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Ecology of *Bacillaceae*

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ABSTRACT Members of the family *Bacillaceae* are among the most robust bacteria on Earth, which is mainly due to their ability to form resistant endospores. This trait is believed to be the key factor determining the ecology of these bacteria. However, they also perform fundamental roles in soil ecology (i.e., the cycling of organic matter) and in plant health and growth stimulation (e.g., via suppression of plant pathogens and phosphate solubilization). In this review, we describe the high functional and genetic diversity that is found within the *Bacillaceae* (a family of low-G+C% Gram-positive spore-forming bacteria), their roles in ecology and in applied sciences related to agriculture. We then pose questions with respect to their ecological behavior, zooming in on the intricate social behavior that is becoming increasingly well characterized for some members of *Bacillaceae*. Such social behavior, which includes cell-to-cell signaling via quorum sensing or other mechanisms (e.g., the production of extracellular hydrolytic enzymes, toxins, antibiotics and/or surfactants) is a key determinant of their lifestyle and is also believed to drive diversification processes. It is only with a deeper understanding of cell-to-cell interactions that we will be able to understand the ecological and diversification processes of natural populations within the family *Bacillaceae*. Ultimately, the resulting improvements in understanding will benefit practical efforts to apply representatives of these bacteria in promoting plant growth as well as biological control of plant pathogens.

The most distinguishing feature of most members of the family *Bacillaceae* (phylum *Firmicutes*) is their ability to form endospores that provide high resistance to heat, radiation, chemicals, and drought, allowing these bacteria to survive adverse conditions for a prolonged period of time. *Bacillaceae* are widely distributed in natural environments, and their habitats are as varied as the niches humans have thought to sample. Over the years of microbiological research, members of this family have been found in soil, sediment, and air, as well as in unconventional environments such as clean rooms in the Kennedy Space Center, a vaccine-producing company, and even human blood (1–3). Moreover, members of the *Bacillaceae* have been detected in freshwater and marine ecosystems, in activated sludge, in human and animal systems, and in various foods (including fermented foods), but recently also in extreme environments such as hot solid and liquid systems (compost and hot springs, respectively), salt lakes, and salterns (4–6). Thus, thermophilic genera of the family *Bacillaceae* dominate the high-temperature stages of composting and have also been found in hot springs and hydrothermal vents, while representatives of halophilic genera have mostly been isolated from aquatic habitats such as salt lakes and salterns, but less often from saline soils (7, 8). The isolates that have been obtained, in particular, from the varied extreme habitats, produce a wide range of commercially valuable extracellular enzymes, including those that are thermostable (9, 10).

Here, we focus on the ecology of selected members of the *Bacillaceae*. We first briefly address the recently revised taxonomy of this family, which encompasses strictly aerobic to facultatively and strictly anaerobic endospore-forming bacteria. Then we will focus on

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the ecological behavior and roles of selected organisms with special reference to members of the genus *Bacillus* that inhabit soil and the rhizosphere. These will be referred to by the name *Bacillus* except where studies focusing on particular species or different genera of the family *Bacillaceae* are discussed. We will also examine the methodology used to study the diversity, abundance, and distribution of *Bacillus* in the environment, in particular, with respect to the benefits and limitations. Finally, we examine the ecological drivers that shape the diversity and evolution of selected members of this genus and address the future goals and needs of the research aimed at furthering our understanding of how *Bacillus* communities are shaped in natural habitats.

BRIEF OVERVIEW OF THE FAMILY *BACILLACEAE*

The family *Bacillaceae* comprises mostly aerobic or facultatively anaerobic chemoorganotrophic rods with a typical Gram-positive cell wall. The majority of the taxa within the family form endospores, although exceptions are found. The aerobic or facultatively anaerobic members of the *Bacillaceae* were, until the early 1990s, positioned within the genus *Bacillus*, which stood next to the strictly anaerobic clostridia. Since then, major taxonomic changes have taken place, and consequently the family now accommodates representatives of the genus *Bacillus* and other newly formed genera with related nomenclature, examples being *Paenibacillus* ("almost" *Bacillus*), *Geobacillus*, and *Halobacillus* (see Galperin [232] for additional details of *Firmicutes* taxonomy). Currently (i.e., September 2014), the family *Bacillaceae* encompasses 62 genera (Table 1) composed of at least 457 species. New genera are continuously being described, as a result of a thorough description of a plethora of new, divergent environmental isolates. In 2009, 31 genera belonging to the *Bacillaceae* were listed in *Bergey's Manual of Systematic Bacteriology* (11), while only 2 years later Logan and Halket (12) indicated the existence of 36 genera in this family. Altogether, 25 new genera have been classified in the past 2 years, for a grand total of a staggering 62 genera (listed in Table 1). The taxonomy of the *Bacillaceae* may be rather confusing for the nonspecialist. For example, *B. pallidus* was reclassified in 2004 as *Geobacillus pallidus* (13), then later (2010) to a new genus, *Aeribacillus pallidus* (14). However, *B. pallidus* was also reclassified in 2009 to a new genus *Falsibacillus* (15). With the exception of *Anoxybacillus*, *Bacillus*, *Halobacillus*, *Geobacillus*, *Gracilibacillus*, *Lentibacillus*, *Lysinibacillus*, *Oceanobacillus*, and *Virgibacillus*, the new genera often include only one or a few species (4). See Table 1. Therefore, and very unfortunately, the taxonomy of the novel genera and species is currently often based on only one isolate per genus or species. Given this low robustness of the novel genera and the lack of sound ecological data, the ecology of these groups will not be further discussed in this review. Thus, the focus of this review will be on those representatives of the genus *Bacillus* (such as members of the *B. cereus sensu lato* and *B. subtilis*/*B. licheniformis* clades), which have gained the most scientific attention because they encompass industrially, agriculturally, or medically important species. Moreover, *B. subtilis* has been a long-standing model or reference organism for the study of gene regulation in Gram-positive bacteria, especially in the context of spore development. It is of utmost scientific interest to link the knowledge of the genetics and biochemistry of this well-studied species to that of its ecology.

The Genus *Bacillus*

The genus *Bacillus* was proposed in 1872 by Cohn, who classified its type species, *Bacillus subtilis*, as an organoheterotrophic aerobic spore-forming rod (16). Since its first description, the genus *Bacillus* has undergone many transformations due to the difficult classification of its member species (17). It is currently the largest genus within the *Bacillaceae*, presently consisting of at least 226 species (September 2014). New strains are constantly added as new species as well as being reclassified into new genera. For example, in the past 3 years, 10 existing species were reclassified into other genera and 39 new species were added to the genus only within the past year (September 2014). The inferred phylogeny of *Bacillus* species is often based on the 16S rRNA gene sequence, but this does not always distinguish species. Therefore, usage of DNA-DNA hybridization or sequencing of core genes is recommended for a better classification. This finer approach is even more important if one studies the *Bacillus* strains at the subspecies level (18, 19). In general, the different species within the genus *Bacillus* show only meager divergence of their 16S rRNA genes and this divergence is poorly correlated with phenotypic characteristics of these bacteria. Recently, Maughan and Van der Auwera (20) calculated the evolutionary relationships among 56 *Bacillus* species on the basis of their 16S rRNA gene sequences and compared these with the phenotypic traits of corresponding isolates. They found that 16S rRNA and phenotypic clustering are not congruent and that

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Bacillus species that form tight phylogenetic clusters are dispersed over the whole phenotypic tree. For example, representatives of the *B. subtilis* cluster comprise 2 subspecies (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*) and 12 species (*B. mojavensis*, *B. valismortis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. pumilus*, *B. licheniformis*, *B. sonorensis*, *B. aquimaris*, *B. oleronius*, *B. sporothermodurans*, *B. carboniphilus*, and *B. endophyticus*). In the light of this discrepancy, which is indicative of the occurrence of fluid genomes across these species, molecular data need to be used to address the phenetic and ecological relationships between closely related taxa.

As with members of the *B. subtilis* cluster, members of the *B. cereus sensu lato* group (*sensu lato* meaning "in the widest sense"), including *B. anthracis*, *B. thuringiensis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (21), are highly related at the genome level. Sequencing of the 16S rRNA gene (22) and even multilocus sequence typing (MLST) indicated high relatedness among the isolates of this group (21, 23, 24). This high relatedness was later confirmed at the level of gene content and synteny of their genomes (25). Traditionally, species of the *B. cereus sensu lato* group have been defined at the phenotypic level. Importantly, a relatively small number of genes, located on plasmids that are shared among *B. cereus*, *B. thuringiensis*, and *B. anthracis* strains, have a disproportionate effect on the ecological behavior (phenotype) of the three species. For example, pXO1 (181 kb) and pXO2 (96 kb) are typically found in *B. anthracis* as they carry major virulence factors associated with anthrax, but recent findings revealed pOX-1- and pOX-2-like plasmids also in *B. cereus* and *B. thuringiensis* (26–28). Interestingly, *B. anthracis* and the other strains carrying pXO1 and causing anthraxlike disease did not undergo major changes of their core genomes after plasmid acquisition, suggesting that there is no subgroup genetically predisposed to anthrax pathogenesis; instead, any number of *B. cereus sensu lato* or possibly even other *Bacillus* may be capable of gaining the ability to produce the lethal toxin (29). Thus, horizontal gene transfer of such plasmids may have a very drastic impact on the ecology of these organisms, potentially resulting in strong shifts in ecological behaviors, from the ability to infect a particular insect species to the ability to cause disease in sheep. Overall, the *B. cereus* group (including especially *B. cereus*, *B. thuringiensis*, and *B. anthracis*) contains strains of key medical and economic importance. Therefore, we advocate that species names are preserved for what we argue are very practical reasons.

