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Social Interactions and Distribution of *Bacillus subtilis* Pherotypes at Microscale[▽]

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Bacillus subtilis **strains communicate through the *comQXPA* quorum sensing (QS) system, which regulates genes expressed during early stationary phase. A high polymorphism of *comQXP′* loci was found in closely related strains isolated from desert soil samples separated by distances ranging from meters to kilometers. The observed polymorphism comprised four communication groups (pherotypes), such that strains belonging to the same pherotype exchanged information efficiently but strains from different pherotypes failed to communicate. To determine whether the same level of polymorphism in the *comQXP′* QS system could be detected at microscale, *B. subtilis* isolates were obtained from two separate 1-cm^3 soil samples, which were progressively divided into smaller sections. Cross-activation studies using pherotype-responsive reporter strains indicated the same number of communication pherotypes at microscale as previously determined at macroscale. Sequencing of the housekeeping gene *gyrA* and the QS *comQ* gene confirmed different evolutionary rates of these genes. Furthermore, an asymmetric communication response was detected inside the two pherotype clusters, suggesting continuous evolution of the QS system and possible development of new languages. To our knowledge, this is the first microscale study demonstrating the presence of different QS languages among isolates of one species, and the implications of this microscale diversity for microbial interactions are discussed.**

Quorum sensing (QS), a widespread phenomenon in the bacterial world, controls a wide range of cell density-dependent behaviors. *Bacillus subtilis* uses QS to control production of antimicrobial peptides, bacteriocins, and antibiotics (20) but also to alternate between two cell types during stationary phase: competent cells, able to take in DNA from the environment, and dormant spores, able to survive harsh environmental conditions (9, 12, 24). Development of genetic competence in *B. subtilis* is controlled by a QS system encoded by the *comQXPA* operon (2, 53, 54). This involves the ComX pheromone that accumulates during exponential growth (25, 46, 47) and is initially synthesized as a 55-residue protein that is processed, modified, and released into the extracellular medium as a 5- to 10-amino-acid peptide. The isoprenoidal modification on the tryptophan residue of this peptide is catalyzed by the ComQ protein (2, 25, 34, 35, 42, 52). Upon reaching the threshold concentration, processed and modified ComX binds to the membrane-associated, histidine protein kinase ComP and triggers the QS response, linking autophosphorylation of ComP and transfer of phosphate to the response regulator ComA (59). The level of phosphorylated ComA is also controlled by dephosphorylation, which is dependent on a separate QS system involving competence sporulation factor (CSF) and the RapC phosphatase (3, 59). Phosphorylated ComA directly controls expression of various genes (6, 33), including the *srfAB* operon that contains the *comS* gene (15, 41), required for development of competence (55).

Previous studies of environmental *B. subtilis* strains indicate a high polymorphism (approximately 56% identity at the nucleotide level) in the QS locus, which is restricted to *comQ*, *comX*, and the N-terminal region of the *comP* gene. Sequences surrounding this locus, downstream gene *comA*, a C-terminal region of *comP*, and the upstream *degQ* gene, are highly conserved (2, 53, 54). Sequence analysis of the *comQXP* loci of 13 strains indicated clustering into four distinct similarity groups (2). These groups were congruent for *comQ*, *comX*, and the N-terminal region of *comP*, indicating coevolution of the three genes. In addition, the similarity groups correlated with four pherotypes, able to communicate efficiently within but not between groups. Similar variation has been reported for the *agr* QS system in staphylococci (19, 56) and in the competence QS system of *Streptococcus pneumoniae* (17, 19, 37, 38, 60).

B. subtilis is often referred to as a soil-dwelling organism, its spores persisting in soil until encountering conditions suitable for germination and growth (10). The basic structural unit of soil ecosystems is the soil aggregate, in which biogeochemical processes occur at scales relevant to microorganisms. Approximately 50% of the volume of a soil aggregate represents open pores, while the remainder consists of mineral particles (sand, silt, and clay) held together by organic material (48), with which *B. subtilis* may be preferentially associated (16, 43). Soil aggregates can be classified as macroaggregates (diameter, >250 μm) and microaggregates (diameter, 2 to 250 μm) (39), but little is known about the distribution of bacteria within aggregates. Structural organization of the soil creates a mosaic of microenvironments, within which water movement and diffusion of nutrients and other molecules play key roles in functioning of the soil microbiota (7, 13, 39). These roles may vary with the scale at which they operate. Tisdall and Oades (51) suggest that scales at which microorganisms are important in the soil aggregation process range between 2 and 2,000 μm, depending on the specific system being investigated (13). Although the microscale distribution of microorganisms and their

