently a new fluorescent particulate tracer was introduced to scientific drilling and that is an aqueous pigment solution that is normally used for paints and plastics (Friese et al., 2016). These pigment particles have the same size range as subsurface microbes (0.25—0.45 μm) and are available in several colors. Their biggest advantage is the price, as they are about three to four orders of magnitude cheaper than normal microspheres. At this price, it is feasible to mix them into the drill mud or even use them in operations where the drill fluid is not recycled. However, it should be noted that at concentrations around 10^9 particles/mL, the tracer stains the drill fluid in a very bright color, similar to dyes (Fig. 9). In their study, Friese et al. (2016) also show the use of flow cytometry for quantification of microspheres, which decreases analysis time considerably. With a new generation of portable flow cytometers, it is even possible to do such analyses right at the drill site. Still, fluorescence microscopy should be employed for calibration.

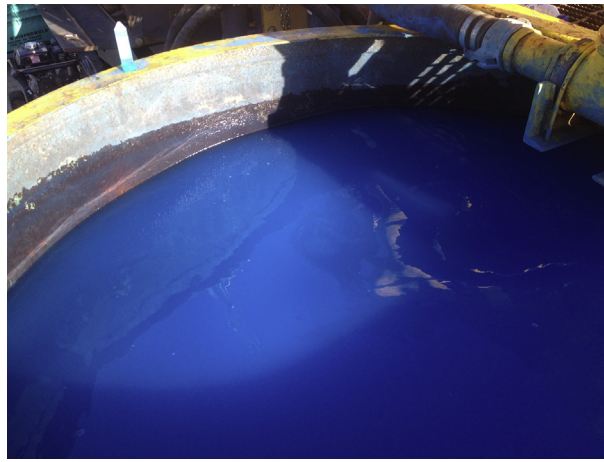

Figure 9 Microsphere tracer mixed into the drill fluid at a concentration of 10^9 particles/mL, which is already a 1000-fold dilution of the stock solution. *Photo: Kallmeyer, GFZ Potsdam.*

3.4 Dissolved Chemical Tracers

Different chemicals have been tested as contamination tracers: Salts like lithium bromide (Haldeman et al., 1995), potassium bromide (Phelps et al., 1989; Russell et al., 1992), barium (Chapelle & Lovley, 1990) or sodium fluoride (Hirtz, Lovekin, Copp, Buck, & Adams, 1993), as well as sulfonic acids (Hirtz et al., 2001). For dissolved salt tracers the choice of a suitable compound largely depends on two factors, pore water chemistry of the formation because the natural background concentration of the ion that is used as tracer is one of the two main factors that determines the minimum detection limit, the other one is tracer concentration in the drill fluid. The former factor limits the use of dissolved salts as tracers mainly to environments with low ionic strength pore waters, i.e., freshwater lakes and terrestrial sites away from geothermal areas or brines. For a tracer study in a hypersaline brine with bromide concentrations in the 100 s ppm range the initial plans for using bromide were abandoned and PFT was used instead (Hirtz et al., 2001). Also, even in environments with low pore water concentrations of the tracer the potential loss through precipitation should be considered, therefore a full pore water analysis should be available prior to drilling. For example, calcium fluoride (CaF_2) has an extremely low solubility in water (15 mg/L) so fluoride might not be a suitable tracer in a lithology with high dissolved calcium concentrations.

The detection method depends on the ion of interest; ion chromatography is usually the method of choice for anions (Russell et al., 1992), whereas for cations, atomic emission spectroscopy or similar techniques are being used (Chapelle & Lovley, 1990). Both techniques have a minimum detection limit between 0.01 and 1 ppm. Sulfonic acids can be detected by fluorescence or by gas or liquid chromatography. In combination with mass spectrometry the detection limit can be as low as sub-ng/mL (Serres-Piole et al., 2012). For salts the maximum tracer concentration in the drill mud is not limited by solubility issues like for PFT, moreover they do not stain the drill mud in a bright color or cause any problems with disposal. Therefore concentrations can be set in the 100 s ppm range to provide three or more orders of magnitude detection range. In closed systems where the drill mud is recycled, dosing such high concentrations of salt is not much of a problem; a kilogram of salt per cubic meter of drill mud will not cause insurmountable logistical or financial challenges. In lake or ocean drilling operations however, where the drill mud is not recycled, the situation is different. As an example of a medium-sized drill rig, the Deep Lake Drilling System of DOSECC Exploration Services, LLC, uses around 20 m^3

of drilling fluid per day. This drilling system does not recirculate the drill fluid, but it exits the drill hole at the lake floor and is lost. For short operations, this will not cause any problems but eventually the amount of salt that has to be purchased and brought to the drill site might become an issue.

Different techniques for extraction of tracer have been used; the choice mainly depends on the volume of available sample and its porosity. For soft and water-saturated samples, centrifugation is the easiest option. Hydraulic pore-water squeezing is much more efficient but requires larger volumes of sediment, which might not be available in all cases. For very small sample volumes the only option might be slurrying the sample in deionized water, followed by centrifugation. Because this technique obviously involves dilution of the sample, it causes a loss in sensitivity.

Other types of chemical tracers that are being used in hydrothermal research are various types of sulfonic acids (Hirtz et al., 1993, 2001; Serres-Piole et al., 2012). The main advantage of these compounds is their thermal stability up to several hundred °C. They are widely used in geothermal research [see (Serres-Piole et al., 2012) for an extensive review] but rarely in scientific drilling (Jackson et al., 2015).

3.5 Microbiological and Molecular Ecological Techniques

Over the last two decades microbiological and molecular biological techniques have gained increased attention. In most cases no tracer was deliberately added but the microbial community composition of the drill fluid, the drilling equipment, and the recovered core samples were analyzed and compared.

The first attempts were culture-based and used *Escherichia coli* as tracer organisms, which were found in the drill mud as serendipitous contaminants (Beeman & Suflita, 1989). Detection was done via cultivation, which only allows for a qualitative assessment, basically providing the information whether viable *E. coli* was in the sample or not. Also, the absolute concentration of *E. coli* in the drill fluid was not determined, which also precludes the chance for a quantitative assessment. In a deep mine hosted in granite, (Pedersen, Hallbeck, Arlinger, Erlandson, & Jahromi, 1997) employed a more quantitative cultivation approach by using viable counts on agar plates as well as Most Probable Number (MPN) counts of sulfate reducing bacteria to estimate microbial abundance in drill fluid and core samples. The numbers were in good agreement with total cell counts.

Using a specific strain of microbes, whether deliberately added or not as a tracer has its advantages, as they most closely resemble the indigenous microbial population. However, the results should be interpreted with caution. It

remains questionable whether the distribution of *E. coli* cells provides a realistic estimate of the distribution of contaminant cells in a drill core because they are about an order of magnitude larger than subsurface microbes (Fagerbakke, Heldal, & Norland, 1996) and may therefore get trapped at pore throats where smaller indigenous cells can easily fit through. Other attempts were the addition of a phototrophic cyanobacterium followed by detection via cultivation and quantification of chl-a (Colwell et al., 1994), or a cultured GFP-expressing *Pseudomonas* strain and detection via a highly sensitive PCR (Juck et al., 2005).

The extreme sensitivity of PCR-based molecular techniques leads to their implementation as contamination tracers. The study of Pedersen et al. (1997) was the first that used comparison of 16s-rRNA gene sequences from drill core, drill mud, and from the surfaces of the tools to assess contamination. This technique was used and further refined in other studies (e.g., Davidson et al., 2011; Watanabe, Watanabe, Kodama, Syutsubo, & Harayama, 2000). The enormous potential of this technique becomes clear in a recent study of 2.5 km-deep submarine coal deposits. Cell abundances were as low as <10 cells/cm^3 and set a new record for both depth of life beneath the sea floor and low cell numbers (Inagaki et al., 2015). Although PFT was employed as a contamination tracer and all cores were checked for mechanical integrity by CT scan, the ultra-low cell abundances make contamination control almost useless as even PFT is on the limit of sensitivity that would be required to render a sample uncontaminated if there are only 10 indigenous cells/cm^3. Because the authors were aware of this issue, they sequenced the V1 to V3 region of 16s-rRNA genes in core samples as well as the drill fluid. They then applied a probabilistic approach to estimate the likelihood that a given taxon would be consistently sampled from a group of samples, either exclusively from the sediment samples or from both drill mud and sediment sample. In this way, those taxa were identified that were either (1) exclusive to sediment samples (**"**most conservative**"**) or (2) consistently found in sediment samples in significant abundance and only occasionally found in contamination controls in low abundance. These two groups of taxa were labeled "most conservative" or "most likely," respectively. Using this approach, correction factors to the raw cell concentrations in the samples were calculated to estimate the corresponding population sizes. So for each sample there is a "raw" cell count and a **"**most conservative**"** and **"**most likely**"** indigenous cell abundance, respectively. This massive amount of work might not be necessary for samples with higher cell abundances, but it shows very nicely the potential

that molecular tools have. Given the ever-decreasing price for sequencing and the rapid advances in automatizing routine steps like DNA extraction, such techniques might become routine in just a few years.