LIFESTYLES AND ECOSYSTEM FUNCTION OF MEMBERS OF THE *BACILLACEAE*

Lifestyles (and thus ecological behavior) of all members of the *Bacillaceae* are tightly defined by their ability to form endospores. Spores allow survival under adverse conditions for shorter or longer time periods, even up to thousands of years (30) (see Fajardo-Cavazos et al. [233] for additional details on the isolation of ancient spores). The formation of endospores in some *Bacillus* species can be induced by nutrient deprivation and high cell population densities, and is also affected by environmental factors such as changes in pH, water content, or temperature (31). Spore survival is also influenced by specific soil parameters, including pH, organic matter content and calcium levels (32, 33).

The remarkable ability of *Bacillaceae* members to form spores and survive presumably allows these bacteria to travel large distances as living entities, even as far as between continents, using airstreams (34, 35). Recently Smith et al. (35) showed that microorganisms are abundant in the upper atmosphere and that transpacific plums can deliver 16S rRNA of *Bacillaceae* from Asia across the Pacific Ocean to North America (35) as well as live *Bacillus* cells (36). It has even been shown that spores survive on spacecraft exteriors especially if protected from UV radiation by soil particles (37) and speculated that spores can travel from Earth to Mars or even beyond (38). Therefore members of *Bacillaceae* are widely distributed across environmental habitats (Fig. 1), presumably also due to the longevity of their endospores. The ubiquity of *Bacillus* spp. is exemplified by the case of the insect-pathogenic *B. thuringiensis*. This organism has been isolated from all continents. Remarkably, an attempt to isolate this bacterium from 1,115 different soil samples from all over the world (using acetate selection) was successful in roughly 70% of the samples (39). Usually, isolation is performed so that the size of the sample is not taken into consideration, and only one or a few isolates are obtained from one sample and then studied further (40). Recently, however, the question about the diversity of the quorum-sensing system encoded by the *comQXPA* locus was addressed by isolation of the multiple strains of *B. subtilis* from two 1-cm^3 soil samples. This approach yielded a unique collection of strains that enabled us to address the ecology and diversity of bacilli at the micrometer distances beyond the quorum-sensing genes and are referred to below as microscale strains (41). In soil, even at micrometer distances, *Bacillus* strains showed differences in various traits and functions, e.g., colony morphology, competence for transformation,

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TABLE 1 Genera in the family *Bacillaceae*

Genus	No. of species	Isolated from	Comments	Reference(s)	Proposal of genus
Bacillus	226	Soils, animals, inner plant tissue, humans, food, domestic, industrial, hospital, marine environment, air, sediment,	38 new species since August 2013	174	1872
Aeribacillus	1	Hot springs, crude oil-contaminated soil	Reclassified from *Geobacillus pallidus*	14	2010
Alkalibacillus	6	Brine, camel dung, loam, mud, salt lake, soil, marine solar saltern, hypersaline soil	Was previously known as *Bacillus haloalkaliphilus* (Fritze 1996)	175	2005
Alkalilactibacillus	1	Cool and alkaline soil		176	2012
Allobacillus	1	Shrimp paste	1 strain	177	2011
Alteribacillus	2	Hypersaline lake		178	2012
Amphibacillus	7	Alkaline compost, lagoon and lake sediment	3 novel species have been proposed since 2011	179	1990
Anaerobacillus	3	Soda lake		180	2009
Anoxybacillus	18	Hot springs, geothermal soil, contaminant of gelatin production, cow and pig manure	6 new species since 2011	181	2000
Aquisalibacillus	1	Hypersaline lake		182	2008
Aquibacillus	3	Hypersaline lake	2 species were reclassified from *Virgibacillus* to *Aquibacillus*	183	2014
Caldalkalibacillus	2	Hot spring		184	2006
Caldibacillus	1	Soil	Reclassified from *Geobacillus debilis*	185	2012
Calditerricola	2	High-temperature compost		186	2011
Cerasibacillus	1	Kitchen refuse		187	2004
Domibacillus	1	Clean room of a vaccine-producing company		188	2012
Filobacillus	1	Sediment of marine hydrothermal vent	Species based on 1 strain	189	2001
Fictibacillus	6	Oyster, bioreactor, wall paintings, arsenic ore	Reclassification of *B. nanhaiensis*, *B. barbaricus*, *B. arsenicus*, *B. rigui*, *B. macauensis*, and *B. gelatini* as *Fictibacillus*	190	2013
Edaphobacillus	1	Hexachlorocyclohexane (HCH) contaminated soil		191	2013
Geobacillus	15	Hot springs, oilfields, spoiled canned food, milk, geothermal soil, desert sand, composts, water, ocean sediments, sugar beet juice, mud, activated sludge	Obligately thermophilic, should be reclassified as *Saccharococcus* due to morphology (Bergey's)	192	2001
Gracilibacillus	11	Salt lakes, desert soil, solar saltern, saline soil, fermented fish		193	1999
Halalkalibacillus	1	Nonsaline soil	Not included in Bergey's *Manual* (2009), only 1 strain	194	2007
Halobacillus	18	Hypersaline environments, salt marsh soils, fermented food, mural paintings		195	1996
Halolactibacillus	3	Decaying marine algae, living sponge	Possesses all the essential characteristics of lactic acid bacteria	196	2005
Jilinibacillus	1	Saline and alkali soil samples		197	2014
Lentibacillus	11	Fish sauce, salt lake, saline soil, solar saltern, saline sediment, salt field		198	2002
Hydrogenibacillus	1	Lake mud		43	2012
Lysinibacillus	10	Forest humus, soil		199	—
Marinococcus	4	Solar salterns, seawater, saline soil		200	1984
Microaerobacter	1	Terrestrial hot spring		201	2010
Natribacillus	1	Soil		202	2012
Natronobacillus	1	Soda-rich habitats (lake sediment)		203	2008

(continued)

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TABLE 1 Genera in the family *Bacillaceae* *(continued)*

Genus	No. of species	Isolated from	Comments	Reference(s)	Proposal of genus
Oceanobacillus	11	Mural paintings, algal mat from sulfurous spring, deep marine sediments, shrimp paste, fermented food, activated sludge, marine animals		204	2001
Ornithinibacillus	5	Hypersaline lake, human blood, pasteurized milk		205	2006
Paraliobacillus	2	Decomposing marine algae, salt lake sediment		206	2002
Paucisalibacillus	1	Potting soil		207	2006
Piscibacillus	2	Fermented fish, hypersaline lake		208	—
Pontibacillus	5	Solar saltern, marine animals, salt field		209	2005
Pseudogracilibacillus	1	Rhizosphere soil		210	2014
Psychrobacillus	3	Soil, mud water	Reclassified from *B. insolitus, B. psychrodurans, B. psychrotolerans*	211	2010
Saccharococcus	1	Beet sugar extraction	1 species reclassified as *Geobacillus*	212	1984
Rummeliibacillus	2	Clean room of Kennedy space center, field scale composter		213	—
Salibacillus	2	Salterns, hypersaline soils	Was reclassified from *B. salexigens*	193	1999
Salimicrobium	5	Salted hides, solar saltern	Reclassified from *Marinococcus albus* and *B. halophilus*	214	2007
Salinibacillus	2	Saline lake		215	2005
Salirhabdus	1	Sea salt evaporation pond		216	2007
Salisediminibacterium	1	Soda lake sediment		217	2012
Saliterribacillus	1	Hypersaline lake		218	2013
Salsuginibacillus	2	Lake sediment, soda lake		219	2007
Sediminibacillus	2	Hyper saline lake		220	2008
Sinibacillus	1	Tropical forest soil and a hot spring sediment		221	2014
Streptohalobacillus	1	Subsurface saline soil		222	2011
Tenuibacillus	2	Hypersaline lake		223	2005
Terribacillus	4	Soil		224	2007
Texcoconibacillus	1	Soil of the former lake Texcoco		225	2013
Thalassobacillus	4	Saline soil, hypersaline lake, tidal flat sediment		226	2005
Thermolongibacillus	2	Hot springs soil and sediment		227	2014
Tumebacillus	2	Permafrost, ginseng field		228	—
Virgibacillus	24	Soils, hypersaline soil and salterns, seawater, salt field, saline soil, mountain soil, salt lake, food, permafrost	6 new species since 2011	229	1999
Viridibacillus	3	Soil	Reclassified from *B. arvi, B. arenosi*, and *B. neidei*	230	2007
Vulcanibacillus	1	Deep-sea hydrothermal vents		231	2006

sensitivity to prophage induction by mitomycin, swarming, and metabolism (P. Stefanic, M. Črnigoj, and I. Mandic-Mulec, unpublished data).

