Chapter 8
Single Genus Approach to Understanding Bacterial Diversity, Niche, Distribution, and Genomics: The *Bacillus* in Cuatro Ciénegas

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Abstract To understand microbial life and get the full picture, we ought to zoom out in order to grasp its magnitud and diversity and zoom in to dissect their genes and functions. We have obtained a significant amount of data on the great diversity of bacteria in the different environments of the Cuatro Ciénegas Basin (CCB), equivalent to obtaining the census of a city. However, what are these bacteria doing and what is their role? How do they get along? We have chosen the genus *Bacillus* to answer these questions. *Bacillus* can form highly resistant spores that disperse readily and can remain dormant for years, which complicates their study. On the other hand, bacteria of the *Bacillus* genus are abundant, diverse, and easily cultivated, so they are a good model, both to understand their biology and to study their interactions in communities. With 2500 isolates from CCB and data obtained from microbiological and genomic approaches, we can now write *Bacillus* tale in Cuatrociénegas.

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Exploring Conservation of Lineage Phenotypic Traits Within the *Bacillus* Genus in a Community Setting

Bacteria of the *Bacillus* genus have been extensively characterized with classical microbiology, biochemistry, genomic, and proteomic approaches. The *Bacillus* species are considered generalists, because they are present in very diverse environments, have versatile metabolic capabilities for exploiting different organic and inorganic substrates, and have excellent dispersion capacity due to their ability to form spores. Spores are quiescent and highly resistant structures able to withstand nutritional and environmental stress that allows them to thrive in different environments. Although *Bacillus subtilis* is the model species, studies have been done in many species within the genus involving sporulation, and other traits have been observed to be deeply rooted among the *Firmicutes*. These traits include Gram-positive staining as well as possessing capabilities for swimming, swarming, and biofilm formation.

Bacteria in the *Bacillus* genus are abundant, diverse, and easily cultivated. From metagenomic studies at the Churince water system, we have (unpublished data), we have found that the *Bacillus* species represent 2.4% of the microbial abundance (unpublished data). *Bacillus* species from CCB do not form a monophyletic group and exhibit ancient divergences, this can be said due to the isolation of lineages that could have inhabited an ancient ocean (Moreno-Letelier et al. 2012). Souza et al. (2006) found that nearly 50% of phylotypes from the CCB were closely related to organisms from marine environments, including some *Bacillus* species. For some of the isolated *Bacillus* strains from Cuatro Cienegas, their closest relatives are from the Gulf of Mexico (*Bacillus* sp. SG-1, *Bacillus* sp. NRRL B-14911), Sea of Korea (*B. aquimaris*), and tidal flat in the Yellow Sea (*B. marisflavi*) or isolated from marine organisms (*B. horikoshii*).

It has also been noted that there are several endemic *Bacillus* species within the Cuatro Cienegas Basin, most of them with very old divergence (Moreno-Letelier et al. 2012). Although we know that many Bacillus lineages co-occur in this environment (Fig. 8.1), we still have many questions: What are these bacteria doing? What is their role? How do they get along? Is taxonomical diversity the same as functional diversity?