3.6 How to Chose the Right Tracer?

Each tracer has its strengths and weaknesses, and many factors have to be taken into account when deciding on a specific method.

Does a particular tracer work under the given circumstances? Not every tracer is suitable for every environment. Some tracers might be affected by the chemistry (pH, salinity) of the water used to prepare the drill. In cases where water with a chemical composition that deviates significantly from normal ocean or tap water is being used, tests prior to ordering any tracer or drill mud additives are highly recommended. As an example of what can happen when such tests were not carried out a drilling operation was carried out in a soda lake, its waters have a pH around 10 and salinity around 20‰. A synthetic thickener and clay minerals had to be used to increase viscosity of the drill mud to stabilize the hole. According to the data sheets of the additives, they should form a stable suspension up to pH 11. However, these tests were only made at low salinities and the suitability of the additives in a combination of high pH and high salinity was not tested. When trying to mix them with actual lake water, they did not form a viscous suspension, but flocculated and settled at the bottom of the mud tank. Due to the remote location, it was not possible to source other drill mud additives, so tens of thousand of liters of freshwater had to be brought from shore to the drilling platform on the lake, causing massive additional operational costs and delays.

Also, the thermal regime in the drill hole should be estimated. In deep holes or in hydrothermal settings the temperature might exceed the thermal stability of a tracer, most fluorescent tracers, either dyes or microspheres are not stable at temperatures above 100°C. In such cases PFT or sulfonic acids might be a better choice. The type of drill mud additives is an intensively discussed issue during the preparation of every drilling operation. When deciding on a specific tracer the additives have to be included in the consideration, as they can substantially alter the properties of the drill fluid and therefore of the tracer as well. For example, the addition of a polymer-based thickener at one of the drill sites of the Ketzin CO_2-sequestration test site led to a decreased infiltration of drill fluid into the core, most probably by clogging the pores on the outside (Wandrey, Morozova, Zettlitzer, Wurdemann, & Grp, 2010).

Is it technically/logistically/legally possible to use a particular tracer? How do I get the tracer mixed into the drilling fluid? This might sound like a simple question, but PFT is highly volatile so they have to be added to the drill mud directly at the intake of the mud pump, which requires dedicated delivery system that varies the delivery rate according to the flow rate of the mud pump. As already discussed in the chapter about PFT, installation of a pump to deliver the PFT into the intake of the drill mud pump has to be discussed with the drilling team well in advance.

Another issue that becomes important is the even distribution of the tracer. In case a tracer is mixed into the drill mud tank, it is of utmost importance to ensure that the tracer becomes well homogenized (Fig. 10). Kallmeyer et al. (2006) provide an example of a time-course measurement of microsphere concentration in a mud pit. It took several tens of hours before a tracer addition led to an increase in tracer concentration at the outflow of the well.

Another issue is the on-site measurement of a tracer. For fluorescent dyes, only a fluorometer is required, which is available as small portable units that can easily be brought into the field. Still, in most cases the sediment has to be slurried to extract the dye and then centrifuged to remove the particles. Centrifugation is also required to measure the dye concentration in the drill fluid. This means that a centrifuge has to be brought into the field as well. Sometimes filtration will also work, but this depends on the grain size of the

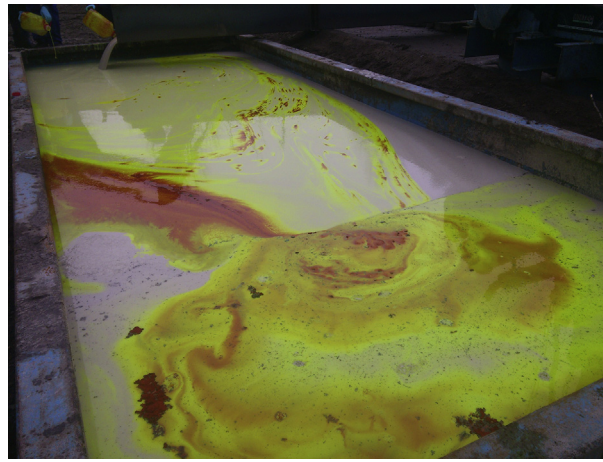

Figure 10 Mixing fresh fluorescein into a batch of drill fluid. The uneven distribution is apparent, and more mixing will be required to ensure homogeneous distribution. *Photo: Alawi, GFZ Potsdam.*

drill core and the composition of the drill fluid. Depending on the amount of liquid that is required for the measurement and the grain size of the material, the size of the centrifuge can vary. A suitable workspace has to be allocated for this equipment. PFT analysis requires a GC-ECD, which might be difficult to operate on site because it requires a carrier gas. Also, samples have to be taken immediately after retrieval of the core, something that might be difficult on small lake drilling barges or other systems with very little working space.

Allocation of workspace becomes even more of an issue when working with fluorescent microspheres. Even when analyzing them on site with a portable flow cytometer, a fluorescence microscope will be required for calibration and spot checks. Due to the relatively weak fluorescence signal a dark room for a microscope is required. If a dark room is not available then a large piece of dark cloth can be hung over the microscope and the head of the person using it, but this is not comfortable for many hours of work and should only be seen as a makeshift solution.

Legal constraints should also be taken into consideration. Depending on the location of the drill site, certain tracers might not be allowed. Also even if a tracer is absolutely nontoxic and does not cause any legal issues, it might raise some attention with the local population if it stains the drill fluid in a bright color. The impact of the local population on the success of a drilling campaign should never be underestimated, and it should be a primary goal of anyone involved in the drilling campaign to maintain a good relationship with the people who live nearby the drill site. Pumping many cubic meters of a brightly colored solution into the ground might not increase their sympathy toward the operation. Adverse reactions can range from revoking access rights over court orders to stop drilling to physical attacks. These are not theoretical considerations, all this did happen during various scientific drilling campaigns. If the choice of a less conspicuous tracer can help running a project more smoothly, then it should be done that way.

Depending on the location of the drill site, disposal of the drill mud after the termination of the drilling operation might also be an issue. Again, even if the tracer is nontoxic and does not pose any harm or additional problems for its safe disposal, if it stains the drill mud in a bright color, it might cause some problems.

What is the lowest concentration of tracer that I can detect in a sample, and is that level sufficient for the material I want to retrieve?

It is of no use to invest lots of resources into a contamination control method that actually works but to find out later that the minimum detection limit is not sufficient and small, but in this case significant, contamination passes unnoticed. Several factors have to be taken into account: concentration of cells in the drill fluid, lowest expected cell concentration in the sample, tracer concentration, and minimum detection limit. Even if these calculations cannot be solved with great accuracy, they will at least provide an order-of-magnitude estimate that will indicate whether the planned approach is feasible or not. There should be a safety margin of at least one order of magnitude.

4. CONCLUDING REMARKS

Drilling is a science in itself and no bio- or geoscientist should feel bad for not being familiar with all the technical details. For the success of every scientific drilling operation, it is therefore of utmost importance to develop a drilling strategy in close collaboration with those people who will eventually run the drilling operation. Most commercial drilling companies have little to no experience working with scientist and vice versa. So it is absolutely necessary for the scientists to clearly formulate their expected goals and requirements and then discuss them with the drilling company. Many scientific drilling operations do not utilize their full potential because of insufficient planning or coordination between the science and the drilling team.

Even as someone with little or no practical experience in drilling, one should be at least familiar with the basic principles and techniques to be able to discuss the goals and technical strategies to achieve them. Perhaps the single most important issue that determines the success of a biogeochemical drilling campaign is to get involved as early as possible. Drilling with contamination control is different from drilling without, but most changes can easily be implemented at an early stage. Nothing is worse and more problematic than trying to implement contamination control in a project at the last minute. It is not just the technical changes, but also the workflow needs to be adjusted to accommodate the additional sampling, plus the extra space for analyses and the potentially different legal situation.

A solid dataset from pilot experiments is also a prerequisite. Will the tracer work under the given conditions and will it provide sufficient

sensitivity. As mentioned previously, there are many things that can go wrong, but most of them can be figured out well ahead of drilling. As a final remark, one should not underestimate the time that is necessary to organize a drilling campaign, irrespective of contamination control. The timeframe can easily be measured in years from the first draft of the project over the planning and permitting process to the actual drilling and later on the closing of the borehole and demobilization of the drill rig. Despite all the efforts that have to be put into drilling campaign, it is the only way to obtain samples from subsurface environments.