Concerning ecosystem function, one usually thinks of biogeochemical cycling processes. Indeed, many members of the *Bacillaceae* are saprophytes that participate in the carbon, nitrogen, sulfur, and phosphorous cycles in natural habitats, e.g., in soil. Some species like *Bacillus schlegelii* isolated from geothermal soil and capable of growth from 59 to 72°C are capable of autotrophic growth by using hydrogen or thiosulfate as an energy source and carbon dioxide as a source of carbon (42). Recently, a proposal was made to transfer *B. schlegelii* to a novel genus with a novel species name

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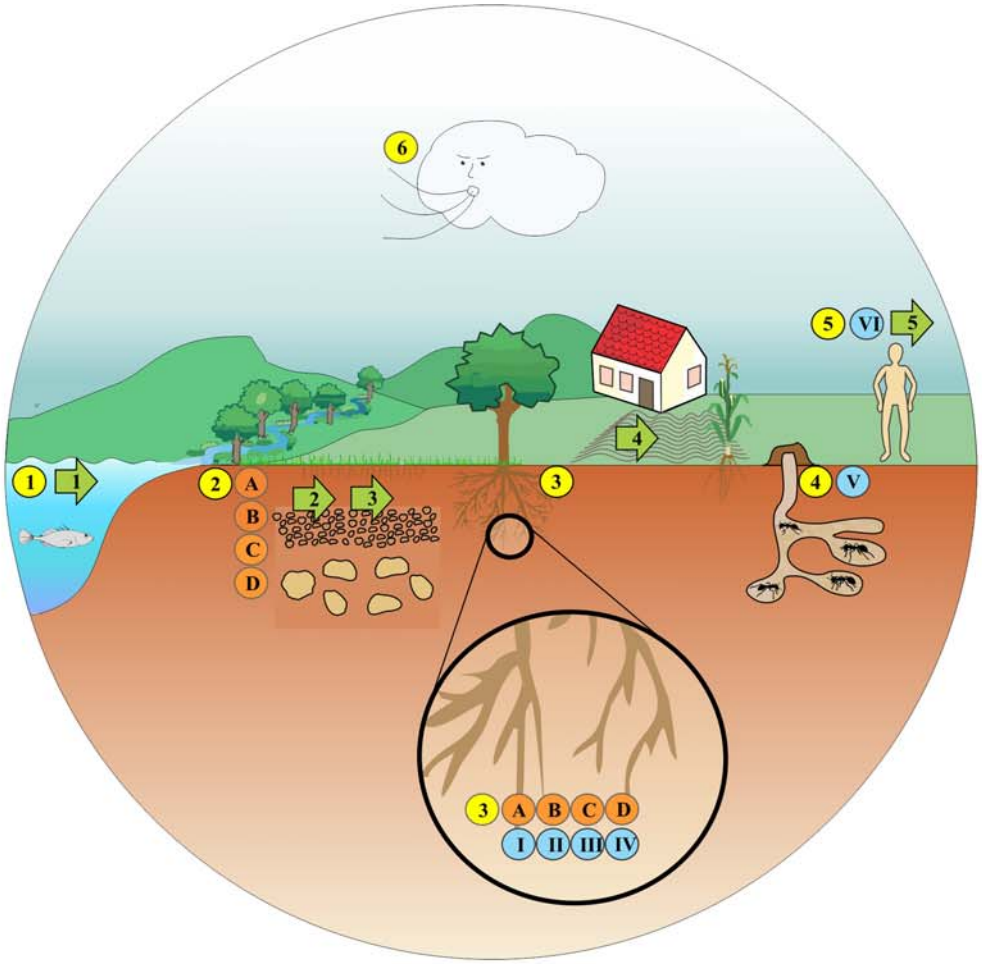

Occurence of Bacillaceae in the environment

1. Marine ecosystems, fresh waters, salt lakes, hot springs
2. Soil
3. Rhizosphere, rhizoplane
4. Soil invertebrates
5. Mammal pathogens
6. Air

Ecosystem functions

- A. Carbon cycle, degradation of soil organic matter
- B. Nitrogen cycle, nitrification, denitrification, nitrogen fixation (biofertilization)
- C. Phosporus solubilization (biofertilization)
- D. Ecoremediation of pollutants

Biotic interactions

- I. Plant defense, microbial pesticide synthesis
- II. Symbiosis stimulation
- III. Fixation of nitrogen, phosporus, Zinc solubilization, iron aquisition
- IV. Phytohormone production
- V. Mutualism (protection from fungus)
- VI. Pathogenicity (toxin production)

Applications

1. Hg Volatilization
2. Phytoremediation of metals from polluted soil (Pb, Mn, Zn, Cr, Ni)
3. Degradation of petrochemicals
4. Biofertilization/Biopesticide
5. Medicine - probiotics

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Hydrogenibacillus schlegelii (43). In addition to *Bacillus*-mediated biogeochemical cycling, other ecologically important functions may also be driven by members of *Bacillaceae*. As indicated in the foregoing, some representatives of the *Bacillaceae* (in particular, *B. anthracis* and *B. cereus*) are important pathogens of mammals (44), whereas *B. thuringiensis* is an insect pathogen. The pathogenicity of the latter organism is linked to plasmid-encoded *cry* genes, which are responsible for the synthesis of an insecticidal protein that interacts with receptors in the gut system of insect larvae (45). In addition, bacilli such as *B. subtilis*, *B. cereus*, *and B. mycoides* are known for their roles as beneficial rhizobacteria that promote plant growth (act as biofertilizers) or protect plants from plant pathogens (function as biopesticides, e.g., *B. subtilis*) (46) as illustrated in Fig. 1.

Involvement of *Bacillaceae* in the Degradation of Soil Organic Matter and Plant Litter

It is generally believed that the primary habitat of many *Bacillaceae* (e.g., *B. subtilis* and *B. cereus*) is the soil. However, almost nothing is known about the intricate mechanisms modulating the *in situ* physiology, germination, growth, and sporulation of these bacteria. Most members of the genus *Bacillus* are aerobic heterotrophic saprophytes (47) that are capable of degrading a range of polymeric carbonaceous substances. They also grow on a variety of simple compounds. Hence, it is generally assumed that actively growing bacteria in soil are associated with soil organic matter or the plant rhizosphere (Fig. 1). Thus, these organisms may prosper in microhabitats where carbon and nitrogen are not strongly limited. Several members of the genus *Bacillus* are known to be typical inhabitants of so-called hot spots for microbial activity in terrestrial habitats, e.g., where organic matter is plentiful. For example, Siala et al. (48) found that vegetative bacilli predominate in the soil A1 horizon, where organic carbon is provided by plant litter or root exudates, while spores predominate in the deeper C horizon of the soil. They also detected colonization by bacilli of organic matter aggregates interconnected by fungal hyphae. In later work in the Jansson laboratory (Uppsala), *B. cereus* was found to interact with soil mycorrhizal fungi (49). Moreover, plant roots (rhizosphere and rhizoplane) are habitats where members of *Bacillaceae* can thrive on plant exudates. *Bacillus* strains were also isolated from animal guts and feces, suggesting that these energy-rich environments may represent suitable habitats for sporeformers from this genus. Indeed, it has been shown that spores of *B. subtilis* can germinate, grow, and go through another cycle of sporulation/germination inside the gut of a mouse (50).

Many *Bacillus* isolates have the ability to break down cellulose, hemicellulose, and pectin (51–54), which suggests their involvement in the degradation and mineralization of plant and humic materials in soil. Also, Maki et al. (55) found that a new *Bacillus* sp. strain was able to modify lignocellulose, and, owing to a variety of cellulase and xylanase activities, it displayed a high potential for lignocellulosic decomposition. In addition, chitinase activity, which facilitates the degradation of fungal cell walls and insect exoskeletons, is also common among many members of soil *Bacillaceae* (56–59). Thus, chitinolytic activity contributes to the role of *Bacillaceae* in the mineralization of soil organic matter and to their ability to protect plants from pathogens (46, 60).

Many members of *Bacillaceae* are also able to degrade proteins in soil, and some proteases produced by these bacteria have gained great scientific attention because of their industrial value. However, only a few studies have addressed proteolytic activity, including that of *Bacillaceae*, in soils. Sakurai et al. (61) showed that proteolytic activity in soil was greater following the addition of organic, rather than inorganic, fertilizer and was also greater in the rhizosphere than in bulk soil. Chu et al. (62) also found evidence for the selection of a *Bacillus*-related organism following treatment of soil with organic manure (but not inorganic fertilizer). This suggested that members of *Bacillaceae* respond to the addition of organic C and N sources to soil and may be important in the degradation of fresh organic matter in the soil.