The results from this first sequenced genome CCB was *Bacillus coahuilensis* m4-4 (Alcaraz et al. 2008), which was a strain isolated from the water column of a desiccation lagoon that no longer exists (Laguna Grande) in the Churince by Cerritos et al. (2008). The sequence revealed that this bacterium had many unexpected features that had never before been observed in the *Bacillus* genus. It lacked teichoic acid, a phosphate-rich cell wall layer always present in Gram-positive bacteria. It also possessed a bacteriorhodopsin gene, which had never been described for *Bacillus*, and, notably, it possessed genes for the biosynthesis of sulfolipids (Alcaraz et al. 2008). These genomic features allowed *B. coahuilensis* to add sulfolipids to its membrane, and, since it had no phosphorus-rich teichoic acid requirement, these bacteria can cope with the limiting phosphorus conditions characteristic
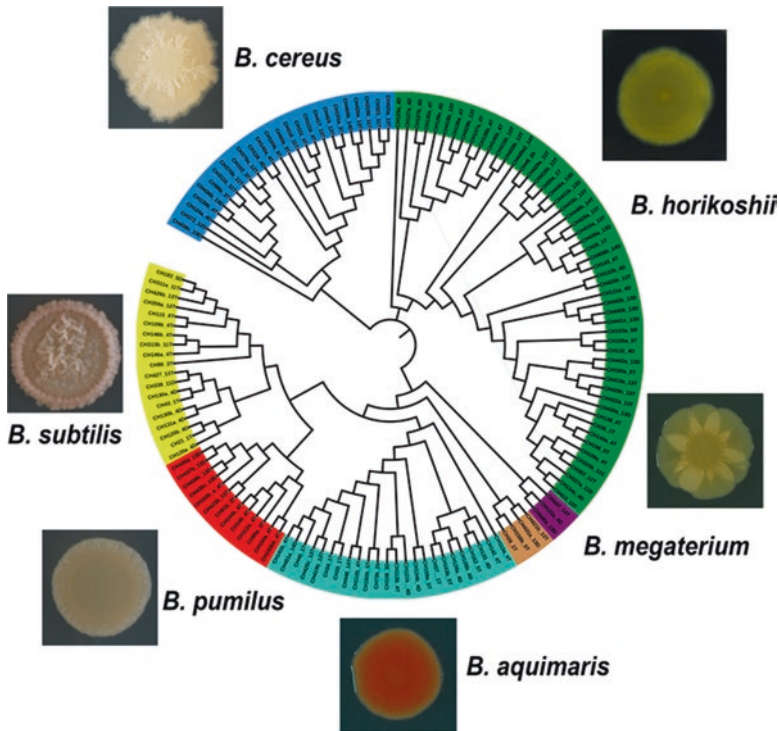
Fig. 8.1 Phylogenetic reconstruction of 465 thermo-resistant isolates from the Churince aquatic system. Most of the isolates are related to *Bacillus* species

of CCB. The results from this first genome were very interesting, as they showed us that the genomic data reflected the metabolic potential of the bacteria and the ecology of the site.

Bacillus m3-13 was the second CCB strain to be sequenced, also from the same extinct lagoon from which *B. coahuilensis* was isolated, and with it, we began the comparative exploration of aquatic CCB *Bacillus*. Before our work, most *Bacillus* studies had been done on the so-called soil species, namely, *B. cereus* and *B. subtilis/pumilus*. We were able to do the first phylogenomic analysis of an aquatic *Bacillus* genus and describe the first core genome for the genus consisting of 870 genes (Alcaraz et al. 2010). We showed that even for the development of the spore—a highly conserved process—the most conserved genes were those driving transcription at different stages, while the signaling and structural genes showed considerable variability (Alcaraz et al. 2010).

The relationship between the phylogeny and the functional features of each member of the *Bacillus* group revealed great variability that limited the predictive power of a microbial composition approach (Alcaraz et al. 2010; Martiny et al. 2013). According to Philippot et al. (2010), the consistency of traits can be maintained up to the phylum level. Nevertheless, other reports support the idea that the

ecological traits may be specific to species or strains, since the microbial genomes are highly dynamic and can change rapidly through gene loss, gene duplication, and acquisition of genes from distant lineages via horizontal gene transfer (HGT, Boon et al. 2014). It has been shown that strains within a given species may have between 30 and 35% gene content differences (Konstantinidis et al. 2006). Bacterial species are known to have a mosaic of transferred genes since their high genetic diversity is obtained in some cases from distantly related organisms (Ochman et al. 2000). If there are at least 1000 different genes at the species level, numerous phenotypic differences can be expected. With these differences, it is not surprising to find species colonizing different habitats.

Most comparative studies on bacterial traits and genomes are based on strains that have been sampled from highly diverse environments. Many functional traits have been shown to be phylogenetically dispersed in bacteria (Martiny et al. 2013). These studies have shown that lifestyle differences correlate with variations in genes required to interact with specific environments. For example, features of free-living bacteria differ from those of pathogens and symbionts. On the other hand, for a community to remain stable, it is suggested that the synthesis of products found in the environment may be sufficient to support a member's loss of genes or functions (McInerney et al. 2011; Morris et al. 2012; Boon et al. 2014).