Every drilling campaign provides new and exciting samples so all the hassle is well worth it.

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CHAPTER FOUR

Microbial Phosphite Oxidation and Its Potential Role in the Global Phosphorus and Carbon Cycles

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Contents

Abstract

Phosphite (HPO_3^{2-}) is a highly soluble, reduced phosphorus compound that is often overlooked in biogeochemical analyses. Although the oxidation of phosphite to phosphate is a highly exergonic process ($E^{o'} = -650$ mV), phosphite is kinetically stable and can account for 10–30% of the total dissolved P in various environments. There is also evidence that phosphite was more prevalent under the reducing conditions of the Archean period and may have been involved in the development of early life. Its role as a phosphorus source for a variety of extant microorganisms has been known since the 1950s, and the pathways involved in assimilatory phosphite oxidation have been well characterized. More recently, it was demonstrated that phosphite could also act as an electron donor for energy metabolism in a process known as dissimilatory phosphite oxidation (DPO). The bacterium described in this study, *Desulfotignum phosphitoxidans* strain FiPS-3, was isolated from brackish sediments and is capable of

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growing by coupling phosphite oxidation to the reduction of either sulfate or carbon dioxide. FiPS-3 remains the only isolated organism capable of DPO, and the prevalence of this metabolism in the environment is still unclear. Nonetheless, given the widespread presence of phosphite in the environment and the thermodynamic favorability of its oxidation, microbial phosphite oxidation may play an important and hitherto unrecognized role in the global phosphorus and carbon cycles.

1. INTRODUCTION

Phosphite (HPO_3^{2-}) is a bioaccessible, reduced phosphorus compound that is present in a variety of environments throughout the world, and yet, its role in biogeochemistry is often overlooked. Phosphate (PO_4^{3-}) is the dominant inorganic P species on Earth, and it is in this oxidation state (P^{5+}) that phosphorus is incorporated into biological molecules (Pasek, 2008; Pasek, Sampson, & Atlas, 2014) (Fig. 1). However, it has been known since the 1950s that certain microorganisms are capable of using phosphite (which has a P^{3+} oxidation state) as a P source by oxidizing it to phosphate, which they then incorporate into their cells (Adams & Conrad, 1953) (Fig. 1). This process is known as assimilatory phosphite oxidation (APO), and its genetic basis and biochemical mechanisms have been extensively studied (Casida, 1960; Costas,

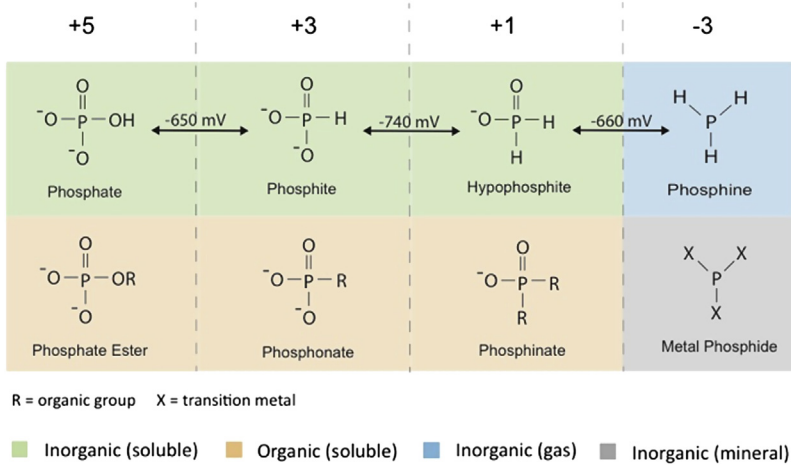

Figure 1 Chemical structures and redox properties of phosphorus compounds discussed in the text. The *number* above each column indicates the P oxidation state of the compounds below. *Arrows* indicate the standard reduction potential at pH 7 of each redox couple. *Based on Roels, J., & Verstraete, W. (2001). Biological formation of volatile phosphorus compounds. Bioresource Technology 79(3), 243–250.*

White, & Metcalf, 2001; Foster, Winans, & Helms, 1978; Malacinski & Konetzka, 1966; Metcalf & Wolfe, 1998; White & Metcalf, 2004b; Wilson & Metcalf, 2005; Yang, Metcalf, & Frey, 2004). More recently, it was shown that phosphite could also act as an electron donor and energy source for microbial growth and carbon fixation in a process known as dissimilatory phosphite oxidation (DPO) (Schink & Friedrich, 2000). The existence of DPO is perhaps not surprising when one considers the chemical properties of phosphite: it is about 1000 times more soluble than phosphate under similar conditions, it is kinetically stable and thus unlikely to participate in unwanted reactions, and its oxidation is very thermodynamically favorable due to the low redox potential (−650 mV) of the phosphate/phosphite couple (Pasek, 2008; Roels & Verstraete, 2001; White & Metcalf, 2007).

Given the benefits of using phosphite both as a phosphorus source and as an electron donor, it is likely that many more phosphite-oxidizing microbes remain to be discovered. Such a prospect raises several questions about the characteristics of microbial phosphite oxidation and its potential impact on the environment. What distinguishes DPO from APO in terms of genetics, physiology, and bioenergetics? In which kinds of environments would we expect to find each of these metabolisms? How prevalent are these metabolisms and what are their phylogenetic distributions? How might phosphite-oxidizing microbes affect global cycling of phosphorus and carbon? In order to address these questions, we will review the current state of knowledge regarding the geochemistry of phosphite and the biology of phosphite oxidation and discuss the roles that microbial phosphite−oxidizing processes might play in the global biogeochemical context.

2. PHOSPHITE GEOCHEMISTRY FROM THE ARCHEAN TO THE ANTHROPOCENE

2.1 Evidence for the Prevalence of Phosphite on Early Earth

Although conditions on early Earth are still a matter of much debate, Pasek et al. have argued that reduced phosphorus compounds, in particular phosphite, were abundant when life first emerged during the Archean period (4−2.5 Gya) (Pasek, 2008; Pasek & Kee, 2011; Pasek et al., 2013). They note the fact that most meteorites contain phosphide minerals (P^{3-} oxidation state), such as schreibersite ($[Fe,Ni]_3P$), which can abiotically corrode in the presence of water to release reduced P compounds such as phosphite, hypophosphite ($H_2PO_2^-$ P^{1+} oxidation state), and phosphine

gas (PH_3, P^{3-} oxidation state) (Bryant & Kee, 2006; Pasek & Kee, 2011; Pasek & Lauretta, 2005) (Fig. 1). Due to the heavy bombardment believed to have occurred 4.5−3.8 Gya, up to 1018 kg (i.e., 10% of the total P on the surface of the Earth) may have been derived from meteorite impacts (Pasek, 2008; Pasek & Kee, 2011; Pasek & Lauretta, 2008). Given that schreibersite corrosion occurs fairly rapidly at geological timescales ($1-10^4$ years) and phosphite can account for >50% of the total soluble reduced P produced, meteoritic impacts would have deposited a substantial quantity of phosphite on the early Earth (Pasek, 2008). Some additional phosphite could also have been derived from lightning discharges associated with volcanic activity since phosphite is known to occur when lightning strikes phosphate-containing minerals and volcanic ash (Glindemann, De Graaf, & Schwartz, 1999; Pasek & Block, 2009). Since phosphite is very kinetically stable (due to the 370 kJ of activation energy needed to break the P−H bond), it would have had a half-life of 10^8-10^{10} years under the reducing conditions of the Archean and could therefore have accumulated in the early ocean to concentrations of up to 10 mM (Pasek, 2008). The recent detection of phosphite at relatively high proportions in 3.5 billion−year-old marine carbonate rocks appears to support this scenario (Pasek et al., 2013).

The idea that reduced phosphorus compounds may have been involved in the development of early life was first proposed by Gulick in the 1950s (Gulick, 1955). He reasoned that phosphate would have been a poor substrate for the phosphorylation of prebiotic organic molecules due to its low solubility and reactivity, whereas reduced P species such as phosphite and hypophosphite, which are significantly more soluble and more reactive toward organic carbon and nitrogen compounds, could have facilitated the emergence of phosphorylated biomolecules (Gulick, 1955). Gulick's theory was dismissed at the time because there was no known source of reduced P that could account for the proposed reactions, but in light of recent evidence for the prevalence of phosphite on early Earth, Pasek and Kee (2011) have revisited this idea. In a series of experiments, they showed that schreibersite corrosion in water not only produces phosphite and hypophosphite but can also lead to the phosphorylation of simple organic molecules such as acetate and ethanol (Pasek, Dworkin, & Lauretta, 2007; Pasek & Lauretta, 2005). Based on these findings, it seems plausible that phosphite could have played a key role in the emergence of life, although further work is needed in order to establish the relevance of these reactions within the context of protobiotic chemistry.