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TABLE 1. *Bacillus* strains used in this study

Strain	Genotype or description	Source or reference
Producer strains		
BD2833	*his leu met srfA-lacZ* (*tet*)	53
BD2913	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*ery*) (*comQ comX comP* replaced by genes from *B. mojavensis* RO-H-1)	53
BD2915	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*ery*) (*comQ comX comP* replaced by genes from *B. subtilis* natto NAF4)	53
BD2936	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. mojavensis* RO-B-2)	53
BD2940	*his leu met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. subtilis* RO-E-2)	2
BD2949	*his leu met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. subtilis* RS-D-2)	2
Tester strains		
BD2876	*his leu met srfA-lacZ* (*tet*) *comQ*::Km	53
BD2877	*his leu met srfA-lacZ* (*tet*) (*comQ*::*phl comX comP* replaced by genes from *B. subtilis* natto NAF4)	53
BD2962	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*ery*) (*comQ*::pED345 *comX comP* replaced by genes from *B. mojavensis* RO-H-1)	53
BD2983	*his leu met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ*::pED345 *comX comP* replaced by genes from *B. mojavensis* RO-B-2)	2
BD3019	*his leu met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ*::pED375 *comX comP* replaced by genes from *B. subtilis* RS-D-2)	2
BD3020	*his leu met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ*::pED375 *comX comP* replaced by genes from *B. subtilis* RO-E-2)	2
Other strains used		
RO-FF-1	*B. subtilis* 168, Mojave Desert, Rosamond, CA	40
B. cereus	Isolated from milk	Gift from S. Smole Mozina

associated functions have rarely been studied, it is becoming recognized that greater knowledge of spatial organization at the scale of a soil aggregate (microscale) is essential for a better understanding of soil ecosystem function and of the mechanisms that generate and maintain diversity, including speciation, extinction, dispersal, and interactions within and between species (7, 13, 26).

The aim of this study was to assess the potential role of QS in generating and maintaining microscale diversity within the soil. This was achieved by determining the genomic and functional diversification of the *B. subtilis* QS system with regard to geographical distance and ecological characteristics. Isolates were obtained from two 1-cm^3 sandy, riverbank soil samples separated by approximately 5 m, allowing assessment of macroscale diversity. In addition, each riverbank soil sample was treated as a separate macroaggregate that was progressively sectioned to obtain subsamples of different sizes, allowing assessment of microscale diversity. The riverbank soil *B. subtilis* isolates were compared with *Bacillus* isolates previously obtained from desert soil samples separated by distances of meters to kilometers (2, 40), representing macroscale distribution. The *Bacillus* isolates were used to (i) correlate geographical distance (microscale/macroscale) with genomic distance of the QS *comQ* gene and the housekeeping *gyrA* gene, (ii) investigate and compare the specificity of the QS response of microscale and macroscale isolates, and (iii) explore dominance of pherotypes inside soil aggregates. To our knowledge, this is the first investigation of a QS system that addresses the genomic and functional diversification of bacterial populations at microscale.

MATERIALS AND METHODS

Soil sampling, strains, and isolation. The strains and genotypes of *Bacillus subtilis* used in this study are listed in Table 1, including *B. subtilis* pheromone producer and tester strains described previously (2, 53). Additional natural isolates of *B. subtilis* were obtained from two structured, sandy soil samples (~1 cm^3) aseptically removed from the surface soil (10 cm) on the bank of the River Sava, Slovenia (grid reference 46°06′N, 14°28′E), approximately 5 m apart in January 2006. Each sample was immediately cut into four equal-size sections, representing one-fourth of the initial aggregate. The one-fourth sections were further divided to give two one-eighth sections and four 1/16 sections. The approximate diameter of the 1/16 samples was 2.5 mm. Soil subsamples were then placed in sterile tubes and brought to the laboratory. On the same day, samples were resuspended in 1 ml of sterile saline solution (0.9% NaCl), and the suspension was heated for 15 min at 80°C to kill vegetative cells but preserve spores. Resultant spore suspensions were plated on tryptose blood agar base (Difco; Becton, Dickinson and Company, Sparks, MD) and incubated for 24 h at 37°C. Emergent colonies were streaked three times to obtain pure cultures, and 30 emerging colonies were examined from each subsection, yielding 420 isolates from both cumulative soil samples, which were then subjected to four metabolic tests (11): the catalase test, the Voges-Proskauer test (demonstrating conversion of pyruvate to acetoin), anaerobic growth on agar, and hydrolysis of starch. *B. subtilis* strains are catalase positive, convert pyruvate to acetoin, do not grow anaerobically, and hydrolyze starch. On the basis of these criteria, 67 isolates were identified as *B. subtilis*.

