

Chapter 2

Diversity of Endospore-forming Bacteria in Soil: Characterization and Driving Mechanisms

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2.1 Introduction

Aerobic endospore-formers have long been considered to be important components of the soil bacterial community. Inoculation of standard nutrient media with soil that has been heat-treated, to kill vegetative cells, leads to growth of high numbers of endospore-formers and the availability of isolates has facilitated detailed taxonomic and physiological studies. Both taxonomic and physiological diversity of soil isolates is high and extrapolation of this metabolic capability and diversity implies important roles in a wide range of soil ecosystem functions and processes. Their heterotrophic life style suggests an obvious role in the carbon cycle but, as a group, aerobic endospore-formers are also important in the soil nitrogen cycle, as denitrifiers, nitrogen fixers and degraders of organic nitrogen; in the sulphur cycle as sulphur oxidizers; and in transformation of other soil nutrients, e.g., through manganese reduction. Their abilities to break down cellulose, hemicelluloses and pectins suggest major roles in mineralization of plant material and humic material, while chitinase activity facilitates degradation of fungal cell walls and insect exoskeletons. Thermophilic bacilli dominate the high-temperature stages of composting and they produce a wide range of commercially valuable extracellular enzymes, including thermostable enzymes. Endospore-formers are important in soil bioremediation, through their ability to degrade BTEX (benzene, toluene, ethylbenzene and xylene) compounds and to methylate mercury. They produce a wide range of antiviral, antibacterial and antifungal compounds, which may be important in interactions with other soil microorganisms and have significant commercial potentials in agriculture and medicine. Many have phosphatase

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activity, releasing phosphate for plant growth, and they possess a range of other plant-beneficial properties that have led to commercial applications as plant-growth-promoting bacteria.

Specific aspects of the ecology of aerobic endospore-formers and their roles in remediation, plant-growth promotion, biological control and other applications are discussed in later chapters. Here we focus on their diversity and community composition in soil, the implications of recent and future methodological developments on diversity studies, and their contributions to our understanding of the mechanisms driving microbial diversity in soil. Most of the studies we discuss preceded recent taxonomic revisions, and aerobic endospore-formers are frequently referred to using the collective term bacilli; this term will be used here, except where studies focus on particular genera or species.

2.1.1 Methods for Determining Diversity of Soil Bacilli

Soil bacilli have been investigated since the first isolation of bacteria from soil in the nineteenth century and methods to study their diversity have followed those used for all soil bacteria. These began with phenotypic characterization of soil isolates, the application of numerical taxonomy and the subsequent introduction of molecular phylogenetic approaches more than 20 years ago. The past 15 years have seen considerable development of cultivation-independent methods for characterizing bacterial communities, based on molecular analysis of nucleic acids extracted directly from the soil, rather than from isolates. These developments will be discussed in this section, considering specific factors related to endospore-formers, examples of their use and their benefits and limitations. Results arising from use of these techniques are discussed in Sect. 2.3.3, while detailed protocols are described in other chapters.

2.1.2 Cultivation-Based Methods

Traditional approaches to determination of soil bacterial community composition and diversity relied largely on cultivation-based methods. Typically, a solid, complex organic growth medium inoculated with dilutions of a soil suspension is incubated and colonies are removed, subcultured and purified. Identification of isolates provides information on the relative importance of endospore-formers within total bacterial communities. More detailed studies exploit the heat-resistance of endospores and select for bacilli by pasteurization of soil, or soil dilutions, to kill vegetative cells. Selection for specific taxonomic or functional groups of bacilli (e.g., nitrogen fixers) is also possible through the use of selective media, based on nutritional or physiological characteristics of the target group. For example, Seldin et al. (1998) describe a method for selection of *Paenibacillus azotofixans* from soil.

2.1.2.1 Phenotypic Characterization

Isolates may be characterized by a range of methods. Traditionally, morphological and physiological characteristics were used for classification and identification. For the large numbers of isolates generated by ecological studies, this process is facilitated by miniaturized methods, such as API strips. While such techniques provided the basis for bacterial, and *Bacillus* taxonomy, additional tests were necessary for confirmation and for fine-scale resolution. For example, analysis of protein or enzyme composition, using multiple-locus enzyme electrophoresis (MLEE), has been used to analyse intraspecific variation in *Bacillus cereus* and *B. thuringiensis* (Helgason et al. 1998; Vilas-Boas et al. 2002) and serotyping, against flagellar antigens, is well-established for distinguishing strains of *B. thuringiensis* (Helgason et al. 1998). Bacterial soil isolates can also be characterized by fatty acid methyl ester (FAME) analysis, which can distinguish bacilli within total bacterial communities, as well as identifying isolates.

2.1.2.2 Identification Using Molecular Techniques

Bacterial taxonomy was revolutionized by the application of molecular techniques and, in particular, analysis of 16S rRNA gene sequences to determine phylogenetic relationships. This approach is much more robust and is now routinely used for classification and identification of soil isolates, including bacilli. Other genes have been employed for phylogenetic analysis and identification. Some increase discriminatory power beyond that of 16S rRNA genes while others have potential ecological relevance and importance and can be used to determine relationships between bacilli and other bacteria sharing particular functions.

16S rRNA and functional gene-based phylogenies are generally useful for delineation to the genus- and, occasionally, species-levels but analysis of chromosome-wide differences increases taxonomic resolution and enable intraspecies discrimination. For example, Meintanis et al. (2008) compared 11 *Geobacillus* and *Bacillus* strains isolated from a volcanic region by *rpoB* sequence analysis, repetitive extragenic palindromic-PCR (REP-PCR) and BOX-PCR; da Mota et al. (2002) compared *Paenibacillus polymyxa* isolates from maize rhizosphere using REP-PCR and randomly amplified polymorphic DNA (RAPD) analysis; and Ryu et al. (2005) used amplified fragment length polymorphism (AFLP) and multilocus variable-number tandem repeat analysis (MLVA) to characterize soil and clinical strains of *Bacillus anthracis*. REP-PCR, BOX-PCR and MLVA are alternative techniques for analysis of interspersed repeated sequences within the genome, while AFLP and RAPD, respectively, amplify genomic DNA using random primers or non-specific primers that are complementary to a number of sites within the genome.

Taxonomic resolution can also be increased using multilocus sequence typing (MLST) and analysis (MLSA). This involves sequencing of short regions of several (typically seven) housekeeping genes distributed throughout the chromosome.

Relatedness of strains is determined by comparison of sequence types of all seven genes. MLST was used by Sorokin et al. (2006) to distinguish 115 *B. cereus* group soil isolates and by Bizzarri et al. (2008) to determine relatedness of 22 phylloplane isolates, comparing results with analysis of plasmid profiles and *cry* gene sequences.

2.1.2.3 Benefits and Limitations

Each of these approaches has its advantages and limitations, with regard to taxonomic resolution, ease of use, cost, and availability, and their values will be evident elsewhere in this volume. An important factor is the amount and type of information they provide. Several of the techniques are valuable in discriminating and grouping strains, but give no useful ecological information. For example, 16S rRNA gene-based methods provide good phylogenetic information to the genus level, but, in themselves, give little information on function. Importantly, traditional approaches that group isolates on the basis of common metabolic properties may be limited in terms of phylogenetic power, but provide clues to environmental factors favouring and selecting for particular groups and can be strong indicators of potential ecosystem function. Classification often requires a multiphasic approach and the different approaches may not agree. For example, groupings indicated by FAME and 16S rRNA gene analysis of rhizosphere communities can differ (Kim et al. 2003). In addition, while phylogenetic analysis may group isolates with similar physiological characteristics, many important ecological traits are borne on plasmids.