2.2 Phosphite on Modern Earth: Where Does It Come From?

It had been previously assumed that reduced P compounds present on early Earth would have been gradually oxidized to phosphate after the Great Oxygenation Event (~2.5 Gya), and therefore, phosphite should be a negligible component of modern environments (Pasek, 2008). However, phosphite has recently been detected in various environments including rivers, lakes, swamps, and geothermal pools (Han et al., 2013; Pasek et al., 2014; Pech et al., 2009). The phosphite concentrations measured in these studies ranged from 0.1 to 1.3 μM and accounted for 1–33% of the total dissolved P in the systems. Although phosphite tended to be more abundant under more reducing conditions, concentrations of up to 1 μM were observed even in some surface water samples (Pasek et al., 2014). The presence of micromolar amounts of phosphite in oxygen-exposed environments is unexpected, given that phosphite reacts with oxygen fairly rapidly at geological timescales (Pasek, 2008). As noted by Pasek et al., meteorite strikes and lightning discharges are relatively rare on modern Earth, making it unlikely that these processes by themselves can account for the amounts of phosphite detected in surface waters (Pasek & Kee, 2011; Pasek et al., 2014). Some of this observed phosphite might be of anthropogenic origin since it can be a byproduct of the industrial production of phosphonates (compounds with C–P bonds), which are used as herbicides, detergents, and chelating agents (Nowack, 2003; Ternan et al., 1998; Yu et al., 2015). Additionally, phosphite itself is used as a reducing agent in some industrial metal electroplating processes (Nagaosa & Aoyama, 2001) and as a fungicide in agriculture (Thi Bich Thao, Yamakawa, & Shibata, 2009). Phosphite can therefore be a component of industrial waste as well as agricultural runoff and has in fact been detected in the influent of wastewater treatment plants (Yu et al., 2015). Han et al. (2013) have also observed higher phosphite concentrations at heavily polluted lake sites compared to less impacted areas.

In pristine environments, geothermal activity may potentially serve as an alternate source of phosphite via the formation and subsequent corrosion of metal phosphides. Similar to other reduced P compounds, phosphide minerals are unstable in the presence of oxygen at geological timescales and are therefore rare on the Earth's surface (Britvin et al., 2015). The deposition of extraterrestrial schreibersite by meteorites and the reduction of phosphorus impurities in iron ore during industrial smelting are typically cited as the only significant sources of phosphides on Earth (Britvin et al., 2015; Glindemann et al., 1998; Pasek, 2008). Nonetheless,

natural terrestrially produced schreibersite has been found in iron-rich basalts in Greenland (Pauly, 1969; Pedersen, 1981), in ultramafic rocks uncovered during continental drilling in China (Yang et al., 2005), and in pyrometamorphic rocks in the Levant (Britvin et al., 2015). Britvin et al. (2015) cite these findings as evidence for "the occurrence of geologically juvenile terrestrial phosphides" and outline the four conditions necessary for the formation of these compounds: (1) the presence of phosphorus, (2) the presence of transition metals such as Fe or Ni, (3) a highly reducing geochemical environment, and (4) temperatures high enough to sustain the reduction process . Based on these criteria it is likely that metal phosphide formation occurs in the subsurface due to the geothermal reduction of phosphate. Indeed, Glindemann et al. (1998) have noted that the strong reducing conditions observed within the Earth's crust should be conducive to the reduction of phosphate minerals to phosphides. The average elemental proportion of phosphorus in the Earth's crust is thought to be about 0.1%, although it may be higher in the oceanic crust due to phosphate deposition into porous subseafloor basalts during hydrothermal circulation of seawater along mid-ocean ridge flanks (Clarke & Washington, 1924; Wheat, Feely, & Mottl, 1996). Subseafloor basalts are also rich in Fe(II) and other reduced chemical species such as H_2, H_2S, and CH_4 (Edwards, Bach, & McCollom, 2005). Furthermore, temperatures at the contact zone between mantle-derived magma and seawater at mid-ocean ridge-spreading zones can be as high as 400°C (Deborah, Baross, & Delaney, 2003). Reduction of phosphate in the presence of metal salts to produce metal phosphides is known to occur within hours at temperatures as low as 400°C in the presence of hydrogen gas (Prins & Bussell, 2012). The subseafloor crust therefore appears to satisfy all the requirements for the formation of metal phosphides, which would subsequently react with seawater at short geological timescales to release phosphite and other reduced P compounds. Since phosphite is highly soluble and kinetically stable, it would likely diffuse up through the porous basalt into the cooler upper layers of the subseafloor and possibly into the water column before being re-oxidized by dissolved oxygen in the ocean.

Phosphite may also be derived from biological processes, such as phosphonate degradation. Phosphonates are organic compounds with C−P bonds, as opposed to the C−O−P esters found in organophosphate compounds, and they have a P oxidation state of +3, as in phosphite (Metcalf & Wanner, 1991; White & Metcalf, 2007) (Fig. 1). They can account for up to 25% of the dissolved organic P in some marine

environments (Clark, Ingall, & Benner, 1999; Kolowith, Ingall, & Benner, 2001). Some of this environmental phosphonate may be derived from industrial processes, but there are also biological routes for phosphonate production. Various organisms can incorporate phosphonates into their cell membranes as phosphonolipids or secrete antibiotic phosphonate compounds such as fosfomycin (Martinez et al., 2011; White & Metcalf, 2007). Although the biosynthetic pathways for these compounds have not been well characterized, the conversion of phosphoenolpyruvate (PEP) to phosphonopyruvate by the enzyme PEP mutase is thought to be a common initial step in the production of phosphonates (White & Metcalf, 2007). Phosphonates can also serve as a source of phosphorus or carbon for a variety of microorganisms, and several pathways for phosphonate degradation have been characterized (White & Metcalf, 2007). For example, some bacteria can use methylphosphonate as a P source in a process that releases methane and inorganic phosphate (Karl et al., 2008). This process is catalyzed by the C—P lyase enzyme and involves a phosphate radical intermediate (Buckel, 2013; Kamat et al., 2013). Under mildly reducing conditions, phosphate radicals can rearrange to form phosphite, making it a possible byproduct of methylphosphonate degradation in anaerobic environments (Pasek, 2008; Pasek et al., 2014). Moreover, phosphonates with carbonyl or hydroxyl groups at the α-carbon, such as phosphonoformic acid, tend to form phosphite rather than phosphate as the product of C—P cleavage even under oxidizing conditions (Freeman, Irwin, & Schwalbe, 1991). Given that C—P lyase enzymes are involved in the degradation of a variety of phosphonates, it is possible that these reactions are a significant source of environmental phosphite.

Biological phosphate reduction has also been posited as a possible source of environmental phosphite. Dévai et al. detected phosphine gas production in wastewater and marsh soils and showed that phosphine production was stimulated by the addition of inorganic phosphate and organic matter, leading them to conclude that phosphate was being reduced to phosphine by microorganisms present in their samples (Dévai et al., 1988; Dévai & Delaune, 1995). Some of this phosphine could subsequently be oxidized to phosphite in the presence of O_2 or UV radiation (Stone & White, 2012; Zhu et al., 2006). However, the conclusion by Dévai et al. that the phosphine they observed was derived from biological phosphate reduction has since been questioned by several researchers (Glindemann et al., 1998; Morton et al., 2005; Roels & Verstraete, 2001, 2004). Roels and Verstraete (2001) have noted that biological phosphate reduction is problematic from a

thermodynamic standpoint, since there is no known biological electron donor with a low-enough redox potential to make the reaction exergonic. Glindemann et al. (1998) have shown that phosphine can be produced during the corrosion of iron, even under sterile conditions. This is due to the fact that iron minerals often contain phosphorus impurities that can be abiotically reduced to iron phosphides during the industrial smelting process and these phosphides can then be released as phosphine gas during corrosion (Glindemann et al., 1998). Subsequent studies have likewise concluded that phosphine is released due to iron corrosion and that the higher rates of phosphine production observed in the presence of microorganisms is likely due to the microbial production of organic acids and hydrogen sulfide, which accelerate the corrosion process (Morton et al., 2005; Roels & Verstraete, 2004).