Studies addressing the growth of *Bacillaceae* in soil or the rhizosphere are scarce. Recently, Vilain et al. (63) assessed the growth and behavior of *B. cereus* in soil extracts and demonstrated that this organism goes through a complete life cycle (germination, growth, and sporulation) in soil-mimicking conditions. Interestingly, *B. cereus* and other related *Bacillus* isolates formed

FIGURE 1 Occurrence of *Bacillaceae*, their ecosystem function, biotic interactions, and applications. The illustration shows different environments from which *Bacillaceae* have been isolated highlights their main ecosystem functions and biotic interactions, and illustrates selected existing and possible applications. doi:10.1128/microbiolspec.TBS-0017-2013.f1

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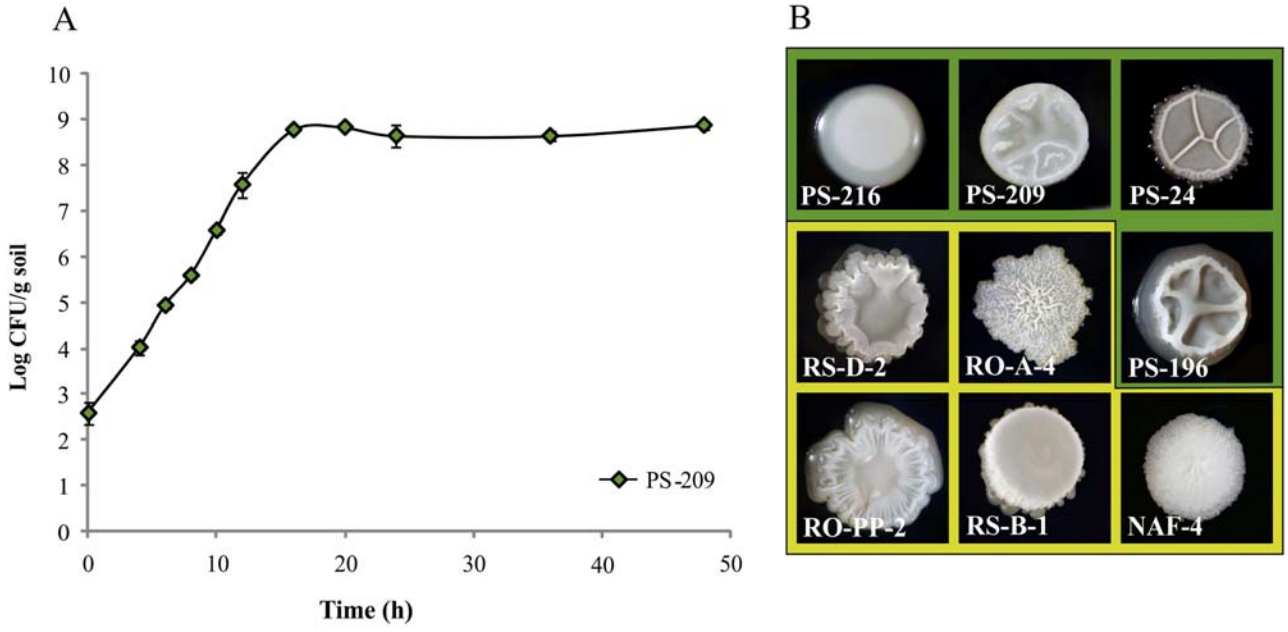
multicellular structures encased in polymeric matrices in soil extracts and soil microcosms, while in LB medium clumping of cells was never observed. This suggested that soil induces a specific physiological adaptation in cells that involves the formation of bacterial aggregates, which may be important for biofilm formation (63). *B. subtilis* isolates also show growth in autoclaved high organic matter soils with rates that are comparable to those in rich nutrient medium (Fig. 2). However, growth of these isolates in unsterilized soil, in which a microbiostatic microbiota was present, was almost undetectable.

Involvement of *Bacillaceae* in the Nitrogen Cycle

Some members of the genus *Bacillus* play key roles in particular steps of soil nitrogen-cycling processes (Fig. 1), such as denitrification and nitrogen fixation, next to their involvement in the mineralization of various nitrogen sources (64–66). Denitrification is an anaerobic process, in which nitrate serves as the terminal electron acceptor during the oxidation of organic matter and is converted to gaseous products such as N_2O, NO, and N_2. This process is important in the removal of nitrogen in biological wastewater treatment and in degradation of organic pollutants (67) and is detrimental for soils, where it depletes nitrogen (68). Soil bacteria capable of denitrification were also found among *Bacillus* isolates (68) that were identified by sequencing their 16S rRNA genes. In addition, gene sequence analysis of *nirS* and *nirK*, both encoding nitrite reductases, was performed on more than 200 cultivated denitrifiers, but only *nirK* was detected in the *Firmicutes* (69). Although the denitrification potential of several *Bacillus* species is well known, many *Bacillus* isolates that are able to use nitrate as an electron acceptor have been misclassified as denitrifiers (68). This is because these isolates more often reduce nitrate to ammonium and not to gaseous products and therefore should be classified as microbes that perform DNRA (dissimilatory nitrate reduction to ammonium), a process that is common in environments that are rich in organic carbon (68). In luvisol soil,

FIGURE 2 Growth of *B. subtilis* in soil and morphology on LB agar media. (A) Growth of riverbank isolate *B. subtilis* PS-209 (41) was grown in an autoclaved soil microcosm at 28°C, and CFU counts were performed at indicated times on Luria-Bertani (LB) medium. The experiment was performed in three replicates. Error bars represent 95% confidence intervals of means calculated from log10 transformed CFU counts (L. Pal, S. Vatovec, P. Stefanic, T. Danevčič, and I. Mandic-Mulec, unpublished data). (B) Colony morphotypes. Colony morphology was visually examined and photographed after incubation at 37°C for 48 h on LB agar medium. Riverbank microscale and desert macroscale *B. subtilis* strains are marked with green and yellow, respectively (Courtesy of P. Stefanic). doi:10.1128/microbiolspec.TBS-0017-2013.f2



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Bacillus spp. were among the most abundant members retrieved among cultured denitrifiers (66). This study tested both, nitrate and nitrite, as electrons that were crucial to evaluate the denitrification potential among soil isolates (66). In general, however, the distribution of denitrifying bacilli in soil and their importance is poorly understood because the existing primer sets that target denitrification genes and allow monitoring of their activity in natural environments through quantitative PCR-based methods are based on sequences from Gram-negative bacteria and, therefore, may not recognize these genes in the Gram-positive *Bacillaceae*.

In addition to the denitrifier and DNRA representatives of the genus *Bacillus*, some members of *Bacillaceae* have been classified as nitrogen fixers. These organisms reduce atmospheric nitrogen to ammonia with the help of the enzyme nitrogenase. Early studies pinpointed the then-called *Bacillus polymyxa*, *Bacillus azotofixans*, and other members of *Bacillaceae* as nitrogen fixers. However, many of these have later been reclassified as *Paenibacillus* species, among which the prominent nitrogen fixers are *P. polymyxa*, *P. macerans*, *P. azotofixans*, and *P. durus* (70–72). One study showed that particular strains of *B. cereus*, *B. megaterium*, and *B. licheniformis* are able to fix nitrogen (73). In contrast, others suggested, on the basis of screenings for *nifH* genes, that nitrogen fixation within the *Bacillaceae* is limited to *Paenibacillus* species (74).

Involvement of *Bacillaceae* in the Phosphate Cycle

Phosphorus is an essential mineral for plant growth. Many of the phosphate anions in soil are in complexes with cations (Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}), hindering the availability of phosphorus to plants. Many bacteria and fungi in soil possess phosphatase or phosphate-solubilizing activity, resulting in the release of phosphate from insoluble polyphosphate and consequently in the improvement of plant nutrition and growth (75–79) (Fig. 1). These phosphate solubilizers include a variety of bacilli (80–82). These naturally occurring phosphorus-solubilizing soil bacilli may serve as potential biofertilizers, either by their introduction in soils that are currently deprived of them or when they are already present by enhancing their prevalence through agronomic measures. Active phosphate solubilization will reduce the environmental phosphate load due to fertilization in phosphate-saturated regions and, in contrast, help to sustain agriculture in regions with severe phosphorus deficiencies across the globe. An example of the latter is found in Ethiopia (78), but several other regions of the world (Pakistan, India, Brazil) are in need of better phosphorus mobilization in their agricultural soils.

Bacillaceae as Plant-Beneficial Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) influence plant health, growth, and development either directly or indirectly. Direct modes of action include nitrogen fixation, phytohormone production, phosphorus solubilization, and lowering of the ethylene concentrations at the plant root to promote lengthening. In addition, indirect effects include an influence on symbiotic relationships between bacteria and plants and the repression of soil-borne plant pathogens (83, 84). Collectively, the PGPR of the genus *Bacillus* participate in nitrogen fixation (85), production of phytohormones, e.g., gibberelins (86), phosphorus solubilization (80–82), and zinc solubilization (87). Members of the *Bacillaceae* family further promote the growth of plant roots by lowering the local ethylene concentrations (88) and by increasing the assimilation of metal ions such as iron through the activation of the plant's own iron acquisition machinery (89). *Bacillus* strains can also stimulate *Rhizobium*-legume symbioses (90) and they possess a range of other plant-beneficial properties that protect plants against pathogens. This ecosystem function may depend on biofilm formation on plant roots and the production of hydrolytic enzymes, various antibiotics, and small molecules such as lipopeptides (surfactins, iturins, fengycins). Some rhizosphere *Bacillus* strains may have the ability to induce systemic resistance in plants, allowing enhanced resistance against phytopathogens. Studies reviewed by Choudhary and Johri (46) and Kloepper et al. (91) revealed that several species of the genus *Bacillus* (*B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, *Lysinibacillus sphaericus*) significantly reduced the incidence or severity of various diseases on tomato, bell pepper, tobacco, *Arabidopsis* sp., and cucumber due to induced systemic resistance. Defense by *Bacillus* spp. has been reported against fungal and bacterial pathogens, systemic viruses, and root-knot nematodes (46, 91). Ryu et al. (92) showed that *Arabidopsis thaliana*, following treatment with plant-growth-promoting *Bacillus pumilus* significantly reduced symptom severity resulting from *Cucumber mosaic virus* (CMV) infections (92).