Cultivation-based analysis of diversity of endospore-formers is facilitated, in one sense, by the ease with which they can be selected, i.e., through pasteurization of samples before cultivation. This is appropriate for qualitative studies of diversity, where the aim is merely to determine “who is there”. However, selection against vegetative cells will introduce bias towards those bacilli that produce the greatest numbers of spores, those producing spores that germinate most rapidly on laboratory media, and those that may have produced large numbers of spores through stress, which makes them inactive in the soil. This approach is therefore severely restricted for studies in which relative abundances of total numbers of bacilli (vegetative cells and spores) are required, or where information is required on active, rather than potentially active organisms. Consequently, it is limited in its ability to link community structure and composition to soil ecosystem processes carried out by bacilli.

Cultivation studies also suffer from the major disadvantages associated with the inability of the majority of soil microorganisms to grow on standard enrichment growth media and under standard laboratory incubation conditions. Selection for particular functional groups (e.g., nitrogen fixers, denitrifiers) is possible by careful design of media and growth conditions, but the broad physiological and metabolic diversity within the bacilli makes it difficult to design media that are selective for particular phylogenetic groups.

2.1.3 Cultivation-Independent Analysis of Diversity

Molecular approaches for phylogenetic analysis of cultivated organisms quickly led to the development and application of similar approaches for analysis of natural soil communities that avoid prior enrichment or isolation of pure cultures. They have transformed our view of soil microbial communities and have uncovered vast and previously unsuspected diversity within groups, such as bacilli, that are well represented among soil isolates. They have also revealed abundant, novel groups of bacteria and archaea performing important soil ecosystem functions.

2.1.3.1 16S rRNA Gene-Based Analysis of Prokaryote Communities

Soil prokaryote community composition is now routinely determined by the amplification of 16S rRNA genes from DNA extracted from soil using primers that are specific for a particular target group. The presence of regions within the 16S rRNA gene with differing degrees of sequence variability allows the design of primers with different resolution. For example, primers are available for amplification of all bacteria or for specific genera and, occasionally, for different functional groups. Amplification products can be cloned and sequenced, for phylogenetic analysis or for identification, by comparison with database sequences obtained from cultivated organisms and 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 analysis of extracted soil DNA (Roesch et al. 2007). These avoid the cloning step, and potential cloning bias, and enable sequencing of hundreds of thousands of amplicons in a single run.

Amplification products can also be analysed using fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP) and single-strand conformation polymorphism (SSCP). DGGE and TGGE separate amplicons on the basis of heterogeneities in GC content and sequence, and consequent differences in melting properties, when run on a gel containing a gradient of a denaturant or temperature, respectively. For T-RFLP, amplified DNA is digested with restriction enzymes, distinguishing amplicons with sequence polymorphisms. SSCP involves electrophoretic separation of single-stranded nucleic acids with differences in secondary structure. All are less expensive than sequencing methods and allow rapid analysis of many samples and assessment of relative abundances of different phylotypes, but provide less information on identity of organisms present. Felske et al. (2003) adopted an alternative approach, multiplex PCR, to amplify simultaneously 16S rRNA genes using primers targeting several groups: *Acidobacteria*, *Verrucomicrobia*, *Bacillus megaterium*, *Paenibacillus* and *Bacillus* RNA groups 1 and 3. Simultaneous detection of many thousands of organisms can now be achieved using microarrays with probes

for functional genes and 16S rRNA genes, including many *Bacillus* representatives (Andersen et al. 2010).

2.1.3.2 Community Composition of Specific Groups

The relative abundance of bacilli in total bacterial communities can be determined by identification of *Bacillus* sequences in 16S rRNA gene clone libraries constructed using universal bacterial primers and using DNA fingerprinting methods. If high numbers of *Bacillus* sequences are obtained, within-group diversity can also be studied. Alternatively, primers targeting specific groups can be used, e.g., for *Bacillus* (Garbeva et al. 2003) and *Paenibacillus* (da Silva et al. 2003), although few have been designed.

Functional genes may also be used to determine the potential contribution of bacilli to specific ecosystem functions. Nitrite reductase (*nirK*) genes can be used to characterize bacteria with potential roles in denitrification (Philippot et al. 2007), and comparison of sequences with those in databases will indicate which *nirK* genes are from bacilli. The value of this approach depends on the number of functional gene sequences in databases and the extent to which they can be linked to taxonomic groups. The latter relies on the ability to link 16S rRNA and functional genes, e.g., by sequencing both genes in cultivated organisms, and on the extent of gene transfer, which is significant for *nirK* genes. Table 2.1 provides

Table 2.1 Examples of functional genes used to classify and identify bacilli isolated from soil and/or cultivated and uncultured bacilli, by amplification from extracted soil DNA

Gene	Function or target group	Reference
*16S rRNA	<i>Bacillus</i>	Garbeva et al. (2003)
*16S rRNA	<i>Paenibacillus</i>	da Silva et al. (2003)
* <i>apr</i>	Alkaline metalloprotease	Sakurai et al. (2007)
<i>cheA</i>	Histidine kinase	Reva et al. (2004)
* <i>cbbL</i>	Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)	Selesi et al. (2005)
<i>cry</i>	Bt endotoxin	Collier et al. (2005)
<i>cspA</i>	Cold shock protein A	von Stetten et al. (1999)
<i>gyrA</i>	Gyrase A	Reva et al. (2004)
<i>merA</i>	Mercury reductase	Hart et al. (1998)
* <i>nifH</i>	Dinitrogen reductase	Rosado et al. (1998)
* <i>nirK</i>	Nitrite reductase	Philippot et al. (2007)
* <i>nosZ</i>	Nitrous oxide reductase	Philippot et al. (2007), Kraigher et al. (2008), Stres et al. (2008)
* <i>npr</i>	Neutral metalloprotease	Sakurai et al. (2007)
<i>panC</i>	Pantothenate synthesis	Guinebretière et al. (2008)
<i>rpoB</i>	β-Subunit of RNA polymerase	da Mota et al. (2004)

*Indicate genes used for cultivation-independent studies

examples of functional genes that have been used in cultivation-dependent and -independent studies.

2.1.3.3 Diversity of Active Organisms

DNA-targeted methods demonstrate the presence of particular groups but not, necessarily, their activity in soil ecosystem processes, which is better achieved by targeting RNA. Extracted RNA is reverse-transcribed to DNA and RNA genes are then amplified as described above. Targeting of 16S rRNA gene sequences in this manner is more sensitive, as cells contain more ribosomes than rRNA genes, but is also believed to characterize active communities. This is based on the assumption that active and growing cells will contain more ribosomes than dormant or inactive cells. This approach was used to target the active soil bacterial community in acidic peat grassland soils (Felske et al. 2000).

Activity associated with specific processes performed by bacteria can be determined by transcriptional analysis of genes controlling specific functions. For example, quantification of *nirK* expression will indicate denitrifier transcriptional activity, and sequence analysis of expressed genes may indicate which denitrifiers are active. Putative activity of uncultured organisms can be assessed by amplification of large sections of chromosomal DNA (typically >100 kb), containing many functional genes and, potentially, several genes encoding a metabolic pathway. This is achieved by constructing bacterial artificial chromosome (BAC) or fosmid libraries, screening to target clones containing the target 16S rRNA gene sequence and full sequencing to determine which functional genes are associated with the target group. Activity can also be assessed by techniques such as stable isotope probing, to determine which organisms are utilizing specific ^{13}C - or ^{15}N -labelled compounds, or BrdU capture, which separates organisms incorporating the thymidine analogue bromodeoxyuridine (Prosser et al. 2010).