Although evidence of biological phosphate reduction remains inconclusive, several theoretical mechanisms by which this process could occur have been proposed. Pasek et al. (2014) have suggested that in addition to being produced during phosphonate degradation, phosphite could also be formed as a byproduct of phosphonate biosynthesis in reducing environments. They determined that the reductive cleavage of PEP by H_2 to form phosphite and pyruvate is thermodynamically feasible under standard cellular conditions (Pasek et al., 2014). Given that PEP is a key intermediate in the production of phosphonates from inorganic phosphate, such a mechanism would be a way of indirectly converting phosphate to phosphite. A more direct mechanism of phosphate reduction has been proposed by Roels and Verstraete (2001), who note that the reduced molybdoferredoxin cofactor of the nitrogenase complex has a redox potential of −1.0 V, which is low enough to reduce phosphate to phosphite. However, they question the usefulness of such a reaction since energy from ATP hydrolysis must be expended in order to achieve such a low reduction potential and the organism would gain nothing from the production of phosphite. Nevertheless, it is possible that phosphite may be formed as an unwanted product of nitrogenase function in the presence of phosphate. This sort of inadvertent phosphate reduction might also occur in photosynthetic organisms, since the redox potentials of excited reaction-center chlorophyll molecules range from −0.8 V (P680) to −1.26 V (P700) (Blankenship & Prince, 1985). Environments dominated by anoxygenic phototrophs may therefore be potential hotspots of biological phosphite production since the absence of strong oxidants in these systems would favor the accumulation of reduced phosphorus species.

3. PHOSPHITE OXIDATION IN BIOLOGY

3.1 Phosphite as a Microbial Phosphorus Source

The process of APO, which allows certain microorganisms to use phosphite as a phosphorus source, has been fairly well studied. At least 20 microbial isolates have so far been shown to carry out APO under laboratory conditions, including proteobacteria, firmicutes, and cyanobacteria (Adams & Conrad, 1953; Foster et al., 1978; Malacinski & Konetzka, 1966; Martinez et al., 2011; Metcalf & Wolfe, 1998; Schink & Friedrich, 2000; Wilson, 2006; Wilson & Metcalf, 2005; Yang et al., 2004). Genetic and biochemical studies of some of these organisms have revealed several enzymes capable of oxidizing phosphite (White & Metcalf, 2007). Some C—P lyases, such as the one found in *Escherichia coli*, are able to metabolize phosphite in addition to phosphonates, although the exact mechanism of the reaction has not been determined (Metcalf & Wanner, 1991). Based on what is known about the mechanism of methylphosphonate degradation by C—P lyase, phosphite oxidation to phosphate most likely proceeds through a radical cleavage of the P—H bond (Buckel, 2013; Frost et al., 2002; Kamat et al., 2013). It is not clear what determines the substrate specificity of these enzymes, since not all C—P lyases are capable of oxidizing phosphite (White & Metcalf, 2007). *Pseudomonas stutzeri*, for example, has two distinct C—P lyase operons, only one of which confers the ability to carry out APO (White & Metcalf, 2004b). Another enzyme known to oxidize phosphite is the bacterial alkaline phosphatase (BAP) of *E. coli*, which is a periplasmic protein encoded by the *phoA* gene that is normally involved in the hydrolysis of organophosphates for P uptake during phosphate starvation (Yang et al., 2004). In addition to its phosphatase activity, however, *E. coli* BAP has also been shown to oxidize phosphite to phosphate in vitro in a reaction that yields molecular hydrogen (Yang et al., 2004). This reaction is thought to proceed in a similar manner to a typical phosphate ester hydrolysis reaction, except that instead of an alkoxide (RO^-) leaving group, the hydride (H^-) from phosphite serves as a leaving group and reacts with a proton to form H_2 (Yang et al., 2004). The ability to oxidize phosphite appears to be a unique property of the *E. coli* BAP, since various other alkaline phosphatases from bacteria as well as eukaryotes have been tested for phosphite-oxidizing activity, and so far, none have yielded positive results (Yang et al., 2004).

The third known APO enzyme is the phosphite dehydrogenase encoded by the *ptxD* gene (Costas et al., 2001; White & Metcalf, 2007). The PtxD enzyme from *P. stutzeri* is capable of oxidizing phosphite in vitro with

NAD^+ as its sole cofactor, yielding phosphate and NADH (Costas et al., 2001). The *P. stutzeri* PtxD has a high affinity for both phosphite ($Km = 53.1$ μM) and NAD^+ ($Km = 54.6$ μM), but no activity has so far been detected in the presence of various potential alternative substrates including hypophosphite, thiophosphite, methylphosphonate, aminoethylphosphonate, D-3-phosphoglycerate, glycerate, lactate, formate, nitrite, arsenite, and sulfite (Costas et al., 2001; Relyea & van der Donk, 2005). PtxD belongs to the NAD^+-dependent D-hydroxy acid dehydrogenase protein family, but is the only member known to have an inorganic substrate (Relyea & van der Donk, 2005). None of the other members of this family tested to date (D-3-phosphoglycerate dehydrogenase, lactate dehydrogenase, glycerate dehydrogenase, and formate dehydrogenase) are capable of phosphite oxidation (Relyea & van der Donk, 2005). The specificity of this enzyme therefore sets it apart from C—P lyase and BAP and suggests that its biological role is limited to phosphite oxidation. Although no crystal structure of PtxD is currently available, Relyea and van der Donk (2005) have used protein alignments, homology models, and site-directed mutagenesis to identify four key residues (Lys76, Arg237, Glu266, and His292) likely to be involved in substrate binding and catalysis. However, further work is needed in order to elucidate the exact enzymatic mechanism by which phosphite oxidation occurs. In addition to *ptxD*, four other genes are found in the *ptxABCDE* operon of *P. stutzeri* (Metcalf & Wolfe, 1998; White & Metcalf, 2004a, 2007) (Fig. 2). The *ptxABC* gene cluster encodes a phosphite ABC transporter, while *ptxE* encodes a transcription factor belonging to the LysR family (Metcalf & Wolfe, 1998). However, deletion of *ptxE* appears to have no effect on the expression of the *ptx* operon or on the ability of *P. stutzeri* to grow on phosphite as its sole P source, so its role remains a mystery (White & Metcalf, 2004a, 2007). Expression of the *ptx* operon in *P. stutzeri* is induced by phosphate starvation and regulated by the PhoBR two-component system common to other phosphate starvation—inducible genes (White & Metcalf, 2004a). Interestingly, the presence of phosphite does not induce the *ptx* operon when excess phosphate is present, which confirms that the only role of this operon is to provide phosphorus for the cell (White & Metcalf, 2004a).

There are currently 601 genomes with predicted *ptxD* homologs in the Integrated Microbial Genomes (IMG) database, which represents roughly 1.5% of the total bacterial and archaeal genomes in the database. Since PtxD appears to be a dedicated phosphite dehydrogenase, it is likely that most, if not all, of these organisms are capable of APO. However, only

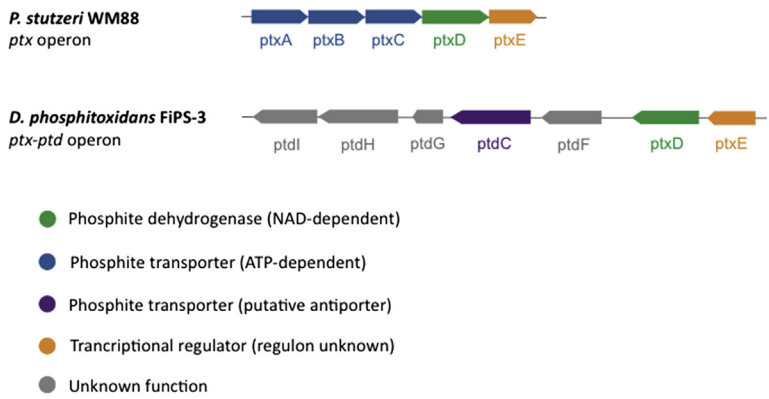

Figure 2 Phosphite oxidation operons of *Pseudomonas stutzeri* WM88 and *Desulfotignum phosphitoxidans* FiPS-3. WM88 is only capable of assimilatory phosphite oxidation, whereas FiPS-3 can carry out both assimilatory and dissimilatory phosphite oxidation.

five of these candidates have so far been tested for their ability to oxidize phosphite and very little work has been done to document the prevalence of APO in the environment (Martinez et al., 2011; Metcalf & Wolfe, 1998; Simeonova et al., 2010; Wilson, 2006; Wilson & Metcalf, 2005). Stone and White surveyed 12 different soil and freshwater sediment samples and found that 10–67% of bacteria were capable of growth with phosphite as the sole P source based on most probable number counts (Stone & White, 2012). Interestingly, they did not find a significant difference in the proportion of APO-capable bacteria when they compared pristine sites to those impacted by human activity (Stone & White, 2012). Martinez et al., (2011) identified a strain of the globally abundant cyanobacterium *Prochlorococcus* that is capable of APO and confirmed the presence of the *ptxABCD* gene cluster in its genome. They also noted the presence of *ptx* clusters in the genomes of other marine bacteria such as *Cyanothece* sp., *Trichodesmium erythraeum*, *Nodularia spumigea*, and *Marinobacter aquaeolei* and concluded that APO may be an important strategy for P acquisition in the world's oceans (Martinez et al., 2011). Furthermore, several researchers have found evidence indicating lateral acquisition of *ptx* genes (Martinez et al., 2011; Metcalf & Wolfe, 1998; White & Metcalf, 2007; Wilson & Metcalf, 2005). Taken together, these observations suggest that the capacity for APO is widespread among microorganisms from a variety of environments and phylogenetic lineages, which makes sense given the competitive advantage that this ability confers, particularly under phosphate-limited conditions.