Many *Bacillus* strains have found commercial applications, mainly as biopesticides (fungicides, bactericides, viricides, actinomyceticides) as well as insecticides (93, 94). For example, tomato plants, inoculated with plant-growth-promoting *B. subtilis* isolated from the tomato rhizosphere, revealed lowered susceptibility to whitefly

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Bemisia tabaci (95). Recently, wild strains of *B. subtilis* and related organisms were shown to have biocontrol efficacy against the potato and tomato wilting agent *Ralstonia solanacearum*. These strains formed robust biofilms both in defined medium and on tomato roots (95). The plant protection phenotype was found to be dependent on the genes required for biofilm formation and matrix production, which was critical for bacterial colonization of plant root surfaces (96, 97) and was induced by plant polysaccharides (98). Similarly, Bais et al. (99) suggested that biofilm formation and the production of the lipopeptide compound surfactin by *B. subtilis* on *Arabidopsis* roots is essential for the plant protection activity of this organism against *Pseudomonas syringae*. Recently, surfactin production by *Bacillus* biofilms was directly imaged on plant roots by matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) (100) and showed that *B. subtilis* strains that originate from the same plant root can dramatically differ in surfactin production, but less so in their ability to form biofilms (101). However, biofilm strength and thickness depends also on the composition of extracellular matrix components and these change in relation to available nutrients (102). It will be interesting to explore whether a good biofilm former on one plant performs similarly on other plants and whether this ability is plant specific, because this may have implications for the development of novel biopesticides. Specific *B. subtilis* strains can also trigger induced systemic resistance (ISR) in plants (103) and change transcriptional response in plants (104), which can have a significant influence on the plant: pathogen interaction and the outcome of infection.

Role of *Bacillaceae* in Soil-Dwelling Invertebrates

Some members of the genus *Bacillus* are key constituents of the bacterial communities in the intestinal systems of soil-dwelling invertebrates that degrade organic polymers (105–108). For instance, cellulose, hemicellulose, and lignin are major constituents of plant material that are broken down in the guts of termites. In the degradative process, three steps ([1] hydrolysis, [2] oxidation, and [3] methane formation) are distinguished. There is evidence that members of *Bacillaceae* may be mainly involved in the initial phases of the degradative process, i.e., steps 1 and 2. For instance, in the termite hindgut, aerobic rod-shaped spore formers have been found in high numbers, occurring in population sizes of up to 10^7 per ml in gut fluid (109). *In vitro*, the isolates obtained could produce a range of hydrolytic or other enzymatic activities that are involved in the degradation of biopolymeric compounds. Although this suggests that such bacilli are involved in degradative processes in the termite intestine, a clear functional link or proof of their involvement is still lacking.

DISTRIBUTION AND DIVERSITY OF *BACILLACEAE* ACROSS DIFFERENT HABITATS

Habitat distribution (biogeography) and diversity of members of *Bacillaceae* in natural and man-made habitats is a fundamental ecological question. They have been investigated in this context since the first isolation of bacteria from soil at the end of the 19th century. Methods used to study diversity have followed those used for all soil bacteria and traditionally included phenotypic characterization, the application of dichotomous and numerical taxonomy, and, later on, molecular phylogenetic approaches (initiated more than 20 years ago) which rely on direct extraction of nucleic acids from the natural habitat (e.g., soil), rather than from isolates. In this review, the benefits and limitations of molecular and cultivation-based methods are critically evaluated.

Cultivation-Based Methods to Study the Distribution and Diversity of *Bacillaceae*

Bacillus strains are commonly obtained from environmental habitats after samples are treated with mild heat (e.g., 80°C for 10 to 20 min), leaving only endospores to form colonies on solid nutrient media. This is a highly selective approach, which has the great advantage of excluding all bacterial cells that are not in the spore form. Aerobic incubation of the resulting plates provides mainly colonies of the genus *Bacillus*, which can be used for further characterization and identification. Traditionally, biochemical tests are applied for the initial characterization of isolates, as described in the classical handbook by Gordon et al. (110). Also, simple miniaturized methods, such as API strips for metabolic differentiation between isolates, are in use (111). In addition, molecular methods that target specific genes (e.g., 16S rRNA) are employed for phylogenetic characterizations, with the caveat that *Bacillus* species are only slightly divergent in this respect. Thus, as mentioned, core genes like *gyrA* may be more useful for phylogenetic identifications (20). Targeting core genes, and using multilocus sequence typing (MLST) and analysis (MLSA) (both methods involving sequencing short regions of several

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[typically seven] housekeeping genes), may result in a better assessment of the relatedness of strains (112, 113).

Two additional rapid classification tools for *Bacillus* strains at the subspecies level are repetitive extragenic palindromic-PCR (REP-PCR) and BOX-PCR, which both target interspersed repeated sequences within the genome (114). Also, fatty acid methyl ester (FAME) analysis has been successfully used for identification of *Bacillaceae* (115). Recently, rapid bacterial identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) targeting ribosomal proteins (S10-GERMS biomarkers) was applied for identification of *Bacillus* strains. The method enables the differentiation of strains at the subspecies level (116). In addition, an alternative procedure to the standard sample preparation protocol was developed that includes microwave-accelerated tryptic digestion of the cellular material. This approach improved the discriminating power of MALDI-TOF MS by increasing the number of strain-specific peaks, in comparison with the standard method (117).

Cultivation-Independent Methods To Study Distribution and Diversity of *Bacillaceae*

We are presently able to culture in the laboratory only 1 to 10% of microbial diversity. Therefore, many as-yet-undiscovered *Bacillus* species may still be hidden within the uncultured majority. Culture-independent molecular methods are thus essential to address the distribution and diversity of *Bacillus* species in a more complete manner. The striking diversity of microbial communities, which has been unraveled in the past two decades by PCR-based molecular methods, has stimulated an unprecedented increase of investigations of various natural environments (terrestrial, aquatic, anthropogenic), including those within humans and animals (118). Representatives of the *Bacillaceae* have also often been detected in these habitats, at varying levels of abundance and richness, besides many other families and fila.

Molecular methods currently enable a direct determination of the diversity and relative abundance of representatives of *Bacillus* in total bacterial communities. Following the isolation of DNA from a particular environment, genes targeted by specific (*Bacillus*–oriented) primer sets are amplified by PCR. Amplification products obtained by PCR can then be cloned and sequenced for phylogenetic analyses, and the results compared with database sequences obtained from cultivated organisms and/or other environmental studies. Sequences can also be used to design probes for *in situ* detection using fluorescence *in situ* hybridization (FISH) or for probing of nucleic acids. Increasingly, high-throughput sequencing techniques are being applied for the direct analysis of extracted soil DNA, which avoids the PCR-dependent cloning step (119). If high numbers of *Bacillus* sequences are obtained with these approaches, within-group diversity can also be studied. Alternatively, primers targeting specific groups can be used, e.g., for a wide suite of species within the genus *Bacillus* (120), although few such primers have been designed. In addition, the population structure and relative abundance of *Bacillaceae* can be addressed by DNA fingerprinting methods. While these cultivation-independent methods have been used only rarely to address the ecology of *Bacillaceae* that have been successfully grown in the laboratory, they are useful when many environmental samples need to be compared in a study, when we aim to decipher the relationships of *Bacillaceae* with other microbial groups *in situ*, and when we target the yet uncultured populations of these families. Fingerprinting methods most often include either denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), or terminal restriction fragment length polymorphism (T-RFLP). DGGE and TGGE separate amplicons (PCR products) on the basis of heterogeneities in GC content and sequence. When run on a gel containing a gradient of a denaturant or temperature, these PCR products, which are of the same size, separate into bands, visible on a gel after staining, due to differences in denaturing or melting properties. For *Bacillus* spp., this method has been applied successfully by Garbeva et al. (120). For T-RFLP, amplified DNA is digested with restriction enzymes, distinguishing amplicons with sequence polymorphisms. All these fingerprinting methods are less expensive than sequencing methods, and they allow a rapid analysis of many samples and the assessment of relative abundances of different phylotypes, but they provide less information on the identity of the organisms present. Identity is only provided by the primer set, which can be either broad (detecting all bacteria) or narrow (specific for genus *Bacillus*).