Growth conditions and general methods. *B. subtilis* strains were grown either in Luria-Bertani (LB) medium; on liquid competence medium supplemented with glucose (0.5% wt/vol), L-histidine, L-leucine, and L-methionine (50 μg ml^{-1}); or on tryptose blood agar base. Tester and producer strains were grown in or on media supplemented with kanamycin (5 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), or tetracycline (20 μg ml^{-1}), as appropriate. All incubations in liquid media were carried out at 37°C with shaking, except for overnight cultures grown for chromosomal DNA extraction, which were incubated at 30°C. DNA manipulation and molecular biological procedures were performed using standard protocols. Conditioned media were prepared and assayed according to the method of Tortosa et al. (53).

PCR amplification. The *gyrA* genes of 39 isolates were amplified by PCR with primers gyrAR1 (5′-GTATCCGTTGTGCGTCAGAGTAAC-3′) (2) and gyrAF (5′-CAGTCAGGAAATGCGTACGTCCTT-3′) (8) in a 50-μl reaction mixture containing 20 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate (Biotools; Madrid, Spain), 2 μl of template DNA, 5 μl of 10× PCR buffer (Biotools), 6 μl of 25 mM $MgCl_2$ (Biotools), and 2 U of *Taq* DNA polymerase (Biotools) (final concentrations). The PCR consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The *gyrA* genes of 15 desert strains were amplified with the same protocol and the same primer set as described above, except for *Bacillus mojavensis* RO-B-2 and *B. mojavensis* RO-H-1, where the reverse primer gyrAR5 (5′-ATCATTGAAGCGCTCTTTGATTTCCGTGAGT TCTTC-3′) was used.

The 16S rRNA genes of 28 isolates were amplified by PCR with primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1406R (5′-ACGGGCGGTGTG TRCAA-3′) (31) in a 50-μl reaction mixture containing 20 pmol of each primer, 20 nmol of each deoxynucleoside triphosphate (Biotools), 1 μl of template DNA, 5 μl of 10× PCR buffer (Biotools), 4 μl of 25 mM $MgCl_2$ (Biotools), and 1 U of *Taq* DNA polymerase (Biotools) (final concentrations). The PCR consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, extension at 72°C for 2 min, and a final extension at 72°C for 10 min.

The *rpoB* genes of seven isolates, for which sequencing of the 16S rRNA genes did not differentiate between *Bacilllus amyloliquefaciens* and *B. subtilis*, were amplified by PCR with primers rpoBF (5′-AGGTCAACTAGTTCAGTATGG ACG-3′) and rpoBRO (5′-GTCCTACATTGGCAAGATCGTATC-3′) (2) in a 50-μl reaction mixture containing 20 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate (Fermentas, Vilnius, Lithuania), 2 μl of template DNA, 10 μl of 5× PCR buffer (Promega), 6 μl of 25 mM $MgCl_2$ (Promega), and 0.4 U of *Taq* DNA polymerase (Promega) (final concentrations). The PCR consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 50 s, and a final extension at 72°C for 5 min.

The *comQ* genes of 39 isolates were amplified by PCR with primers Uni-comQ1 (5′-GGGAGGGGGGAAGTCGTTATTG-3′) and P1 (5′-AAGAACCG AATCGTGGAGATCGCG-3′) (53) in a 50-μl reaction mixture containing 10 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate, 1 μl of template DNA, 5 μl of 10× PCR buffer (Promega, Madison, WI), 3 μl of 25 mM $MgCl_2$ (Promega), 200 nM primers, and 5 U of *Taq* DNA polymerase (Promega) at the final concentration. The PCR profile of the *comQXP* locus amplification consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 3 min, and final extension at 72°C for 5 min. The 3-kb *comQXP* PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) prior to sequencing. All PCRs were carried out in a Biometra Uno-Thermoblock. The resulting amplicons were examined by electrophoresis on a 1% agarose gel.

DNA sequencing. The *comQXP* locus was sequenced with the forward primer Uni-comQ1 (5′-GGGAGGGGGGAAGTCGTTATTG-3′), and the *gyrA* gene was sequenced using the reverse primer gyrAR1 (5′-CAGTCAGGAAATGCG TACGTCCTT-3′), except for the desert strains *B. mojavensis* RO-B-2 and *B. mojavensis* RO-H-1, where primer gyrAR5 (5′-ATCATTGAAGCGCTCTTTG ATTTCCGTGAGTTCTTC-3′) was used. The 16S rRNA genes were sequenced using the reverse primer 1406R (5′-ACGGGCGGTGTGTRCAA-3′), and the *rpoB* genes were sequenced with the primer rpoBF (5′-AGGTCAACTAGTTC AGTATGGACG-3′). PCR products were sequenced by Macrogen Inc. (Seoul, Korea).