3.2 Phosphite as a Microbial Energy Source and Electron Donor

Desulfotignum phosphitoxidans strain FiPS-3 is currently the only known isolate capable of DPO (Schink & Friedrich, 2000; Schink et al., 2002). This bacterium was isolated from brackish canal sediments in Venice, Italy, and is able to grow by coupling phosphite oxidation to the reduction of either sulfate to sulfide, carbon dioxide to acetate, or nitrate to ammonia (Poehlein, Daniel, Schink, & Simeonova, 2013; Schink et al., 2002). As seen in Eqs. (1)–(3) (Table 1), the oxidation of phosphite coupled to sulfate, carbon dioxide, or nitrate reduction is exergonic and yields enough energy to drive ATP formation, which requires approximately 40–50 kJ mol^{-1} ATP under typical intracellular conditions (Thauer, Jungermann, & Decker, 1977). The ability to conserve this energy and grow with phosphite as the

Table 1 Chemical equations and free-energy values for reactions discussed in the text

Reactions	$\Delta G^{o\prime}$ (kJ·mol^{-1} HPO_3^{2-})	References
$4HPO_3^{2-} + SO_4^{2-} + H^+ \rightarrow 4HPO_4^{2-} + HS^-$ (1)	−91	Schink et al. (2002)
$4HPO_3^{2-} + 2CO_2 + 2H_2O \rightarrow 4HPO_4^{2-} + CH_3COO^- + H^+$ (2)	−77	Schink et al. (2002)
$4HPO_3^{2-} + NO_3^- + H_2O + H^+ \rightarrow 4HPO_4^{2-} + NH_3$ (3)	−89	This review (based on Poehlein et al. (2013); Thauer et al. (1977))
$HSO_3^- + 3H_2 \rightarrow SH^- + 3H_2O$ (4)	−172	Badziong and Thauer (1978)
$HSO_3^- + 3NADH + 3H^+ \rightarrow SH^- + 3NAD^+ + 3H_2O$ (5)	−118	This review (based on Badziong & Thauer, 1978; Thauer et al., 1977)
$HPO_3^{2-} + NAD^+ + ADP^{3-} \rightarrow NADH + ATP^{4-}$ (6)	−14	This review (based on Schink et al., 2002; Thauer et al., 1977)

$\Delta G^{o\prime}$ = Gibbs free energy under standard conditions at pH 7. Equations and values were taken from the studies cited under references or calculated based on data from these studies.

sole electron donor is what distinguishes a DPO-capable organism from one that merely uses phosphite as a P source by means of APO. Still, one would expect any organism capable of DPO to also be capable of APO, since the phosphate produced by this metabolism could be subsequently incorporated into biomass. This is the case with FiPS-3, which can use phosphite as its sole P source in the presence of an alternative electron donor such as fumarate (Simeonova et al., 2010). However, the amount of phosphite needed to drive the growth of FiPS-3 to high cell densities (~10 mM) is two orders of magnitude greater than that required for use as a P source (~0.1 mM), which leads to the accumulation of phosphate in the medium during DPO (Schink et al., 2002; Simeonova et al., 2010). No other APO-capable organism tested so far has been able to grow with phosphite as its sole electron donor or to accumulate phosphate in the medium, which indicates that DPO is a distinct metabolic process (White & Metcalf, 2007).

FiPS-3 belongs to the *Desulfobacterales*, an order within the Deltaproteobacteria comprising sulfate-reducing bacteria (Schink et al., 2002). Interestingly, the closest known relative of FiPS-3, *Desulfotignum balticum* strain SaxT, is not capable of either DPO or APO even though the two strains have 99% 16S rDNA identity (Schink et al., 2002). One salient difference between the two strains is that homologs of *ptxD* and *ptxE* are present in the genome of FiPS-3 but not in that of SaxT or other members of the *Desulfobacterales* (Poehlein et al., 2013). In FiPS-3, *ptxDE* are part of a seven-gene operon distinct from the *ptx* operon of *P. stutzeri* (Poehlein et al., 2013; Simeonova et al., 2010) (Fig. 2). The FiPS-3 operon lacks the *ptxABC* genes, which encode an ATP-dependent phosphite transporter and are typically found in APO gene clusters (Simeonova et al., 2010; White & Metcalf, 2007). Although the PtxD enzyme from FiPS-3 has not yet been purified and characterized in vitro, its sequence indicates that it is the most divergent of known PtxD homologs; yet, it still retains the predicted catalytic residues and NAD-binding site (Martinez et al., 2011; Simeonova et al., 2010).

In addition to *ptxDE*, there are five other genes in the operon, *ptdFCGHI*, that show no homology to genes found in known APO gene clusters (Simeonova et al., 2010) (Fig. 2). Although the functions of these genes have not yet been experimentally determined, their annotations in the IMG database offer some insight into their possible roles. PtdC (IMG locus tag: Dpo_11c01230) is annotated as a glycerol-3-phosphate transporter belonging to the major facilitator superfamily. The glycerol-3-phosphate transporter from *E. coli*, GlpT, functions as an antiporter, which

couples the export of inorganic phosphate from the cell to the import of glycerol-3-phosphate (Lemieux, Huang, & Wang, 2005). PtdF (IMG locus tag: Dpo_11c01240) is annotated as an UDP-glucose 4-epimerase, an enzyme that in *E. coli* converts UDP-galactose to UDP-glucose and uses NAD^+ as a cofactor (Bauer et al., 1992). PtdG (IMG locus tag: Dpo_11c01220) is annotated as a nucleotide-binding universal stress protein from the UspA family. In *E. coli*, UspA is a phosphoprotein that is expressed in response to a wide variety of stresses including growth arrest during stationary phase, exposure to heat, and carbon, nitrogen, phosphorus, and sulfur starvation (Kvint et al., 2003). UspA can autophosphorylate with ATP or GTP as phosphate donors, but its exact biochemical function in cellular stress response remains unknown (Kvint et al., 2003). PtdH (IMG locus tag: Dpo_11c01210) is predicted to be a B12-dependent radical S-adenosylmethionine (SAM) family protein. Members of this protein family are characterized by the presence of both an SAM-binding domain and a cobalamin-binding domain and are able to generate radical intermediates that may be involved in a variety of reactions such as methylations, dehydrogenations, bond cleavages, molecular rearrangements, and substrate activations (Broderick et al., 2014). PtdI (IMG locus tag: Dpo_11c01200) is a hypothetical protein with no homologs of known function in the IMG database.

Simeonova et al. (2009) have shown that expression of the PtdF protein is increased in the presence of phosphite. However, when they heterologously expressed the *ptxD—ptdFCG* cluster in SaxT, they found that, although it gained the ability to use phosphite as a P source, it was still unable to grow with phosphite as an electron donor (Simeonova et al., 2010). Furthermore, when they transformed SaxT with different versions of the *ptxD—ptdFCG* plasmid containing single deletions of each of the genes, they found that deleting either *ptxD* or *ptdC* abolished the ability of transformants to grow with phosphite as sole P source but deleting *ptdF* or *ptdG* did not (Simeonova et al., 2010). These results indicate that *ptxD* and *ptdC* are necessary and sufficient for phosphite uptake and oxidation, but additional genes are needed in order to couple phosphite oxidation to cell growth. Presumably, PtxD carries out the phosphite-oxidizing step via an NAD-dependent mechanism similar to that described in *P. stutzeri*. Based on its homology to GlpT, Simeonova et al. (2010) have posited that PtdC acts as a phosphite/phosphate antiporter in lieu of the ATP-dependent transporter typically encoded by *ptxABC* in APO-capable bacteria. What role the other four *ptd* genes may play in DPO remains unclear. It is likely that *ptdH* and *ptdI*, which were not included in the expression

plasmid, are required for energy conservation along with *ptdF* and *ptdG*. However, it is also possible that additional genes outside of the *ptx—ptd* operon are involved in the metabolism. Future work is needed in order to conclusively answer this question, but based on the available evidence, it appears that the *ptx—ptd* operon harbors the genes responsible for DPO in FiPS-3.