In addition to 16S rRNA genes, functional genes (e.g., those for the degradation of aromatic compounds, cellulose, proteins, and nitrate reduction) could be used to estimate the potential contribution of *Bacillaceae* members to the specific relevant ecosystem functions, although primers that target *Bacillus*-specific functional genes have not been developed yet. Moreover, by targeting RNA, it is possible to monitor the active populations in soil and the rhizosphere. The extracted RNA is reverse-transcribed into complementary DNA (cDNA),

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which is then amplified as described above. Targeting 16S rRNA sequences in this manner is sensitive, because cells contain more ribosomes than rRNA genes. This approach has been used to target the active soil bacterial community in acidic peat grassland soils (121). On the other hand, activity can also be assessed by techniques such as stable isotope probing (SIP) to determine which organisms are utilizing specific ^{13}C- or ^{15}N-labeled compounds, or bromodeoxyuridine (BrdU) capture, which separates organisms incorporating the thymidine analogue bromodeoxyuridine (122). Again, SIP has not been applied to *Bacillus*-specific functions yet.

Benefits and Limitations of Molecular Methods Used to Investigate *Bacillaceae* in Natural Settings

The major advantage of the nucleic acid-based techniques is their lack of dependence on laboratory cultivation of soil bacilli. Because growth media and cultivation conditions are inherently selective, it is inevitable that some strains will not be successfully cultured in the laboratory. However, molecular methods also have potential biases. Prosser et al. (122) discuss the benefits and limitations of molecular techniques and developing methods for the assessment of bacterial community diversity and activity. Nevertheless, there is now a suite of quantitative PCR methods available to identify representatives of soil *Bacillaceae*, but there is a need for better primer sets to be used to measure changes in their diversity and community structures and determine their abundance.

The presence of spores in *Bacillus* populations can introduce biases that can significantly influence specific types of studies. For example, molecular techniques are based on the extraction of nucleic acids, which requires lysis of vegetative cells. Lysis of spores, however, requires very severe conditions, and most studies achieve this through physical disruption by bead-beating. The methods for cell lysis and nucleic acid extraction require a balance between conditions and lengths of treatment that are sufficiently rigorous to optimize the lysis of cells and spores and minimize DNA degradation (which will be increased by the length of treatment). This balance is particularly difficult to achieve for *Bacillaceae* because of significant differences in the conditions required to lyse vegetative cells and spores.

Diversity of *Bacillaceae* in Natural Settings Such as the Soil and Rhizosphere

Members of the family *Bacillaceae* are often detected in soil habitats. Studies based on clone libraries of 16S rRNA genes from various soils revealed only a few *Bacillus* sequences (below 3%), indicating the generally low abundance of *Bacillaceae* (123, 124). However, in desert soils, endospore formers are usually highly abundant and diverse (124) in comparison with many other soils.

16S RNA gene-based libraries invariably contain sequences closely related to *Bacillaceae*, but the proportion varies between studies. This is not only because of the differences in environmental conditions, but it is also a result of the differences in nucleic acid isolation techniques, primers, and analytic methods. Our sequence databases have developed considerably over time, as molecular methods have continuously generated new sequence data over the past 15 to 20 years. In general, sequences matching to *Bacillus* represent 1 to 15% of total clone sequences in traditional clone libraries from soil samples (125). For example, a meta-analysis of 32 soil clone libraries (125) indicated that members of *Bacillus* encompassed less than 1 to 2% of the soil bacterial 16S rRNA gene sequences. In contrast, representatives of *Bacillus* comprised 5 to 45% of isolates from traditional cultivation-based studies. As mentioned above, DNA extraction procedures may have a very strong influence on the recovery of *Bacillus* sequences. Kraigher et al. (123) extracted DNA without bead beating and found that only 2 of 114 partial 16S rRNA sequences from a high organic grassland fen soil belonged to *Bacillus*. While high-throughput sequencing methods greatly increase the depth of coverage of soil bacterial diversity, easily providing information on more than 20,000 sequences, representation of *Bacillaceae* remains low. Roesch et al. (119) used pyrosequencing of up to 50,000 16S rRNA fragments from soils, and *Firmicutes* still comprised only 2 to 5% of the sequences. In contrast, denaturing gradient gel electrophoresis (DGGE) analysis with four primers targeting the 16S rRNA V6 region indicated that *Firmicutes* constituted between 19% and 32% of sequences in a grassland soil, and the majority of these (76 to 86%) were members of genus *Bacillus* (126). This suggests that many factors may affect measurements of *Bacillus* diversity in soils. Most diversity studies have targeted DNA, providing information on "total" communities, i.e., assessing active and dormant cells, including spores. Felske et al. (127) characterized the active soil bacterial community in acidic peat grassland soils by targeting RNA, rather than DNA. Sequencing of these clones indicated that the active community was dominated by bacilli and around 65% of all bacterial ribosomes originated from *Firmicutes*. Among these, *Bacillus* species were the most

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active and represented half of the detected sequences (127). Recent analyses of 16S rRNA gene sequences in the RDP database revealed many uncultivated representatives, even within the *B. cereus* or *B. subtilis* groups. In addition, 16S rRNA genes obtained from cultured representatives showed less diversity than environmental sequences classified as *Bacillus* (20). This suggests that there is still undiscovered diversity in this genus and that novel approaches are needed to study its ecology.

Diversity of *Bacillaceae* in the Rhizosphere

The rhizosphere of plants is a special habitat where microbial communities are under the influence of the plant root exudates, which serve as chemoattractants and nutrient sources for bacteria. Approximately 10^7 to 10^9 CFU of culturable rhizosphere bacteria per gram of soil have been reported (128). The rhizoplane bacteria colonize the root surface with 10^5 to 10^7 bacteria per gram of fresh root (128) and are attracted by plant exudates to the rhizosphere and rhizoplane. Specifically, malic acid has been reported to induce colonization and biofilm formation by *B. subtilis* (129). Several studies have compared the bacterial communities in bulk soil and in the rhizospheres of different plants, to assess the influence of root exudates and plant species on bacterial diversities (130–135). The prevalence of *Bacillus* representatives in the rhizosphere varied significantly between studies. Smalla et al. (136) compared bulk and rhizosphere soil microbial communities of field-grown strawberry (*Fragaria ananassa* Duch.), oilseed rape (*Brassica napus* L.), and potato (*Solanum tuberosum* L.) by DNA-based DGGE profiling. *B. megaterium* dominated the bulk soil and potato rhizosphere communities, whereas it was only detectable in strawberry and oilseed rape rhizospheres. Garbeva et al. (137) also found differences in the composition of *Bacillus* in soils under different plant species: *Bacillus* spp., *B. thuringiensis* and *B. cereus* were in particular associated with maize, *B. benzoevorans* and *B. pumilus* with pasture grass, and *Bacillus* sp. and *B. fumarioli* with oats and barley.

Differences in soil microbial communities were also associated with different plant cultivars as well as with the age of plants – young plants versus flowering plants and plants in senescence stages (138–140). Interestingly, root colonization by *Bacillus* was found to be not only plant specific but also root area specific. For example, different types of *B. amyloliquefaciens* FZB42 preferred to colonize root hairs of maize, while primary root tips and lateral roots were favored on *Arabidopsis* roots. On *Lemna*, *Bacillus* accumulated preferably along the grooves between epidermal cells of the roots (141). Interestingly, successful colonization of lettuce rhizosphere by *B. amyloliquefaciens* FZB42 did not show durable effect on the rhizosphere community, while at the same time it did decrease disease severity caused by the pathogen *Rhizoctonia solani* (142).

Mechanisms Driving the Diversity of *Bacillaceae* in Natural Settings

Diversity in microbial communities is theoretically driven by immigration, speciation, horizontal gene transfer (HGT) and extinction. However, the shaping of these communities is also tightly linked to interactions between their members (118, 143, 144). The role of diversity in ecosystem function has attracted much scientific attention over the past 2 decades. This has been driven, in part, by the application of molecular approaches (recently including high-throughput sequencing methods) that allow us to study microbial communities without cultivation. However, molecular methods have only rarely been applied to the analysis of *Bacillaceae* population structure and diversity; most studies rely on cultured isolates. These isolates have been used as models to study the mechanisms of diversification. Recently, within defined species of the *Bacillaceae*, ecologically distinct groups, termed putative ecotypes, were identified (145). An ecotype is defined as an ecologically distinct group of organisms that fall into distinct sequence clusters (lineages), sharing a common evolutionary path. Diversity is then periodically purged by selection. Sequence clusters are initially viewed as putative ecotypes and have to be evaluated further at a more specific genetic or phenetic level or at the level of geographical distribution to be considered real ecotypes (146). Ecotype formation is thought to be countered by frequent recombination (145), which is known to occur between closely related members of *Bacillus*. To determine the actual rates of recombination, Roberts and Cohan (40) analyzed the restriction patterns of three housekeeping genes among closely related *Bacillus* isolates. Recombination within *B. subtilis* or *B. mojavensis* was too low, however, to prevent adaptive divergence between ecotypes (147, 148). Diversification of ecologically distinct populations will also increase due to neutral sequence divergence and differences in restriction/modification systems between the two populations (111, 149).