2.1.3.4 Benefits and Limitations

The major advantage of nucleic-acid-based techniques is their lack of dependence on laboratory cultivation of soil bacilli. All growth media and cultivation conditions are selective, leading to inevitable bias, and only a small fraction of soil bacterial communities grow on enrichment and isolation plates. However, molecular methods also have potential biases. (See Prosser et al. (2010) for a discussion of benefits and limitations of molecular techniques and of developing methods for assessment of bacterial community diversity and activity.) Nevertheless, there is now a suite of techniques available to identify soil bacilli, measure changes in their diversities and community structures and determine their abundance, using quantitative PCR methods.

Although molecular techniques eliminate the major restrictions of cultivation-based techniques for analysis of diversity of soil bacilli, spore production will

introduce bias which may be important for some studies. Molecular techniques are based on extraction of nucleic acids, which requires lysis of vegetative cells. Lysis of spores requires more severe conditions and most studies achieve this through physical disruption by bead-beating. All methods for cell lysis and nucleic acid extraction require a balance between conditions and lengths of treatment that are sufficiently rigorous to optimize lysis of cells and spores and minimization of DNA degradation, that will be increased by length of treatment. This balance is particularly difficult to achieve for bacilli, because of significant differences in the conditions required to lyse vegetative cells and spores. Consequently, many of the problems associated with cultivation-based analysis of soil bacilli diversity apply equally to molecular methods.

2.2 Diversity of Endospore-Formers Within Soil Bacterial Communities

Investigations of the diversity of soil bacilli fall into two classes. In the first, bacilli are studied as members of the total soil bacterial community. In the second, diversity within specific groups of bacilli is determined. This distinction is followed here, combining both cultivation-dependent and -independent approaches. Again, the term “bacilli” will be used to represent aerobic endospore-formers, particularly for older studies; in addition, some molecular studies do not distinguish bacilli from other bacteria within the *Firmicutes*. Although characterization of isolates provides clues to the roles of bacilli, and the studies described below provide evidence for the influence of a range of factors on their diversities, it is rarely easy to link isolates to soil ecosystem function. For example, *Geobacillus* is typically isolated from thermophilic environments, but is commonly found in soils from temperate environments (Marchant et al. 2002). Isolates from these environments can grow at temperatures up to 80°C, but not below 40°C, calling into question their role in these environments (see Banat and Marchant 2011).

2.2.1 Bacilli Within Soil Bacterial Communities

The introduction of molecular techniques led to a reassessment of soil bacterial diversity, mainly through analysis of 16S rRNA gene clone libraries. Libraries invariably contain sequences closely related to bacilli, but the proportion varies between studies – not only because of differences in environmental conditions, but also through the use of different techniques for isolation of nucleic acids, use of different primers and analysis methods, and differences in timing; sequence databases have developed considerably as molecular methods have generated new sequence data over the past 15–20 years. Early studies were limited by the resources

(cost and time) required for sequencing, and involved libraries containing only tens of clones. The frequency of *Bacillus* sequences in these libraries was often low. For example one *Bacillus globisporus*-related sequence was found in a grassland soil library with a total of 275 clones (McCaig et al. 1999), but 8 of 52 clones from an oilseed rape rhizosphere fell within a *Bacillus* group related to *B. megaterium* (Macrae et al. 2000). DGGE analysis also shows the presence of bacilli, for example, a *Bacillus* sp. in bulk soil and *B. megaterium* in barley rhizosphere and rhizoplane (Normander and Prosser 2000). More recently, DGGE analysis with four primers targeting the V6 region indicated that *Firmicutes* constituted between 19% and 32% of sequences in a grassland soil, and the majority (76%–86%) of these were bacilli (Brons and Van Elsas 2008).

Liles et al. (2003) amplified 16S rRNA gene sequences from DNA extracted from soil using a bead-beating method and from BAC libraries. The latter were constructed by extracting high-molecular weight genomic DNA using successive freeze–thaw cycles and cloning into an *E. coli* vector. Traditional clone libraries from soil samples indicated that bacilli comprised 3–15% of total clone sequences, while one of the 28 BAC clones harbouring 16S rRNA genes contained a *Bacillus*-related sequence, closely related to *Bacillus licheniformis*. Thus, the two different DNA extraction processes led to significant differences in recovery of *Bacillus* sequences, presumably because the gentler freeze–thaw method (required for recovery of high-molecular weight DNA) was less efficient at lysing spores and, possibly, vegetative cells of bacilli. Kraigher et al. (2006) also extracted DNA without bead-beating and found that 2.6% of 114 partial 16S rRNA sequences from a high organic grassland fen soil belonged to *Firmicutes* with only two, closely related sequences from the *Bacillus* group, possibly through poor extraction from spores.

High-throughput sequencing methods greatly increase the depth of coverage of soil bacterial diversity. Roesch et al. (2007) used pyrosequencing to obtain 26,140–53,533 16S rRNA gene sequences from each of four soils. This approach removes potential cloning bias and *Firmicutes* comprised 2–5% of sequences from the four soils. Microarray systems also provide the potential to obtain detailed information on soil diversity and have been used to determine differences in bacterial community structure between a soil that was suppressive against a plant-parasitic nematode and an adjacent non-suppressive soil (Valinsky et al. 2002). Bacilli were tenfold more abundant in the non-suppressive soil, but reliable quantification was difficult.

Most diversity studies have targeted DNA, providing information on “total” communities, i.e., assessing active and dormant growth forms, including spores. Felske et al. (1998) characterized the active soil bacterial community in acidic peat grassland soils by targeting RNA, rather than DNA. TGGE analysis and sequencing of clone library representatives indicated dominance of the active community by bacilli. More than 50% of sequenced clones were related to bacilli, and 20% were closely related to a previously uncultivated strain, *Bacillus benzoovorans*, including one clone which gave the strongest band on TGGE gels, implying high relative abundance.

A meta-analysis of 32 soil clone libraries (Janssen 2006) indicated that bacilli encompassed less than 1% of soil bacterial 16S rRNA gene sequences. In contrast, bacilli comprised 5–45% of isolates from traditional cultivation-based studies. Similar patterns were found for other bacterial groups that were traditionally considered to be “dominant” soil organisms. This may reflect difficulties in cultivating truly dominant soil organisms but, for bacilli, this analysis is confounded by lack of information on contributions by spores and vegetative cells.

2.2.2 Diversity of Plant-Associated Bacilli

Root exudates are the major source of organic matter input to soil and lead to high microbial biomass in the rhizoplane and rhizosphere, where their compositions are likely to influence the composition and diversity of the root-associated bacteria, including bacilli. Several studies have therefore compared bulk soil bacterial communities and rhizosphere communities of different plants, to assess selection and the influence of plant root exudates as a driver of bacterial diversity. An understanding of rhizosphere bacterial diversity is also important for the commercial development of plant-growth-promoting bacterial inocula and biocontrol agents.

The reported importance of bacilli in the rhizosphere varies significantly between studies. For example, Chin et al. (1999) characterized nine isolates from anoxic rice paddy soils using different isolation media with xylan, pectin or a mixture of seven mono- and disaccharides as the growth substrates. Isolates were obtained from terminal dilutions of most probable number counts, to obtain the most abundant organisms; *Bacillus* was only obtained on the sugar mixture and had 16S rRNA gene sequences similar to sequences from other rice paddy studies. Garbeva et al. (2001) also found that bacilli were not major components in endophytic bacterial populations of potato plants, and the only isolate was *Paenibacillus pabuli*.