3.3 Bioenergetics of Phosphite Oxidation

The fact that PtxD alone does not confer the ability to grow with phosphite as an electron donor raises an intriguing question: Why are other genes besides PtxD required for DPO? The PtxD of *P. stutzeri* is known to produce NADH during phosphite oxidation and *P. stutzeri* carries out APO under aerobic conditions, meaning it should be capable of generating about 2 mol ATP per mol NADH oxidized by the electron transport chain (Rich, 2003). Why, then, is this organism not capable of growing by DPO? The answer probably has to do with the way phosphite is transported into the cell. Recent work has shown that ABC transporters hydrolyze two molecules of ATP for each molecule of substrate they import (Patzlaff, van der Heide, & Poolman, 2003). Since *P. stutzeri* uses the ABC transporter PtxABC to bring phosphite into the cell, the cost of transport would consume the two ATP produced from phosphite oxidation. Furthermore, the expected ATP yield of phosphite oxidation would actually be lower during active growth since some of the NADH produced would have to be diverted for use in anabolic reactions. APO in *P. stutzeri* would therefore be an energy-neutral or net energy-consuming process (Fig. 3a). FiPS-3, on the other hand, uses PtdC as its phosphite transporter instead of PtxABC. If PtdC does, in fact, function as a phosphite/phosphate antiporter as has been proposed, then there would be no energy cost associated with phosphite uptake in FiPS-3 (Fig. 3b).

However, when both PtxD and PtdC were expressed in SaxT, it still did not gain the ability to grow by DPO, which indicates that an additional mechanism of energy conservation, possibly mediated by the *ptdFGHI* genes, is required in this organism. In contrast to *P. stutzeri*, FiPS-3 and SaxT growing by sulfate reduction would gain substantially less energy from NADH oxidation. During sulfate reduction, two ATPs must be initially expended in order to activate and reduce sulfate to sulfite, which can then be further reduced to sulfide in an exergonic reaction (Badziong & Thauer, 1978). Sulfate-reducing bacteria growing on H_2 typically generate three ATP from the sulfite reduction step for a net overall

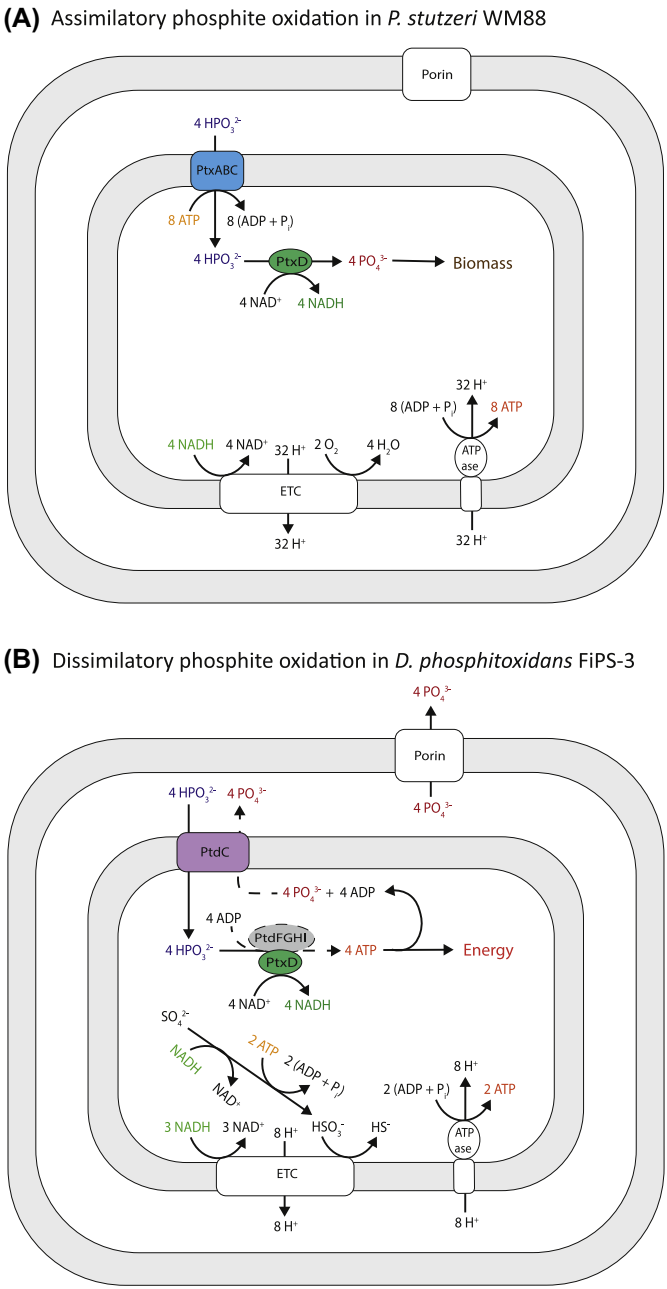

Figure 3 Cellular energetics and transport during aerobic assimilatory phosphite oxidation in *Pseudomonas stutzeri* WM88 (A) and dissimilatory phosphite oxidation coupled to sulfate reduction in *Desulfotignum phosphitoxidans* FiPS-3 (B). *Dotted lines* denote putative mechanisms based on physiological and genomic observations that have yet to be confirmed by direct biochemical evidence.

production of 1 mol ATP per mol sulfate reduced, which corresponds to the expected yield based on Eq. (4) (Table 1) (Badziong & Thauer, 1978). However, if sulfite reduction were instead coupled to NADH oxidation according to Eq. (5) (Table 1), the expected yield would only be 2 mol ATP per mol sulfite reduced, which would result in no net ATP production from the overall reduction of sulfate (Fig. 3b). In order to grow by DPO, therefore, FiPS-3 and SaxT would not only need to save energy on phosphite uptake but also conserve more of the free energy available from the oxidation of phosphite. The NAD^+/NADH couple has a redox potential of −320 mV under standard physiological conditions (Thauer et al., 1977), which means that the reduction of NAD^+ coupled to phosphite oxidation ($E^{o'} = -650$ mV) releases 63.7 kJ mol^{-1} phosphite. This additional energy is presumably lost in traditional APO-capable organisms, but there is evidence that it is conserved in FiPS-3. Schink et al. (2002) observed substantially higher cell yields when FiPS-3 was grown on phosphite and sulfate (12.1 g mol^{-1} phosphite) versus formate and sulfate (4.85 g mol^{-1} formate). Since phosphite and formate both donate two electrons and the redox potential of the CO_2/formate couple ($E^{o'} = -432$ mV) is actually higher than that of NAD^+/NADH (Thauer et al., 1977), the higher yields seen on phosphite are not consistent with NADH oxidation being the sole means of ATP production during DPO. Furthermore, the growth yield of FiPS-3 on phosphite and CO_2 via the Wood−Ljungdahl pathway (12.8 g mol^{-1} phosphite) was about 10 times higher than the yields typically observed for other Wood−Ljungdahl acetogens growing on H_2 ($E^{o'} = -414$ mV) and CO_2, such as *Acetobacterium woodii* (1.1 g mol^{-1} H_2) and *Acetogenium kivui* (1.3 g mol^{-1} H_2) (Schink et al., 2002; Thauer et al., 1977; Tschech & Pfennig, 1984; Yang & Drake, 1990). These results suggest that FiPS-3 can in fact take advantage of the extremely low redox potential of phosphite, although it is unclear how this is accomplished since there are no known biological redox carriers that can accept electrons at such a low potential (Schink et al., 2002). Schink et al. have proposed that ATP is generated from phosphite oxidation by means of substrate-level phosphorylation in addition to the reduction of NAD^+, thus yielding both energy and reducing equivalents for each molecule of substrate utilized (Schink et al., 2002). Such a reaction would be thermodynamically feasible according to Eq. (6) (Table 1). Therefore, the function of the *ptdFGHI* genes may be to facilitate substrate-level phosphorylation during phosphite oxidation (Fig. 3b). Relyea and van der Donk have suggested that one of the possible mechanisms of phosphite oxidation by PtxD may involve the creation of

a phosphorylated enzyme intermediate that is subsequently hydrolyzed to release phosphate (Relyea & van der Donk, 2005). PtdFGHI might interact with PtxD in order to facilitate the transfer of this phosphoryl group to ADP, either directly or by means of additional phosphorylated intermediates. This is a promising avenue for future inquiry, but more work is currently needed in order to determine whether phosphite acts as a phosphoryl donor for ATP synthesis during DPO and what role, if any, the *ptdFGHI* genes play in this process.