Adaptations to Deal with Very Low Concentrations of Phosphorus in CCB

Phosphorus (P) is an essential element for multiple processes and is found in different essential molecules, such as DNA, cell membranes, and cell walls (teichoic acid in Gram-positive bacteria), and in molecules for energy transfer. Different *Bacillus* taxonomic groups coexist in the Churince water system of CCB, a site with high concentrations of sulfates, magnesium, and calcium but low concentrations of NaCl and carbonates and, notably, extremely low concentrations of phosphorus (0.3 µM). Therefore, this water system is categorized as an ultra-oligotrophic environment (Cerritos et al. 2008).

Tapia-Torres et al. (2016) investigated how bacteria in CCB managed to obtain P. They measured the availability of P and the enzymatic activity associated with P release in soil and sediment from the Churince and Pozas Azules systems. They also evaluated the use of different sources of phosphorus for culturable bacteria. The patterns of enzymatic expression showed substantial differences in the amounts and types of exoenzymes in the different microbial communities in the study sites. Phosphodiesterase activity was higher in soil samples, phosphomonoesterase activity was higher in sediment, and phosphonatase activity was detected in soil samples but not in sediment samples.

Regarding the use of different sources of phosphorus for culturable bacteria, such as DNA, calcium phosphate, phosphite, and phosphonates (2PA or 2AEP), Tapia-Torres et al. (2016) results showed no correlation between taxonomic group and the ability to use different P sources. This lack of correlation suggests that the

genes involved in the utilization of P can transfer laterally among strains. This was corroborated by our studies on the gene encoding the Pst transporter, that suggests that it moves among bacteria through lateral transfer (Moreno-Letelier et al. 2011).

Since the different *Bacillus* species can break down and use P forms in different oxidation states, they contribute to P cycling in the ecosystem, suggesting a dynamic movement of P utilization traits among bacteria in microbial communities. Most bacteria can use potassium phosphate, calcium phosphate, and DNA and to a lesser extent phosphite, 2PA, or 2AEP. Interestingly, members of the *B. cereus* group isolated from aquatic sediments at the Churince could use more phosphorus sources compared to members isolated from soils. The ability to use phosphite was high in members isolated from sediment. This phosphite utilization preference among *B. cereus* from sediment reflects the variable occurrence of the different P sources in soil and sediment and shows evidence for the presence and importance of phosphite in natural environments (Tapia-Torres and Olmedo 2018). On the other hand, the dispersion of traits related to P acquisition within and across taxonomic groups is suggestive of niche partitioning strategies that also explain how genetic diversity is maintained.

Other physiological adaptations have been observed in *Bacillus* species that live in the phosphorus-limited CCB environment. The best characterized strains are those of *B. coahuilensis*, which were isolated from the water column of a shallow desiccation lagoon in the Churince system (m4-4 and m2-6) and Pozas Azules (p1.1.43) (see Chap. 5 in this book). These strains possess genes that encode key enzymes in the synthesis of sulfolipids (sulfoquinovose synthase and glycosyltransferase). Sulfolipids can be integrated into the membrane and can replace some phospholipids. *B. coahuilensis* also lacks genes involved in the synthesis of teichoic acid, a polymer located within the Gram-positive cell wall, and involved in the regulation of cell division and Gram-positive bacterial physiology (Gómez-Lunar et al. 2016; Brown et al. 2013). This finding helped to reveal that some *Bacillus* taxonomic groups related to marine bacteria environments and isolated from CCB were Gram-negative (unpublished data).

Although *B. coahuilensis* m4-4 and m2-6 strains share the same environment as *Bacillus* m3-13, *Bacillus* m3-13 shares many strategies with the m2-6 strain for dealing with the poor nutrient environment, but not the m4-4 strain. *Bacillus* m3-13 encodes phosphonate ABC importers, permeases, and phosphonate lyases that can take up and assimilate phosphonates (see Chap. 5 in this book). In this sense, the *Bacillus* species contain adaptations that reveal different strategies for using phosphorus in order to survive in the oligotrophic CCB environment.