In contrast, Smalla et al. (2001) compared bulk soil and rhizosphere communities of field-grown strawberry (*Fragaria ananassa* Duch.), oilseed rape (*Brassica napus* L.), and potato (*Solanum tuberosum* L.). DGGE profiles from bulk soil and potato rhizosphere were dominated by two *B. megaterium*-related bands, which were also found in strawberry and oilseed rape rhizospheres. Pankhurst et al. (2002) found higher *Bacillus* populations in roots growing within macropores in subsoil than in bulk soil and Duineveld et al. (2001) found several *Bacillus*-related DGGE bands in the *Chrysanthemum* rhizosphere, with little difference with growth stage or between rhizosphere and bulk soil. The number of bands amplified when targeting RNA was less than that from DNA, suggesting that not all strains present were active and that active organisms were phylogenetically diverse. Garbeva et al. (2008) found differences in bacilli under maize (*Bacillus* sp. and *B. thuringiensis*), a commercial grass mix (*B. benzoeverans* and *B. pumilus*) and oats and barley (*Bacillus* sp. and *B. fumarioli*). *Bacillus*-specific primers generated most phylotypes from grass and maize rhizosphere, while diversity and abundance were greatest in

permanent grassland and arable land originating from grassland. Reva et al. (2004) classified 17 *Bacillus* root isolates on the basis of 16S rRNA, gyraseA (*gyrA*) and the *cheA* histidine kinase sequences. The isolates were closely related to two *Bacillus subtilis* strains and *B. mojavensis*. They were also related to, but distinct from, *Bacillus amyloliquefaciens* – which showed greatest rhizosphere colonization with oilseed rape (*Brassica napus*), barley (*Hordeum vulgare*) and thale cress (*Arabidopsis thaliana*).

Risk assessment of genetically modified crops requires assessment of impact on rhizosphere communities and a number of studies have found differences between *Bacillus* communities colonizing rhizospheres of wild-type and genetically modified plants. For example, Tesfaye et al. (2003) found fewer *Bacillus* clones in alfalfa when it was over-expressing a nodule-enhanced malate dehydrogenase, and Siciliano and Germida (1999) found differences in root-associated *Bacillus* communities of three Canola (rapeseed cultivar) varieties, one of which was genetically engineered to tolerate the herbicide glyphosate. The significance of these and other reported differences is unclear, however; both in terms of the mechanisms driving community composition, and the impact on the colonized plant.

2.2.3 Effects of Fertilizer Application and Removal on Diversity of Bacilli

Changes in soil nutrients through fertilization, or changes in plant communities associated with different management strategies, are likely to influence soil bacterial communities. Soil fumigation appears to select for endospore-formers, with high proportions (81%) of bacilli among isolates (Mocali et al. 2008). In many studies, however, changes in aerobic endospore-former communities are not great. Smit et al. (2001) investigated seasonal changes in isolates from a wheat field soil and only found *Bacillus* isolates in July, but *Bacillus* sequences were not detected in clone libraries. Chu et al. (2007) found some evidence for selection of a *Bacillus*-related strain following treatment with organic manure, but not inorganic fertilizer, and Sturz et al. (2004) found increased diversity in rhizosphere isolates following sulphate fertilization to control potato common scab, with increased antibiosis of isolates against *Streptomyces scabies*.

Studies of soil bacterial diversity focus on changes in community structure; i.e., changes in the relative abundances of different groups, but not in actual abundance. Therefore there is often a need for quantification, using qPCR or alternative techniques, to understand the effects of environmental change on bacterial communities. Most studies also give no information on bacterial activity, with the exception of a small number of studies targeting rRNA. Felske et al. (2000) addressed these issues when investigating the effects of grassland succession on bacterial communities. Bacterial RNA, quantified by RNA probing (dot-blot hybridization), doubled for several years after cessation of fertilization of a grassland

soil, probably owing to increased levels of root residues following replacement of *Lolium perenne* by other grasses. rRNA levels then decreased, but no changes were detected in major phylogenetic groups. Multiplex PCR, however, coupled with TGGE showed changes in the 20 dominant bacterial ribotypes, including several representing bacilli. Thus, four *Bacillus* types increased during the final stage of succession, one increased during the early stage, three showed no clear pattern with succession but fell to low relative abundances during the later stages, and two decreased with grassland succession. These results demonstrate an ability to follow dynamics of soil *Bacillus* communities in soil. In this study, changes were accompanied by changes in plant communities, earthworm activity and soil processes, but establishment of links between these changes, and of links to physiological characteristics of different ribotypes, was not possible and remain difficult to discern in most studies.

2.2.4 Links Between Diversity and Ecosystem Function

Several studies have investigated bacterial diversity by the use of growth conditions selective for particular metabolic processes, or by amplifying functional genes associated with specific processes. The ability to identify functional genes derived from bacilli depends on the size and reliability of sequence databases, which are much smaller than those for 16S rRNA genes. Also, phylogenies of 16S rRNA and functional genes may not be congruent, making identification difficult, and important functional genes may also be plasmid-encoded and subject to gene transfer.

Bacilli have roles in two soil nitrogen cycle processes: denitrification and nitrogen fixation. Denitrifying bacilli have been targeted either by enrichment on selective media or by targeting functional genes, rather than by using 16S rRNA genes. For example, 138 denitrifying isolates from three soils, classified using ARDRA and 16S rRNA gene sequence analysis, fell within five groups, one of which comprised bacilli (Chèneby et al. 2000). *Bacillus* and *Paenibacillus* strains have been found among diverse communities of nitrogen fixers associated with *Drosera villosa*, a Brazilian carnivorous plant (Albino et al. 2006), wheat rhizosphere (Beneduzi et al. 2008a) and rice rhizosphere and bulk soil (Beneduzi et al. 2008b), where there was evidence of a relationship between different RFLP groups and soil pH. Most isolates were members of *Bacillus* or *Paenibacillus*. Wheat rhizosphere and bulk soil strains were dominated by *Paenibacillus*, particularly *P. borealis* and *P. graminis*, the remainder being identified as *Bacillus* sp.

Communities involved in degradation of proteins were characterized by Sakurai et al. (2007) in fertilized soils planted with lettuce. Proteolytic activity was greater following addition of organic, rather than inorganic, fertilizer and was greater in rhizosphere than bulk soil. Sequences of alkaline (*apr*) and neutral metalloprotease (*npr*) genes associated with DGGE bands were closely related to those of *Pseudomonas fluorescens* and *B. megaterium*, respectively, with homology of one band to *Bacillus vietnamensis*. Fertilizer type affected *apr* community composition in the

rhizosphere, but not bulk soil, and affected *npr* composition in both. Rhizosphere and bulk soil communities were different for both genes. Multiple regression analysis of protease activity and DGGE profiles of both genes showed significant relationships: the *apr* community was affected by fertilizer treatment and the rhizosphere, while the *npr* community was affected mainly by fertilizer. The results were interpreted as indicating different roles for pseudomonads and bacilli in the rhizosphere and bulk soil, and showing that community structure has an important influence on soil protease activity.

Chitinolytic activity contributes to the role of bacilli in mineralization of soil organic matter and Hallmann et al. (1999) found *Bacillus* and *Arthrobacter* to be the dominant genera in the soil and rhizosphere of cotton; but they were not detected as endophytes. Addition of chitin to soil, to reduce fungal pathogens, decreased the frequency of *Bacillus* among isolates suggesting a role for other organisms in chitin degradation in this habitat. Degradation of cellulose, hemicellulose and aromatic compounds by bacilli is also believed to be important in the guts of soil invertebrates, which contain a diverse range of bacilli (see König 2011).