Environmental Factors Driving Diversification in Soil *Bacillaceae*

We still understand very little about the influence of environmental factors on the diversity and community

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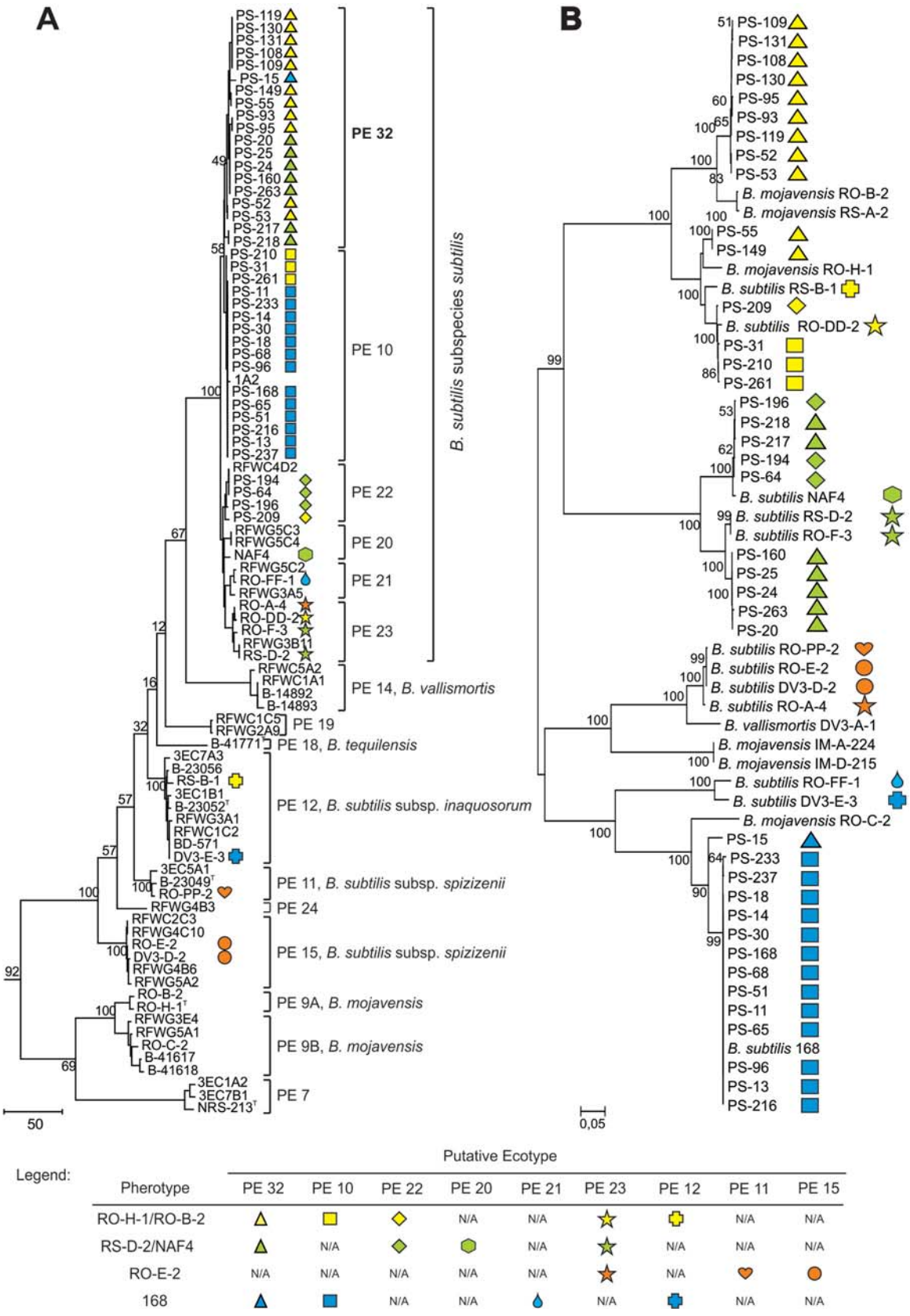

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composition of soil *Bacillaceae*. Recently, an ecotype simulation algorithm (150) has been used to model the evolutionary dynamics of bacterial populations. *B. simplex* isolates obtained from the "Evolutionary Canyons" in Israel, with three major habitats: north-facing (European) slopes, south-facing (African) slopes, and the canyon bottom with largest access to water (151) were used to test the ecotype model (150). Ecotypes demarcated within the *B. simplex* collection showed a strong association with habitats of isolation (150). In agreement with this, isolates taxonomically classified as members of the *Bacillus subtilis–Bacillus licheniformis* clade sampled in the south and north slopes of Death Valley also diversified into several ecotypes, showing adaptation to solar exposure and soil texture (152). This suggests that temperature and soil type may be important environmental parameters that shape the ecological divergence of *Bacillus* ecotypes. These studies also provided evidence for the contention that ecotype clustering observed at the level of housekeeping genes and ecological distinctness may correlate.

Temperature also determines the distribution of the psychrotolerant *B. weihenstephanensis* and other *B. cereus sensu lato* representatives, which grow in the range 7 to 46°C. Von Stetten et al. (153) studied the distribution of about 1,000 mesophilic and psychrotolerant isolates obtained from tropical or temperate soil or two alpine habitats, with average annual temperatures of 28, 7, 4, and 1°C, respectively. Isolates were characterized phenotypically, in terms of their growth responses to temperature and psychrotolerance, as well as genotypically, at the level of 16S rRNA and *cspA* gene sequences. The proportions of psychrotolerant isolates in these four habitats were 0, 45, 86, and 98%, respectively, indicating strong temperature selection. Moreover, psychrotolerant and mesophilic strains exhibited growth at low or moderate temperature, respectively, and possessed psychrotolerant or mesophilic *cspA* genotypes.

Studies addressing ecotype diversity within *Bacillaceae* thus indicate a significant impact of environment (including solar exposure and soil type) on their formation, but information is still required to determine whether the variation between ecotypes is correlated with ecosystem function such as nutrient cycling or other functions such as plant protection, virulence, sporulation, development of competence, biofilm formation, and many other functions that promote survival or may involve biotic interactions.

Recently Stefanic et al. (19) tested whether quorum-sensing types of *B. subtilis* encoded by the *comQXPA* locus differ ecologically and whether they correlate with ecotypes defined by the ES model (19) (Fig. 3). *B. subtilis* strains and relatives encode a polymorphic quorum-sensing system involving the signal-processing enzyme ComQ, the signal ComX, the ComP receptor, and the response regulator ComA (19, 41, 154–159). Previous studies indicated that the *comQXP* loci show high intraspecies (within species) diversity (154). Similarity analysis of *comQXP* loci in desert and other *B. subtilis* strains indicated that they cluster into four distinct groups (19, 41, 156). Strains within the group produce similar peptide pheromones and have similar ComP receptors and are therefore able to induce quorum-sensing responses in each other. We can classify the strains that productively exchange signals as belonging to the same pherotype or communication group. In contrast, strains with divergent *comQXPA* loci (from different groups) cannot induce each other's QS response and are thus of different pherotypes (41, 154, 156, 160, 161). This exemplifies functional diversification with a potential ecological role. The study by Stefanic et al. (19) addresses the ecotype: pherotype correspondence using a collection of highly related *B. subtilis* strains that were isolated from two 1-cm^3 soil samples. Because of the relatively small size of these samples, it was assumed that *B. subtilis* isolates had been exposed to the same environmental conditions and therefore had had, at least theoretically, a history of interactions. We refer to this collection (39 *B. subtilis* strains) as microscale strains. They diverged into three different pherotypes

FIGURE 3 Phylogenetic and ecotype simulation analyses of the *B. subtilis-B. mojavensis* subclade and minimum evolution tree of *com* sequences. (A) The phylogeny of *B. subtilis* isolates from riverbank microscale and desert soils is based on a maximum parsimony analysis of the recombination-free concatenation of *dnaJ*, *gyrA*, and *rpoB*, rooted by strain C-125 of *B. halodurans* (19). (B) Minimum evolution tree of *com* sequences (*comQ*, *comX*, and partial *comP* sequences, 1,402 bp) depicts four sequence clusters that correspond to previously identified pherotypes or communication groups within *B. subtilis-B. mojavensis* clade. Strains are marked with a shape representing their putative ecotype (PE) and by color representing pherotype (yellow, pherotype ROH1/RO-B-2; green, pherotype RS-D-2 /NAF4; orange, pherotype RO-E-2; and blue, pherotype 168). Unmarked strains were used as additional reference strains for tree construction (19). doi:10.1128/microbiolspec.TBS-0017-2013.f3