Traits Deeply Rooted in Taxonomic Lineages: Exploring Conservation of Lineage Phenotypic Traits Within a *Bacillus* Genus in a Community Setting

One of the most important characteristics of the *Bacillus* species when facing nutrients scarcity is their ability to form endospores: structures highly resistant to heat, radiation, desiccation, and oxidizing agents. Most of the *Bacillus* species obtained

from Cuatro Cienegas were isolated by heating environmental samples, so it was assumed that the strains readily sporulated. However, when a representative group of different taxonomic groups were subjected to different culture media to trigger sporulation, the strains related to aquatic members (i.e., *B. marisflavi*, *B. aquimaris*, and *B. horikoshii*) had sporulation values below 1% (unpublished data), whereas *Bacillus* related to *B. subtilis*, *B. thuringiensis*, or *B. pumilus* had a sporulation frequency that was above 50% and could reach 100% (unpublished data). Based on genomic data, Alcaraz et al. (2010) observed that less than one-third of the genes involved in the sporulation of *B. subtilis* were conserved across other *Bacillus* species with essential differences in the signaling genes.

Genomic and phenotypic insights of *B. coahuilensis* showed that its genome is a genetic mosaic. It has acquired genes involved in phosphorus utilization efficiency and genes relevant to adapting to high-light environments possibly through HGT from cyanobacteria (Alcaraz et al. 2008). These traits depend on a few genes, like other traits related to nutrition that are known to be easily transferred among bacteria (Martiny et al. 2013). In this sense, analysis of the utilization of carbon sources in *B. coahuilensis* strains showed that m2-6 and p1.1.43 had similar utilization patterns of poly- and oligosaccharides, carboxylic acids, and an aromatic compound, but m4-4 and m2-6 were more similar in their choice of monosaccharide utilization. In general, *Bacillus* species exhibit some differences in their preference for different carbon sources, according to genomic predictions made from the presence of transporters in genomes belonging to (1) soil members (*B. subtilis*/*B. licheniformis* and *B. pumilus*), (2) aquatic members (*B. marisflavi*, *B. aquimaris*, and *B. horikoshii*), and (3) *B. cereus*-related species (Alcaraz et al. 2010). However, experimental evaluation of members from these groups showed diverse capabilities to utilize glucose, raffinose, trehalose, xylose, and sorbitol. These capabilities were sparsely distributed and did not cluster with any particular *Bacillus* lineage (Rodriguez-Torres et al. 2017).

The *Bacillus* species possess other essential traits associated with specific growth or developmental stages. Swarming and swimming are traits related to motility (Fig. 8.2). Both traits depend on a multi-protein complex that forms the flagella. Swimming in an aqueous medium allows an individual bacterium to move toward chemoattractants or to get free from a biofilm. Swarming allows bacterial groups to move on surfaces with less water, so this trait is tested on semisolid agar. Swarming requires additional flagella as well as surfactants to break the superficial tension (Kearns and Losick 2003). The ability to make biofilms is another conserved trait in bacteria. Biofilms allow bacteria to attach to surfaces and to grow as a community so as to produce a barrier against environmental hazards (i.e., antibiotics). Biofilm production depends on multiple genes considered deeply rooted in taxonomic lineages, such as exopolysaccharides. Swarming and biofilm formation are multicellular traits. These traits are considered social features (González-Pastor et al. 2003), since they provide benefits at a group level and depend on the coordinated expression of genes and signals (Stoodley et al. 2002; Daniels et al. 2004). Swimming and sporulation may also respond to group signals.