2.2.5 The Influence of Environmental Factors on Diversity of Bacilli

One obvious factor favouring selection of endospore-formers is their ability to survive temperatures that kill vegetative cells. This is of little advantage under normal soil conditions, but can be important following forest fires. For example, *Bacillus aminovorans*-related sequences were detected in a spruce-dominated boreal forest ecosystem one year after a large wildfire (Smith et al. 2008). Incineration of municipal solid waste material, before disposal to landfill soil, also leads to selection (Mizuno et al. 2008). *B. cereus* and *B. megaterium* were the most common isolates from forest and cultivated soils, while buried ash contained *B. licheniformis*, *Bacillus firmus*, *Bacillus thioparas* and *Bacillus krulwichiae*, the first two species also being found in the overlying forest soil. Incinerated ash has high concentrations of Na, Ca, K and Cl, and a high pH, and phenotypic analysis indicated that selection was due to ability to grow under anaerobic conditions and at high pH, in addition to selection of *Bacillus* during incineration.

Flooding reduces oxygen availability in soil and might be expected to select for groups carrying out processes such as denitrification, ferric iron reduction, sulphate reduction or methanogenesis. Graff and Conrad (2005) determined the effects of flooding on bacterial communities both in bulk soil and soil associated with roots of poplar trees, grown in microcosms. *Bacillus*-related sequences represented 16% of the bacterial community in unflooded bulk soil and the rhizosphere, but this proportion was reduced following flooding, although a *Paenibacillus*-related sequence increased in relative abundance. Rhizosphere soil contained 42% *Bacillus* sequences, but sequence types were different to those in bulk soil. Although some

of these findings are consistent with other studies, no mechanistic basis for the changes was proposed. Kim et al. (2005) also found significant effects of flooding in rice fields, with reduction in biodiversity of bacilli from >10% to undetectable, and domination by *Arthrobacter*. Clone sequences were less diverse and contained a lower proportion of bacilli than isolates.

Lear et al. (2004) investigated the effect of applying an electrical field to soil, to increase biodegradation of pollutants. Although this had no detectable effect on the majority of the members of the bacterial community, some effects were seen in the immediate vicinity of the anode. In control soil, *Bacillus mycoides* and *Bacillus sphaericus* dominated isolates. *B. sphaericus* was not detected in the electrokinetic cells and *B. megaterium*, which was not found in control soil isolates, was the most abundant *Bacillus* isolate. This was explained in terms of protection afforded by production of a cell capsule and greater stress at the anode arising from changes in soil characteristics, particularly increased acidity.

2.2.6 Effects of Soil Contaminants on Diversity of Bacilli

Mercury resistance in soil bacilli is widespread and is encoded through a chromosomally borne *mer* operon. Hart et al. (1998) found that 5% of bacilli in a mercury-contaminated soil were resistant to mercury. RFLP analysis of mercuric reductase (*merA*) genes gave 14 RFLP groups from soil isolates and 11 from directly extracted soil DNA, with three common to both, reflecting difficulties in cultivation of dominant organisms. Phenotypic analysis indicated significant physiological diversity among isolates, suggesting high levels of genetic exchange. There is also evidence for selection of a *Bacillus* strain in auriferous soils (Reith and Rogers 2008), supporting earlier observation of a 1,000-fold increase in *B. cereus* spores (Reith et al. 2005). In contrast, there was no evidence of selection of members of the *Firmicutes* following spiking of soil with mercury (Nazaret et al. 2003), in mine tailings, or in cultures grown on media containing cadmium (Zhang et al. 2007). A lead-enriched community contained both *Paenibacillus* and *Bacillus* sequences, but there are no reports of metal resistance in *Paenibacillus* (Zhang et al. 2007).

Bodour et al. (2003) screened 1,305 isolates from soil contaminated with metals and/or hydrocarbons and found greater numbers of Gram-positive, biosurfactant-producing isolates in metal-contaminated or uncontaminated soils and more Gram-negative isolates in hydrocarbon-contaminated or co-contaminated soils. Of the 45 isolates, eight were *B. subtilis* and three *B. licheniformis*, both of which produce a range of biosurfactants. A decrease in the proportion of bacilli among isolates was seen following contamination of soil with toluene (Chao and Hsu 2004) or long-term contamination with the organophosphate pesticide methylparathion (Zhang et al. 2006).

2.2.7 *Intraspecific Diversity Within Soil Bacilli*

In general, studies of diversity of bacilli within “total” prokaryote soil communities are descriptive, giving information on which groups are present and, in some studies, estimates of the relative abundance of bacilli. The physiological characteristics of bacilli, notably spore formation but also degradative capacities, provide the potential for an understanding of mechanisms determining diversity and ecosystem function. This potential is, however, severely limited by inability to distinguish the relative contributions of spores and vegetative cells and ignorance of which bacilli are active, and the nature and extent of their activity. In contrast, studies of within-species diversity of soil bacilli have given clues to mechanisms that control diversity within this group, and provide model systems with which to investigate the mechanisms controlling general prokaryotic diversity and community structure. This section therefore provides descriptions of diversity within species of soil endospore-formers. For several species, these descriptions link closely with the mechanistic studies that are described in the following section.

2.2.8 *Bacillus benzoovorans* and *Bacillus niacini*

Molecular techniques uncover and demonstrate the potential importance of groups with no cultivated representatives, and can thereby direct work towards the isolation of these groups. Two examples of this are detection of *B. benzoovorans* in a Dutch soil by FISH and qPCR and the presence in soil clone libraries of sequences associated with *B. niacini*, constituting 15% of the uncultured *Bacillus* community in soil. Screening of 4,224 soil isolates by multiplex PCR generated several hundred novel *B. benzoovorans* relatives (Felske et al. 2003). A similar search, using several enrichment media, led to isolation of 64 isolates of the *B. niacini* group from different Dutch soils (Felske et al. 2004). The isolates grew best on acetate and were very diverse, but their metabolic diversities gave no clues to their likely ecosystem functions. Metabolic potentials of isolates on laboratory media give good information on their genetic potentials, but it is difficult to predict the ecosystem function of soil isolates because metabolic genes may be responsive to environmental cues that may significantly change the gene expression patterns from those observed in the laboratory.

2.2.9 *Paenibacillus polymyxa*

Mavingui et al. (1992) isolated 130 *Bacillus* (now *Paenibacillus*) *polymyxa* strains from rhizosphere, non-rhizosphere and rhizoplane soils by immunotrapping and characterized them phenotypically, serologically, by RFLP analysis of total DNA,

and by hybridization with a rRNA probe. Phenotypic analysis placed isolates in four groups. Two contained isolates from non-rhizosphere soil, one contained isolates from the rhizosphere soil and the fourth contained only rhizoplane isolates. Serological and molecular methods indicated greater diversity among isolates, with lowest diversity in rhizoplane isolates. Von der Weid et al. (2000) isolated 67 *B. polymyxa* strains from maize rhizosphere and analysed them phenotypically and by hybridization with a *nifKDH* probe and by BOX-PCR. The different methods gave different numbers of major clusters, with BOX-PCR showing greatest discrimination, and communities changed during plant development. Finer scale molecular methods (RADP-PCR and BOX-PCR) indicated high diversity of *P. polymyxa* strains colonizing rhizospheres of four maize cultivars, with evidence of selection by different plants for different strains, and implications for choice of inocula to improve plant growth (da Mota et al. 2002).