4. THE POTENTIAL ROLE OF MICROBIAL PHOSPHITE OXIDATION IN GLOBAL PHOSPHORUS CYCLING AND PRIMARY PRODUCTION

Given that FiPS-3 is currently the only organism known to carry out DPO, the environmental prevalence of this metabolism remains unclear. However, the presence of transposase genes flanking the *ptx—ptd* cluster in FiPS-3 as well as the fact that this cluster has not been found in SaxT or other members of the *Desulfobacterales* are strong indicators that these genes were horizontally acquired (Poehlein et al., 2013). This raises the possibility that, similar to APO genes, DPO genes may be propagated by lateral gene transfer and thus present in a phylogenetically diverse subset of microorganisms. Since growth by DPO requires higher concentrations of phosphite, it is probably less prevalent than APO on a global scale, although it may have a greater impact on P cycling in areas of phosphite enrichment due to the substantially higher rates of phosphate production during DPO compared to APO. Marine or estuarine sediments, such as the one from which FiPS-3 was isolated, are likely sites of phosphite accumulation due to the presence of biogenic phosphonates as well as the sulfidogenic nature of most marine sediments, which would help protect phosphite from abiotic oxidation. Furthermore, many coastal sediments are impacted by industrial wastewater and agricultural runoff, which might serve as additional sources of reduced phosphorus. Since abiotic phosphite oxidation is a slow process even under aerobic conditions, microbial oxidation is likely the principal mechanism by which phosphite is converted to phosphate at biologically relevant timescales. Given that phosphorus tends to be the limiting nutrient for marine life due to the low solubility of phosphate in the oceans (Redfield, 1958), phosphite oxidizers could play a key role in stimulating eutrophication in coastal waters as well as in promoting the growth of primary producers in the oligotrophic open ocean (Fig. 4).

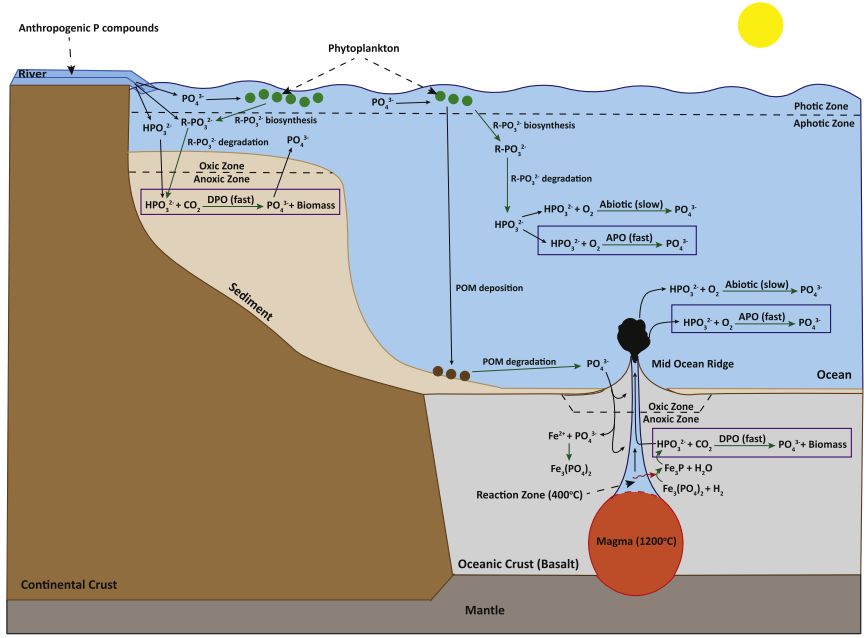

Figure 4 Potential sources and sinks of phosphite in marine environments and proposed role of microbial phosphite oxidation in phosphorus and carbon cycling. APO = assimilatory phosphite oxidation, DPO = dissimilatory phosphite oxidation, POM = particulate organic matter. *Solid black arrows* indicate transport, *green arrows* indicate chemical reactions, *red arrows* indicate heat, and *dotted black arrows* indicate labels.

In addition to its role in P cycling, DPO could also serve as an energetic electron-donating process in aphotic zones. Phosphite oxidation can be coupled to sulfate, nitrate, and carbon dioxide reduction in FiPS-3 and, given its extremely low redox potential, could theoretically be coupled to the reduction of any other known electron acceptor while yielding more energy per electron than any other known electron donor. This property would make phosphite especially attractive as a donor in aphotic environments such as the deep ocean and the subseafloor basaltic crust, where phosphite might be geochemically produced at biologically relevant timescales. Since no light and very little organic matter reaches the deep ocean, energy and organic carbon availability are thought to be the main factors limiting growth in these regions (Edwards et al., 2005; Lever, 2011). At hydrothermal vents and in the upper layers of subseafloor basalts, chemoautotrophic growth may be driven by the oxidation of geothermally derived reduced species such as H_2, CH_4, H_2S, Fe(II), and Mn(II) coupled to the reduction

of O_2 or NO_3^- present in seawater (D'Hondt et al., 2004; Edwards et al., 2005). However, organisms living below this redox transition zone do not have access to strong oxidants and have to depend on less energetically favorable metabolisms such as sulfate reduction, methanogenesis, and acetogenesis (Edwards et al., 2005; Lever, 2011). Deep subseafloor microbes are thought to exist in a near-constant state of starvation, so autotrophic growth poses a huge challenge even for organisms that use the Wood–Ljungdahl pathway, which is the most energy efficient of the known carbon fixation pathways (Lever, 2011). However, the use of phosphite as an electron donor could help overcome this energetic obstacle, as evidenced by the 10-fold higher cell yields seen during growth of FiPS-3 on phosphite and CO_2 compared to those typically observed for homoacetogenic autotrophs. If it were indeed prevalent in subseafloor basalts, phosphite would provide a substantial competitive advantage to DPO-capable autotrophs and could serve as a key driver of primary production in the deep biosphere (Fig. 4). Porous oceanic basaltic crust covers approximately 60% of the Earth's surface and extends down to 500 m deep, making it the largest aquifer in the world (Edwards et al., 2005; Nielsen & Fisk, 2010). By some estimates, this subseafloor biosphere may contain up to a third of the Earth's total living biomass (D'Hondt et al., 2004; Whitman, Coleman, & Wiebe, 1998). Subsurface carbon fixation driven by phosphite oxidation could therefore play an important and currently unrecognized role in the global carbon cycle.

5. CONCLUDING REMARKS

Over the last 20 years, the study of reduced phosphorus compounds and their role in nature has grown from a series of curious observations and intriguing theories into an exciting new frontier in biogeochemistry. In particular, recent discoveries regarding the geochemistry and biology of phosphite have highlighted the potential significance of this compound both as a facilitator for the emergence of life on ancient Earth and as a modern driver of microbial processes that continue to shape the global biosphere. Phosphite has been detected in several environments at concentrations that suggest the current existence of a phosphorus redox cycle occurring at short geological timescales. Several anthropogenic sources of phosphite have been identified, and there is evidence that phosphite may also be produced by natural processes such as biological phosphonate metabolism and geothermal phosphate reduction. The presence of the genes responsible for APO in

hundreds of microbial isolates from a variety of environments indicates that this process is widespread and may have a substantial impact on the global P cycle. The discovery of DPO and its ability to sustain carbon fixation while providing an energetic benefit raises the possibility of phosphite as a key driver of primary productivity in aphotic environments. Nonetheless, many aspects of microbial phosphite oxidation remain poorly understood. Only one organism capable of DPO has been isolated to date, and the genetic and biochemical factors that allow energy conservation during this metabolism and thus differentiate it from APO have not been conclusively determined. The paucity of DPO-capable organisms discovered so far compared to those capable of APO is somewhat puzzling, but may simply be due to the fact that most efforts to identify phosphite-oxidizing organisms have so far been carried out in aerobic freshwater and soil environments in which phosphite concentrations tend to be relatively low. We believe that DPO-capable microbes are more likely to be found in anoxic marine environments in which phosphite from anthropogenic and/or natural sources might accumulate to high enough levels to support growth by DPO, such as coastal sediments or deep subseafloor basalts. The prospect of finding novel DPO-capable organisms among the microbial "dark matter" of the subsurface is particularly exciting, and we hope that future work in this area will be undertaken. More importantly, we hope that awareness of the existence and potential significance of phosphorus redox cycling will continue to grow among geochemists and biologists alike and spur the further development of this promising area of research.

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