The results of the swarming tests of the *Bacillus* isolates from Churince were negative for aquatic members, but positive for soil members, while a smaller number of *B. cereus*-related species presented this ability (Rodriguez-Torres et al. 2017). The three *B. coahuilensis* strains lacked swarming capabilities, and their genomic analysis showed that they lacked the *srfABCD* operon that encodes surfactin (Gómez-Lunar et al. 2016). Regarding swimming evaluation—depending only on flagella synthesis—most species showed this ability, except for *B. coahuilensis* m4-4 and 9 out of 23 strains related to the *B. horikoshii* (Fig. 8.2).
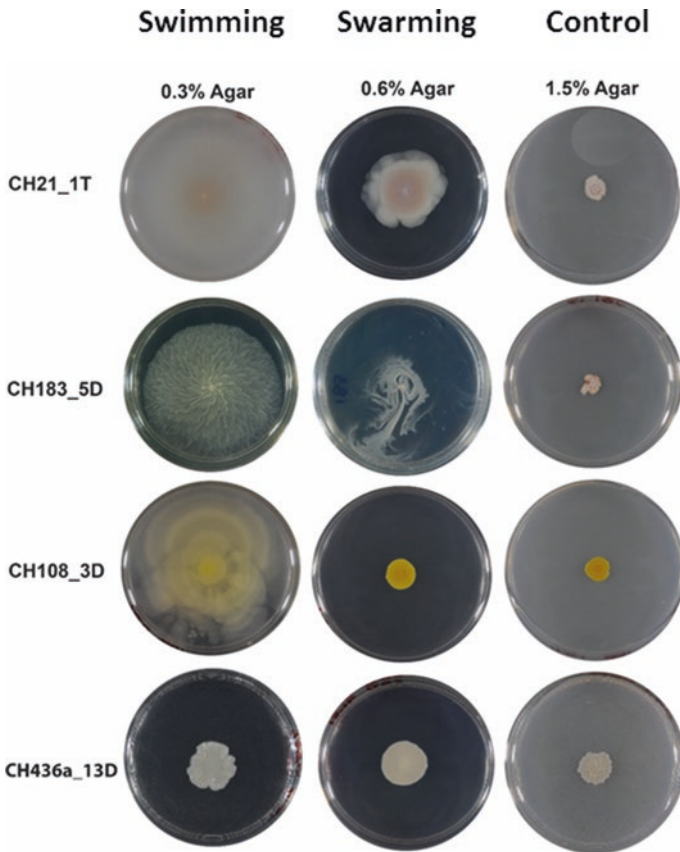

Fig. 8.2 Swarming and swimming motility evaluated in *Bacillus* strains from the Churince. The plates show photographs of strains that exhibit swarming and swimming motility compared with normal agar (1.5%: first column), the second column shows plates with 0.6% agar (to evaluate swarming), and the third column shows with 0.3% agar (to evaluate swimming). We show three types of results: (1) strain CH21_1T y CH183_5D exhibited swarming and swimming, (2) strain CH108_3D exhibited swimming but not swarming, and (3) strain CH436a_13D did not present either swarming or swimming motility

Biofilm formations are organized populations that depend on the expression of an extracellular polymeric substance (Spoering and Gilmore 2006). In natural environments, biofilm formations typically provide benefits at the group level (Stoodley et al. 2002; Daniels et al. 2004). Biofilm results of *Bacillus* isolated from CCB showed results similar to swarming, since it was usually present in members of the soil *Bacillus* and the *B. cereus* group but absent in most aquatic members (Fig. 8.2).

Bacteria also possess metabolic routes for producing their amino acids. Isolates that can produce all amino acids are called prototrophs, while those that cannot produce one or more amino acids are called auxotrophs. The evaluation of amino acid biosynthesis capabilities revealed this to be another variable trait among members of the communities. There were numerous auxotrophs found in *B. cereus* and also among aquatic *Bacillus*. In contrast, *B. subtilis/pumilus* members were almost always prototrophs.

We analyzed the genomes of some bacteria to further understand the nature of auxotrophs that lacked particular genes in the amino acid biosynthetic pathways. For *B. coahuilensis*, some auxotrophs correlated with the absence of genes in the conventional routes (Gómez-Lunar et al. 2016). The biosynthesis routes appeared to be complete for *B. cereus*, but surprisingly some auxotrophs were observed (unpublished results). This lack of consistency between genotypes and phenotypes has to be further explored.

How can this trait variation in *Bacillus* from the communities be explained? The variation observed could be attributed to compensated trait loss occurring in communities which allows an individual, even with a function loss, to continue obtaining the trait from the community or an adaptive trait that responds to specific cues that cannot be assayed in culture (Ellers et al. 2012).