2.2.10 *Bacillus cereus*–*Bacillus thuringiensis* Group

This group contains a wide diversity of strains, including six closely related species: *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *B. cereus* (*sensu stricto*). They inhabit diverse soil habitats and have many economically important representatives, notably *B. thuringiensis*, but also many plant-growth-promoting bacteria. Diversity is greatest in *B. cereus* and *B. thuringiensis*, but low in *B. anthracis*. For example, Helgason et al. (1998) found high diversity in soil isolates collected from five geographic regions in Norway ranging from coastal to the Arctic, while the diversity of *B. anthracis*, but not *B. cereus* or *B. thuringiensis*, isolates collected worldwide was low (Keim et al. 2000). *B. cereus* or *B. thuringiensis* strains appeared to co-exist in a French forest soil (Vilas-Boas et al. 2002), but were genetically distinct and diverged to a greater extent than strains of the same species isolated from geographically different locations. MLST (rather than MLEE) analysis of these strains and 19 strains from elsewhere separated them into three clusters, one exhibiting frequent exchange between strains, while the other two were clonal (Sorokin et al. 2006). These differences may reflect differences in life style between strains. *B. weihenstephaniensis* isolates are psychrotolerant and the role of temperature in determining their distribution is discussed in Sect. 2.3.5.

2.2.11 *Bacillus simplex* and *Bacillus subtilis*

Both *B. simplex* and *B. subtilis* are common members of the soil endospore-former community and are important model organisms for mechanistic studies. Their diversity is described in greater detail in Sect. 2.4.

2.2.12 *Paenibacillus*

Both cultivation-based and molecular methods have been used to investigate associations between *Paenibacillus* strains and plant roots. *Paenibacillus durus* (previously *P. azotofixans*) is a common member of the rhizospheres of maize, sorghum, sugarcane, wheat and forage grasses, where it fixes nitrogen and produces antimicrobial compounds. Rosado et al. (1998) isolated 53 *P. azotofixans* strains from the rhizoplane and rhizosphere of different grasses and from soil, and characterized them using a *nifKDH* probe, RAPD, BOX-PCR and API. There was little evidence of specific plant associations, although some clusters were isolated more frequently from wheat and sugarcane. Subsequent work (Rosado et al. 1998; de Albuquerque et al. 2006) placed *P. durus* isolates in two clusters, the first associated with wheat, maize and sugarcane rhizospheres, and the second with all plant species investigated and bulk soil. The different clusters showed different patterns of carbohydrate metabolism: wheat isolates could metabolize sorbitol, and sugarcane isolates could metabolize starch and glycogen, suggesting a selective role for root exudates.

Paenibacillus communities in the rhizospheres of four maize cultivars have been investigated in two soils. Cultivation-based methods (Rosado et al. 1998; da Mota et al. 2002) indicated that *Paenibacillus* diversity was determined by soil type, rather than by cultivar. This was confirmed by molecular methods based on 16S rRNA genes (da Silva et al. 2003) and *rpoB* genes (da Mota et al. 2005). A clone library constructed using *Paenibacillus*-specific 16S rRNA gene primers indicated high diversity and clustering into 12 groups, with *P. azotofixans* the most abundant (19% of clones). DGGE analysis showed clear differences in rhizosphere communities from the different soils.

Vollú et al. (2003) classified cyclodextrin-producing *P. graminis* root-associated isolates from wheat, maize and sorghum sown in Australia, Brazil and France using *rpoB*-RFLP and *gyrB*-RFLP and *rpoB* gene sequencing. Brazilian isolates clustered separately from Australian and French isolates and strains fell within four clusters. For the Brazilian isolates, soil type was more important than plant host in determining the rhizosphere community.

2.3 Mechanisms Driving Diversity

2.3.1 General Mechanisms Driving Microbial Diversity in Soil

A combination of speciation, extinction, dispersal and microbial interactions is responsible for the creation and maintenance of diversity (Horner-Devine et al. 2004a; Ramette and Tiedje 2007). Speciation is driven by natural selection, acting on a pool of genotypes in a specific environment. The rate of speciation in bacteria is high owing to large population size and high reproduction rates. Diversity in

bacteria may also be high because of low extinction rates, and the ability to form highly resistant life forms such as the spores that are typical of bacilli. Dispersal rates of spore-formers are also high (Roberts and Cohan 1995), reducing extinction rates further by giving spore-formers opportunities to colonize new habitats and escape harsh conditions.

Prokaryotic species have been discriminated by divergence in 16S rRNA gene sequences of 2.5–3%. Isolates that diverged more at the 16S rRNA gene level also show less than 70% DNA–DNA re-association values and are thus placed in different species (Stackebrandt and Goebel 1994) but ecologically distinct groups, termed ecotypes, can be discerned within such species from protein coding sequences (Cohan and Perry 2007) and phenotypic characteristics. An ecotype is defined as an ecologically distinct group of organisms that fall into distinct sequence clusters (lineages), sharing a common evolutionary path, with diversity limited by periodic selection and genetic drift. Ecotype formation is purged by frequent recombination (Cohan and Perry 2007), which may occur between closely related bacilli. Genetic exchange among closely related bacilli occurs at high frequency in laboratory soil microcosms (Graham and Istock 1978; Duncan et al. 1995). In addition, linkage disequilibrium and genetic recombination among wild isolates of *B. subtilis* obtained from a microsite (200 cm³) of surface desert soil was higher than in *E. coli* populations, based on MLEE, phage and antibiotic resistance and RFLP analysis (Istock et al. 1992). The potential for genetic exchange is further increased by transformation, which has been observed within natural populations of *B. subtilis* and closely related species isolated from various desert soils, whose transformation rates range over three orders of magnitude (Cohan et al. 1991). To determine actual, rather than potential, rates of recombination Roberts and Cohan (1995) analysed restriction patterns of three housekeeping genes among natural closely related *Bacillus* isolates. Recombination within *B. subtilis* or *B. mojavensis* was too low to prevent adaptive divergence between ecotypes (Cohan 2002; Maynard Smith and Szathmari 1993). Diversification between ecologically distinct populations increases, due to sequence divergence and differences in restriction modification systems between donor and recipient (Dubnau et al. 1965; Roberts et al. 1994).

2.3.2 Environmental Factors Driving Diversification in Bacilli

Bacteria are highly adaptable and can exploit a wide range of environmental opportunities for diversification (Horner-Devine et al. 2004b; Ramette and Tiedje 2007), but little is known of the influence of environmental factors on diversity and community composition of soil bacilli. However, an ecotype simulation algorithm (Koepfel et al. 2008) has been used to model evolutionary dynamics of bacterial populations and was tested using *Bacillus* ecotypes belonging to two clades isolated from two “Evolutionary Canyons” in Israel. These sites show interesting topographies, with three major habitats: north facing (European) slopes, south facing, more

stressful (African) slopes and a canyon bottom with greater access to water (Nevo 1995). Nine and 13 ecotypes were identified among isolates belonging to the *B. simplex* and *B. subtilis*–*B. licheniformis* clades, respectively (Fig. 2.1). *B. simplex* strains within each ecotype were exclusively or sometimes predominantly isolated from one of the habitats (Sikorski and Nevo 2007). Similar, but less strong, associations between habitat and ecotype were also detected within the *B. subtilis*–*B. licheniformis* clade (Roberts and Cohan 1995; Koepel et al. 2008). This suggests that specialization for environmental conditions associated with one of the three habitats can be discerned at the level of sequence clustering and provides strong evidence that ecotype clustering and ecological distinctness may correlate. However, ecotypes did not correlate well with physiological characteristics, determined using Biolog, suggesting that energy metabolism is not determined by soil characteristics or by solar radiation, temperature and drought, which are considered to be the major abiotic features of this environment (Sikorski et al. 2008).