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and three distinct ecotypes (19). The majority of strains that share the ecotype also have the same pherotype. This distribution suggested that ComX-mediated communication within ecotypes is preferred as opposed to communication between ecotypes and even other *Bacillus* species (like *B. licheniformis*). However, each ecotype also harbored a few strains representing other pherotypes (19). Based on these observations, it was hypothesized that pherotype diversity within ecotypes is driven by social interactions among strains within the ecotype. Why would this be the case? At high cell density, cells induce quorum sensing responses, which enable them to adapt to adverse environmental conditions by secreting public goods (extracellular enzymes, surfactins, antibiotics, surfactants) and develop genetic competence for transformation. These adaptive responses are costly but promote survival. If a member of this cooperative group obtained a different set of pherotype genes through horizontal gene transfer (HGT), this may confer adaptive value to the recipient organism in the next growth cycle. Then the recipient of another pherotype locus would be at low frequency and would therefore fail to induce the quorum-sensing response that requires high cell density and high concentration of the signaling molecule. However, it would still be capable of feasting on the public goods (e.g., extracellular enzymes, surfactants) produced by its ancestors. In the context of social evolution (162) the recipient of another pherotype will be a cheater and in social conflict with its ancestors. However, the competitive advantage will last only as long as its frequency increases and it is also forced to produce public goods. This hypothesis based on conflict also proposes the cycling of pherotypes within the ecotype (19) and is in agreement with the social conflict model proposed recently by Eldar (163). The model suggests that social conflict arises when the signal blind ComP mutant arises in the population of the QS wild-type cells. According to social evolution theory (162) the QS receptor mutant is a cheat (164), which, by remaining unresponsive to the signal, takes advantage of the QS wild-type cells that provide the QS-regulated public goods (extracellular enzymes, antibiotics, surfactants). By cheating, the signal blind mutant gains fitness advantage, rises in frequency, and may purge its cooperative ancestors from the population. However, the advantage of the signal blind mutants is presumably short lived because it may soon face conditions where QS is essential for survival. In this case, it is adaptive for the ComP receptor mutant to obtain suppressive mutations in any one of the *comQXP* genes that can restore the QS response (163, 165). Therefore, this evolutionary game between cheating, frequency-dependent selection, and adaptation could be responsible for pherotype diversification under conditions that favor quorum sensing. Future experiments will show whether a different pherotype of the same ecotype is able to rise in frequency in the population dominated by another pherotype or whether the *comP* mutant has an advantage when surrounded by the wild-type cooperative cells. Our preliminary results suggest that the *comP* null mutant in competition with the wild type has an advantage (P. Stefanic and I. Mandic-Mulec, unpublished data) but it will be important to show that this holds also in conditions where QS is beneficial. It will also be interesting to test the coexistence and competition of these microscale *Bacillus* strains in soil microcosms under conditions where growth depends on quorum sensing. This would ultimately test the hypothesis of social conflict (19, 163) driving pherotype diversification. However, the observation that different *Bacillus* species such as *B. subtilis*, *B. mojavensis*, and *B. amyloliquefaciens* can share pherotypes (19, 41, 156) strongly supports the notion that HGT is also important in the distribution and evolution of pherotypes. Recently we showed that the *comQXPA* quorum-sensing genes are widespread within *Bacillaceae* and that polymorphism of this locus typical for *B. subtilis* clade is evident also in the non-*B. subtilis* clade, suggesting grossly similar evolutionary constraints in the underlying quorum-sensing systems (159, 166). Interestingly, the ComXPA QS system has built in a molecular mechanism, which by acting as a private link between signal and response in *B. subtilis* quorum sensing, may stabilize cooperative communication (167). A signal-deficient mutant showed lowered fitness in the presence of signal-producing wild-type ancestors because it overinvested into production of public goods (e.g., into production of lipopeptide antibiotic and surfactant surfactin) and became surfactin sensitive. While this punishment mechanism probably originally evolved to fine-tune the QS response, it also has implications for the stability of cooperation within a pherotype (167).

B. subtilis is one of the best-studied bacteria with respect to its molecular make-up (168). However, surprisingly little is known of its ecology and diversity in soil. Strains within the *B. subtilis* clade form two subclusters that represent two subspecies: *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*. They show DNA relatedness of 58 to 69%, and differ in cell wall composition (169). Among these two subspecies, strains 168 and W23 have been most often used in research addressing various aspects of *B. subtilis* physiology (40,

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169). The level of genetic diversity based on multilocus sequence typing (MLST) within subcluster W23 was found to be higher than that within 168, or in the closely related *B. mojavensis* cluster. The ecological significance of this diversity is not understood. However, microarray-based comparative genomic hybridization (M-CGH) (168) confirmed closer genomic tightness within the subclusters than between them. The level of gene sequence divergence within the species was 30%. Sequence diversity was highest for genes involved in the synthesis of secondary metabolites and of teichoic acids and for genes involved in the adaptive response to alkylating DNA damage. Recently, Earl et al. (170) published genome sequences of several closely related *Bacillus* strains. This study indicated strain-specific regions that were spread throughout the core genomic backbone. A majority of these variable sequences were smaller than 5 kb but some were also up to 100 kb in size. Many of them encoded genes involved in adaptive functions (e.g., antibiotic synthesis, competence, sporulation), which is in agreement with the high adaptive plasticity of these bacteria to diverse ecological niches.

The locus encoding the first three genes of the *comQXPA* quorum sensing system is one of the regions that is highly diverse in closely related strains of the *B. subtilis – licheniformis* group (154, 161). This diversity is present within organisms isolated from a small soil sample (1 cm^3 or even 0.25 cm^3) (41) as well as those that were isolated from soils separated by large geographical distances (156). The high heterogeneity of isolates living in close proximity may be due to a very high spatial heterogeneity of soil. Soil has a high surface-to-volume ratio and therefore provides a tremendous contact surface area for microorganisms to grow on. For example, 1 g of clay minerals, which are the smallest solid-phase component of soil and are classified as a size fraction of <2 μm in diameter, provide a surface of 93 to 800 m^2. Besides clay particles and organic matter, soils also contain the silt fraction (2 to 50 μm) and the sand fraction (50 to 2000 μm). The proportion of each fraction defines the soil texture, which influences the other two soil phases: the gaseous (soil-air) and the aqueous ones (soil-water or soil solution). The size of the soil pore increases with increased proportion of sand in soil and decreases with the increased content of clay. The soil texture affects water percolation and evaporation and influences the composition of microbial communities (171). It is believed that the soil matrix, due to its huge reactive surface, is a habitat where geographic isolation and niche differentiation of microorganisms is already possible at small distances. Therefore, it is not that surprising to find a high diversity of *B. subtilis* in soil even at distances of 250 μm (19) or below (172). The ecological and evolutionary principles, including the competition between bacteria for natural resources, acting at this scale are poorly understood and studies addressing this field have recently been reviewed by Vos et al. (173).

CHALLENGES AND GOALS

We may safely state that we currently have gathered only a rough understanding of the lifestyle of key members of the genus *Bacillus* in their natural habitat, e.g., the soil and the plant rhizosphere. Given the fact that members of *Bacillaceae* are mostly aerobic or facultatively anaerobic heterotrophic organisms with the capacity to show a rapid growth response to available organic carbon, one would expect a role of such bacteria in carbon-rich sites in nature, such as in the rhizospheres of actively exuding plants or in soil sites where organic matter is being degraded. In such sites, bacteria may become active and play roles in local processes. However, we know very little of *Bacillaceae* life dynamics in soil, because most of them are able to form spores and studies monitoring the activity of vegetative cells in soil are lacking. For example, we have no knowledge of how quorum-sensing processes operate in soil; how stable the signaling peptides are; whether the adaptive processes, such as competence development, sporulation, extracellular enzyme production, known to be controlled by quorum sensing at the laboratory conditions, are regulated in the same manner in soils. Even less is known about the temporal dynamics of these adaptive processes in soil and other natural environments. Are members of *Bacillaceae* mostly represented in soils by spores? Is vegetative growth a rare event? Do representatives of *Bacillaceae* actively compete and affect other members of the soil and rhizosphere community? How important are adaptive responses such as competence development or antibiotic production for the success of the *Bacillus* in soils and/or the rhizosphere? What is needed in future studies are direct observations, on the basis of sensitive tools from the "omics" area, of the differentiated cells and their constituents. On the methodological level, the focus should thus certainly be on the development of additional molecular tools to target such facets of *Bacillus* species in their natural habitat (e.g., soil). This may, for instance, boil down to an improvement of the methods for isolation of DNA/RNA from soil, followed by an investment in the development of sensitive tools for the detection of cell types as well as particular cellular

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constituents (e.g., mRNAs of different types, target proteins) that depict the make-up of the *Bacillus* population in its natural setting.

Independent studies reveal that members of the genus *Bacillus* are not usually the most abundant bacterial species in soil. Nonetheless, some *Bacillaceae* and, often, *Bacillus* species, are almost invariably present at levels of 10^6 to 10^8 per g of dry soil, which is between a thousandfold to a hundredfold less than the total bacterial density. Is the predicted role of *Bacillus* as a driver of soil organic matter mineralization in agreement with its abundance? Answers to these question call for development of *Bacillus*-specific molecular tools which will enable us to quantify the active members of the soil *Bacillus* community and also follow *in situ* their adaptive response, which have been extensively studied *in vitro*.

We here postulate that, in the light of the great diversity in ecological roles found across members of the *B. cereus* cluster, a similar great diversity of roles may be present across soil saprotrophic bacilli. This may extend into the plant rhizosphere, where such bacilli may have beneficial roles as a result of their activities as saprotrophs in this habitat. It is in this key environment, where interactions with the plant root take place that improving our understanding of *Bacillus* ecology will have the most impact. How can we promote the plant-beneficial functions exerted by such rhizospheric bacilli? A key need is to further our understanding of the plant root colonization and cell differentiation processes that presumably direct which *Bacillus* strain will be favored in the rhizosphere. It will be important to decipher cell-cell interactions within and among *Bacillus* populations *in vitro* and *in situ*. Placing such observations in the context of local conditions in the rhizosphere will ultimately pave the way to an ecology-guided strategy for the application of *Bacillus* biocontrol agents.

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