Antagonistic Interactions Influence Community Assemblage at Each Site

As explained above, the absence of some traits among strains of the sediment communities suggests that particular product loss can be afforded if these can be obtained by sharing with other community members. However, sharing it is not the only thing happening in communities. It has been suggested that the interactions among culturable bacteria are dominated by competition (Foster and Bell 2012).

Pérez-Gutiérrez et al. (2013) analyzed the interactions between several coexisting lineages of *Bacillus* species from five physicochemically similar sampling sites from the Churince system. They determined the antagonism potential of the isolates and compared them against each other, as well as within and across the different sampling sites to understand the assembly of sediment communities and the influence of interfering interactions. A directed network could describe the 6000 scored interactions with a hierarchical structure that revealed a food chain-like structure in which different *Bacillus* taxonomic groups occupied specific positions. Fewer
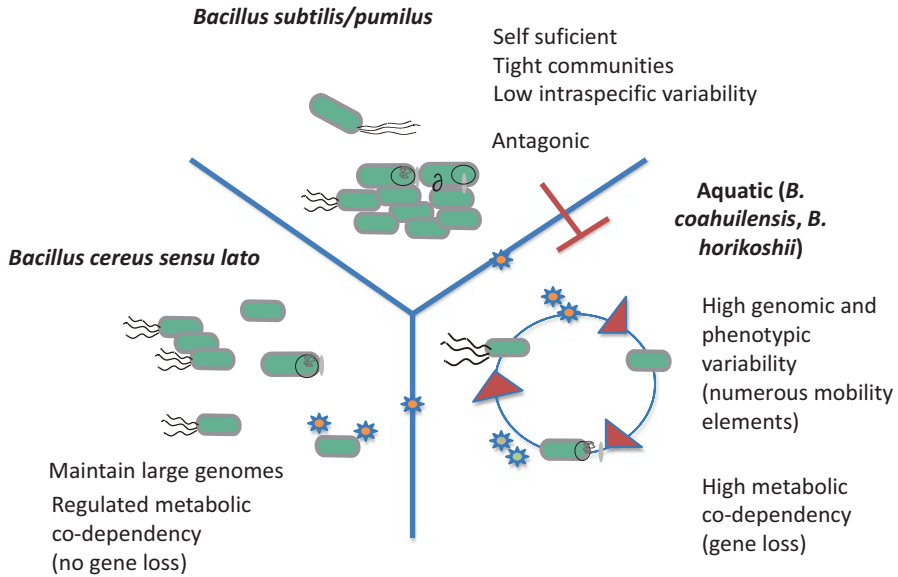
Fig. 8.3 Distinct traits associated to the different *Bacillus* taxonomic groups from sediment communities. Three main strategies were observed among the *Bacillus* taxonomic groups studied. The *Bacillus subtilis/pumilus* exhibits low intraspecific variability and are also the most antagonistic. The aquatic bacteria are generally sensitive to antagonism and exhibit great variability in social traits. The group of the *B. cereus* is generally resistant to antagonism, maintains large genomes, but exhibits dependency on the communities to complement nutritional and social traits

antagonistic interactions were detected within sites than across sites, suggesting that antagonistic interactions influenced community assemblage at each site. Furthermore, antagonism and sensitivity could be associated with specific *Bacillus* taxonomic groups, and the data could be represented as a hierarchical network representation. For instance, the *B. subtilis/pumilus* group was usually antagonistic toward the aquatic *Bacillus aquimaris/horikoshii*, while the *B. cereus* group members were resistant to antagonism and were not antagonistic (Fig. 8.3).

In summary, our in-depth analysis of a single genus, the *Bacillus* from CCB, allowed us to unravel wide intraspecific variability among community members at the genomic and phenotypic level focusing on substrate utilization traits and social traits (Fig. 8.3). We also showed that the ecological strategies are different at the species level. Although there is competition, different members diversify their micro-niche allowing them to coexist in complex communities. New studies need to elucidate how those complex communities get build, which is the role of historic vs change in the community assemblage, not only of the coexisting *Bacillus* but of all the community. This is a dauntingly fascinating challenge.

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