The study by Koepel et al. (2008) demonstrates a significant impact of environment on *Bacillus* diversity, but more ecological and physiological data are

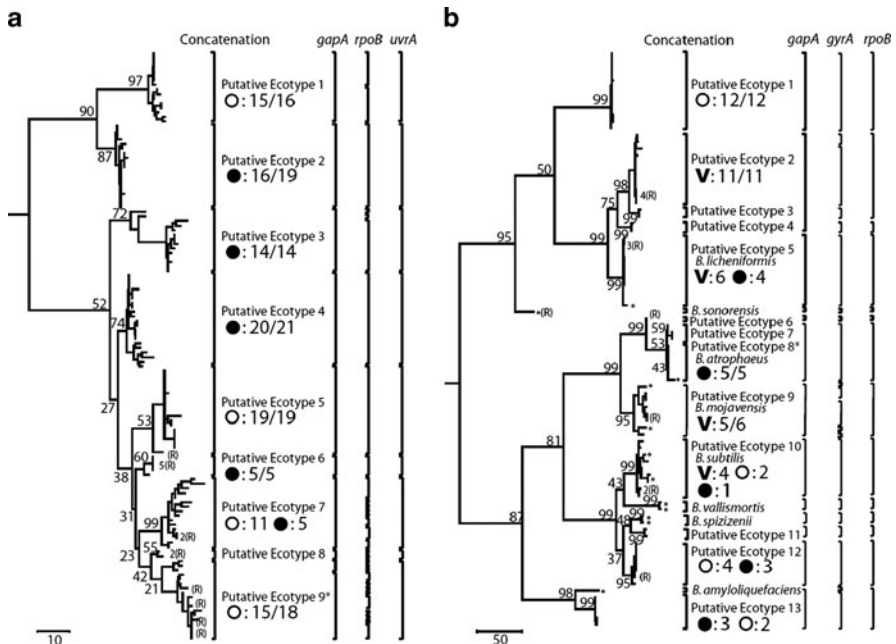


Fig. 2.1 Phylogeny and ecotype segregation of the *B. simplex* and *B. subtilis*–*B. licheniformis* clades. Analysis was (a) *gapA*, *rpoB* and *uvrA* genes for the *B. simplex* clade, resulting in nine putative ecotypes, and (b) *gapA*, *gyrA* and *rpoB* genes for the *B. subtilis*–*B. licheniformis* clade, giving 13 putative ecotypes. Bacilli were isolated from the south facing slope (open circle), the north facing slope (filled circle) and the canyon bottom (V) at Evolutionary Canyon (Israel). Habitat source is indicated for ecotypes represented by at least four isolates and only one habitat is depicted if >80% of isolates originated from one habitat. All habitat sources are indicated for clusters not dominated by isolates from one habitat (From Koepel et al. (2008), with permission)

required to understand the specific factors leading to ecological distinctness of each putative ecotype (Koeppel et al. 2008). More information is also required to determine whether variation between ecotypes is correlated with ecosystem function and whether ecotypes identified by sequence analysis correspond to ecologically distinct groups.

Temperature has been shown to determine the relative distributions of the psychrotolerant *B. weihenstephaniensis*, which can grow below 7°C and up to 38°C, and the mesophilic *B. cereus sensu stricto*, which grows in the range 7°C–46°C. The psychrotolerant and mesophilic phenotypes are reflected in genotypic differences in the cold shock protein A (*cspA*) gene. Von Stetten et al. (1999) studied the distribution of 1,060 mesophilic and psychrotolerant isolates obtained from a tropical soil, a temperate soil and two alpine habitats, with average annual temperatures of 28°C, 7°C, 4°C and 1°C, respectively. Isolates were characterized phenotypically, in terms of their growth–temperature responses and psychrotolerance, and genotypically (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. Psychrotolerant strains were able to grow at temperatures below 7°C and up to 38°C, while mesophilic strains could grow at temperatures above 7°C and possessed psychrotolerant or mesophilic *cspA* genotypes.

Only *B. cereus* isolates were obtained from the tropical habitat and isolates from the alpine habitats were heavily dominated by *B. weihenstephaniensis*. These isolates also contained the corresponding psychrotolerant or mesophilic *cspA* genotype. Both groups were found in the temperate habitat together with isolates named “intermediate thermal types”. The latter carried the psychrotolerant *cspA* gene, but showed mesophilic phenotype or carried the mesophilic *cspA* gene and had the psychrophilic phenotype, and sometimes even had mesophilic and psychrotolerant 16S rRNA operon copies within a single isolate. These intermediate thermal types may represent ongoing adaptation to prevalent temperatures. In addition, diversity was greater in temperate soils, potentially reflecting greater variation in temperature around annual means, leading to co-existence of psychrotolerant and mesophilic organisms.

2.3.3 Diversification Within Species of Aerobic Endospore-Formers

B. subtilis is one of the best-studied bacteria at the molecular level, but relatively little is known of its ecology and diversity. Strains within the *B. subtilis* clade form two subclusters, 168, delineating *B. subtilis subsp. subtilis*, and W23, representing *B. subtilis subsp. spizizenii* (Roberts and Cohan 1995; Nakamura et al. 1999). The level of genetic diversity within W23 is considerably higher than within 168 or the closely related *B. mojavensis* cluster. The ecological

significance of this diversity is not understood, but microarray-based comparative genomic hybridization (M-CGH) (Earl et al. 2008) confirmed closer relations within than between subspecies, with 30% divergence of genes within species. Diversity was highest for genes involved in the synthesis of secondary metabolites, teichoic acid and the adaptive response to alkylation DNA damage, but there was variation in all functional groups for genes of potential ecological importance in adaptation to different environments, including environmental sensing and carbohydrate or amino acid metabolism. In contrast, genes belonging to the core genome, previously identified as essential under laboratory conditions in *B. subtilis* 168, were highly conserved. Divergence was greater in germination than sporulation genes, suggesting that environmental cues for outgrowth might vary between strains. In addition, genes involved in the ability of *B. subtilis* to become naturally competent and take up DNA from the environment were highly conserved, except for the first three genes in the *comQXPA* operon that have been previously indicated as highly polymorphic (Tran et al. 2000; Tortosa et al. 2001) (see below).

2.3.4 Cell–Cell Signalling Driving Diversification

Soil bacilli provide an interesting system with which to investigate the influence of microbial interactions on diversification. Competence in *B. subtilis* is controlled through a population-density dependent, quorum-sensing system, encoded by the *comQXPA* operon for enzymes involved in the synthesis, processing and recognition of the extracellular pheromone ComX. The genomic diversity of *comQXP* genes results in functional diversity, so that strains producing similar pheromones are able to induce competence in each other, while divergent strains do not (Ansaldi et al. 2002; Mandic-Mulec et al. 2003; Stefanic and Mandic-Mulec 2009). This therefore provides an example of functional diversification with a potential ecological role. Polymorphism of competence-signalling may act as a sexual isolation mechanism (Tortosa et al. 2001; Ansaldi et al. 2002), lowering the frequency of recombination even among members of the same species, and so increasing diversification among strains of different phenotypes. It would be interesting to examine the diversity of members of different phenotypes at other loci that may be adaptive in certain environments.

Stefanic and Mandic-Mulec (2009) examined polymorphism of highly related *B. subtilis* isolates from soil aggregates that were exposed to the same environmental conditions. All four phenotypes previously found among strains isolated from distant geographical locations were present in microscale samples taken from within aggregates, but only three were specific for *B. subtilis* (Fig. 2.2). The fourth, previously associated with *B. subtilis* and the closely related *B. mojavensis*, was detected in *B. amyloliquefaciens* isolates, suggesting a role for horizontal gene transfer in interspecies distribution of the phenotypes.

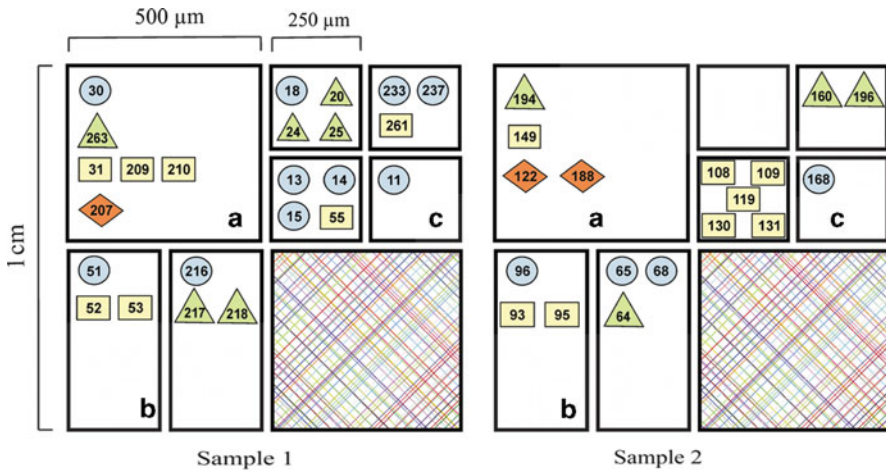


Fig. 2.2 The distribution of *B. subtilis* and *B. amyloliquefaciens* phenotypes in two 1-cm³ samples of river bank soil that were sectioned to progressively smaller subsamples, comprising 1/4, 1/8 and 1/16 of the initial soil sample. From each sub-sample 30 spore-formers were isolated but, among those, only two, three and sometimes more were identified as *B. subtilis* and were analysed further. Each phenotype is depicted by colour and shape: the 168, RS-D-2/NAF4, RO-B-2/RO-H-1 and RO-E-1 phenotype with blue circles, green triangles, yellow rectangles and orange diamonds, respectively (From Stefanic and Mandic-Mulec (2009), with permission)

2.3.5 Diversification and Biogeography of Bacilli

Similarities in macroscale and microscale diversity of *B. subtilis* phenotypes are consistent with the cosmopolitan nature of bacilli. Global distribution of spore-forming bacteria such as *B. mojavensis* and *B. subtilis* is indicated by analysis of protein coding genes, which show similar linkage disequilibrium ($D = 0.50 - 0.87$) in local populations and global ($D = 0.71$) populations. A similar linkage disequilibrium, which is the non-random association of genetic loci, suggests high migration rates and lack of geographical isolation (Roberts and Cohan 1995). Migration rate increased with geographical scale, but even populations separated by the greatest distances were not sufficiently isolated to demonstrate genetic drift. This suggests that, for the genes investigated, evolutionary processes that require continued geographical isolation are unlikely to occur, although it would be interesting to see whether diversification due to geographical isolation would be detectable in faster-evolving genes. Diversification of the fast-evolving *comQXP* loci has been observed even within soil aggregates, where spatial isolation might not be expected, and the data indicate that diversity decreases with aggregate size (Fig. 2.3) (Stefanic and Mandic-Mulec 2009). The spatial heterogeneity of the soil environment may therefore lead to geographic isolation and niche differentiation even at the 250-µm scale. Evolutionary principles and competition between bacteria for natural resources acting at this scale are poorly understood and warrant further studies (Grundmann 2004).

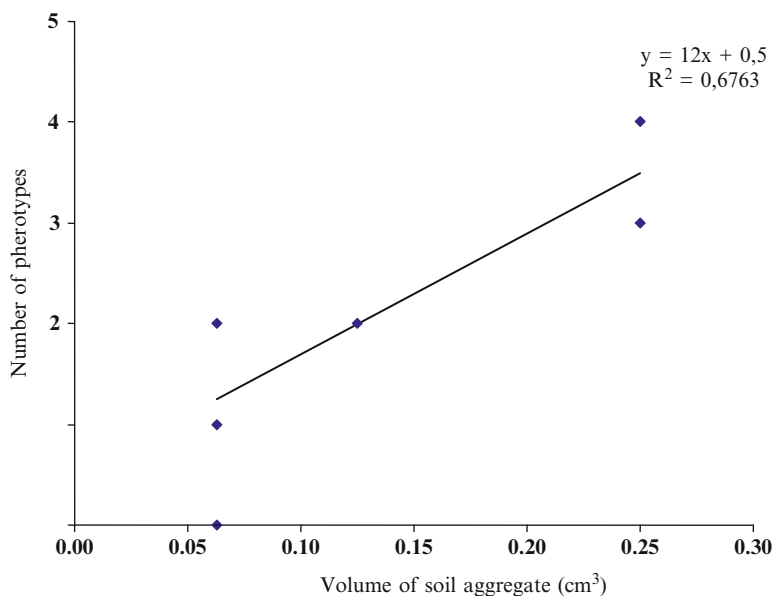


Fig. 2.3 The correlation between number of pherotypes and sample size in the study of Stefanic and Mandic-Mulec (2009) (see legend to Fig. 2.2). The number of pherotypes decreased with soil sample size

2.4 Conclusions

Aerobic endospore-forming bacteria are taxonomically and physiologically diverse, and are ubiquitous members of soil microbial communities. Understanding diversity patterns in soil is of particular interest, given their cosmopolitan nature and high biotechnological potentials that range from the biological degradation of pollutants, through the production of industrially interesting enzymes and bioactive chemicals, to their roles as biopesticides. The nature of these patterns and the importance of this group for soil ecosystem function are, however, difficult to assess. Few studies attempt to determine quantitative activity of bacilli in soil, or the specific processes which they carry out, in relation to the activities of the total microbial community. To some extent this reflects methodological limitations. Cultivation-based approaches are highly selective, and will underestimate abundance, and they give information on potential, rather than actual activity. Molecular techniques avoid cultivation bias, but very few studies have utilized primers that target bacilli or specific genera, species or ecotypes. None of these has been used to investigate in situ activity. Design and application of group-specific primers is required therefore, combined with molecular techniques that assess activity rather than just presence. The next generation of molecular techniques, based on high-throughput sequencing, genomics, metatranscriptomics and proteomics, should also be exploited. More importantly, there is a need for studies that aim to assess

the links between diversity, community structure, physiological diversity and ecosystem function, rather than merely characterizing the presence, absence and identity of strains present.

A major conceptual and technical issue is the distinction between vegetative cells and spores. For cultivation-based methods, the presence of spores is likely to lead to overestimation of the importance of bacilli in soil processes. This could be addressed by comparisons with and without heat-treatment of samples, but is rarely attempted. Consequently, although the ability to eliminate non-spore-formers makes it easy to study spore-formers, there is little attempt to address the relative sensitivities of spores of different organisms, the relative importances of spores and vegetative cells for cultivation, and the relative culturabilities both of spores (from spore-formers) and vegetative cells of non-spore-formers.

Molecular techniques cannot currently distinguish spores and vegetative cells, and nucleic acid extraction techniques represent a compromise between lysis efficiency and nucleic acid degradation. Therefore, differences between cultivation-based and molecular studies may be exaggerated for spore-formers, in comparison with other microbial groups. These problems and biases can actually be used to advantage, depending on the ecological question being addressed, but in general these issues are not considered. While these issues limit our knowledge of the ecology of soil bacilli, the study of specific genera and species has generated important and generic advances in our understanding of the mechanisms driving microbial diversity. These include studies of biogeography and the impact of environmental factors, signalling, spatial scale and horizontal gene transfer on evolution of bacterial species, ecotype formation and speciation. These studies have been based mainly on the analysis of laboratory isolates and it will be interesting to see how the results obtained correlate with diversity patterns detected directly in soil and how communities respond spatially and temporally to changing environmental parameters.

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