Phylogenetic analyses. Phylogenetic analyses were conducted using MEGA version 4 (50) for neighbor-joining and minimum-evolution analyses using Tajima-Nei (49) and Tamura-Kumar (50) models of evolution with heterogeneous patterns among lineages and gamma distributed rates among sites. All positions containing gaps and missing data were eliminated from the data set. Since conservation of topology among the resulting trees was independent of the applied method, only minimum-evolution trees are shown. Bootstrap support was calculated from 1,150 replicates.

β-Galactosidase assay. β-Galactosidase assays were performed as described previously (53). Briefly, tester strains containing the *srfA-lacZ* reporter were grown in conditioned medium and samples were taken 2 h after the end of exponential growth. Cell suspensions were centrifuged, and cells were assayed for β-galactosidase activity with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. β-Galactosidase activities were calculated from the slopes of the reaction curves.

Nucleotide sequence accession numbers. Accession numbers of the riverbank *comQ* and *gyrA* nucleotide sequences have been deposited in GenBank under the accession numbers FJ172555 to FJ172593 and FJ72594 to FJ172632, respectively. The accession numbers of the extended desert *gyrA* genes are deposited under accession numbers FJ546326 to FJ546340. The 16S rRNA genes and *rpoB* genes have been deposited in GenBank under the accession numbers FJ489838 to FJ489867 and FJ546319 to FJ546325, respectively.

RESULTS

Characterization of *Bacillus subtilis* isolates. More than 400 spore-forming bacteria obtained from microscale samples from the two riverbank soil samples were characterized biochemically according to the identification key proposed by Gordon et al. (11). Using this approach, 38 and 29 potential *B. subtilis* strains were identified in the first and second samples, respectively. Sequencing of the *gyrA* gene of 39 of these isolates confirmed their identity, as indicated by phylogenetic analysis (Fig. 1A). Desert and riverbank *B. subtilis* sequences formed two similarity clusters, with 92 to 95% identity between the two clusters. One cluster contained all the riverbank soil sequences and also a few desert sequences, the *B. subtilis* natto NAF4 *gyrA* sequence and *gyrA* from the laboratory strain *B. subtilis* 168, which were phylogenetically classified as *Bacilllus subtilis* subsp. *subtilis*. Sequences of the *gyrA* gene inside the cluster were highly conserved, with 98 to 100% identity among these isolates. The *gyrA* sequences from the two riverbank soil samples showed the same identity inside the sample as between the two samples. However, the second cluster was comprised of exclusively desert sequences representing *Bacillus subtilis* subsp. *spizizenii* (29). The average GC content of *gyrA* from riverbank soil isolates was 42.4%. For the remaining 28 putative *B. subtilis* strains isolated from the riverbank soil, *gyrA* could not be amplified with the existing primers and the phylogeny of these strains was investigated by targeting the 16S rRNA gene. This revealed that 15 of the 28 strains belonged to the *Bacillus pumilus* species, two belonged to *Bacillus licheniformis* species, one belonged to *Cronobacter dublinensis*, three strains showed highest homology to *Bacillus cereus* or *Bacillus thuringiensis* species, and seven strains clustered with *B. subtilis* and/or *B. amyloliquefaciens* species, which had identical 16S rRNA partial sequences. These were further analyzed by sequencing the *rpoB* genes. The *rpoB* sequences showed 99% identity with *B. amyloliquefaciens* and 93% identity with *B. subtilis*, suggesting that these seven strains might belong to the *B. amyloliquefaciens* species.

Polymorphism of *comQ*. Previous studies of *Bacillus* isolates from desert soils indicated a striking polymorphism in *comQ*, *comX*, and the N-terminal sequence of *comP*, indicating their coevolution (2, 53, 54, 61). Polymorphism in isolates obtained from the riverbank sandy soil was examined by partially sequencing *comQ* of the 39 *B. subtilis* isolates and by comparison with database sequences. As observed in previous studies, these *comQ* sequences were highly polymorphic, showing only 53 to 62% identity at the nucleotide level among riverbank isolates with an average GC content of 32.4%. The similarity tree obtained by minimum-evolution analysis of the 39 new *comQ* sequences and the *comQ* genes of previously analyzed desert strains formed four distinct clusters with three out of four clusters containing mixtures of both desert and riverbank sequences and one cluster (RO-E-2) containing only desert *comQ* sequences (Fig. 1B).

Diversity was very high between and within clusters (Table 2): for example, 67 to 100% identity was observed among

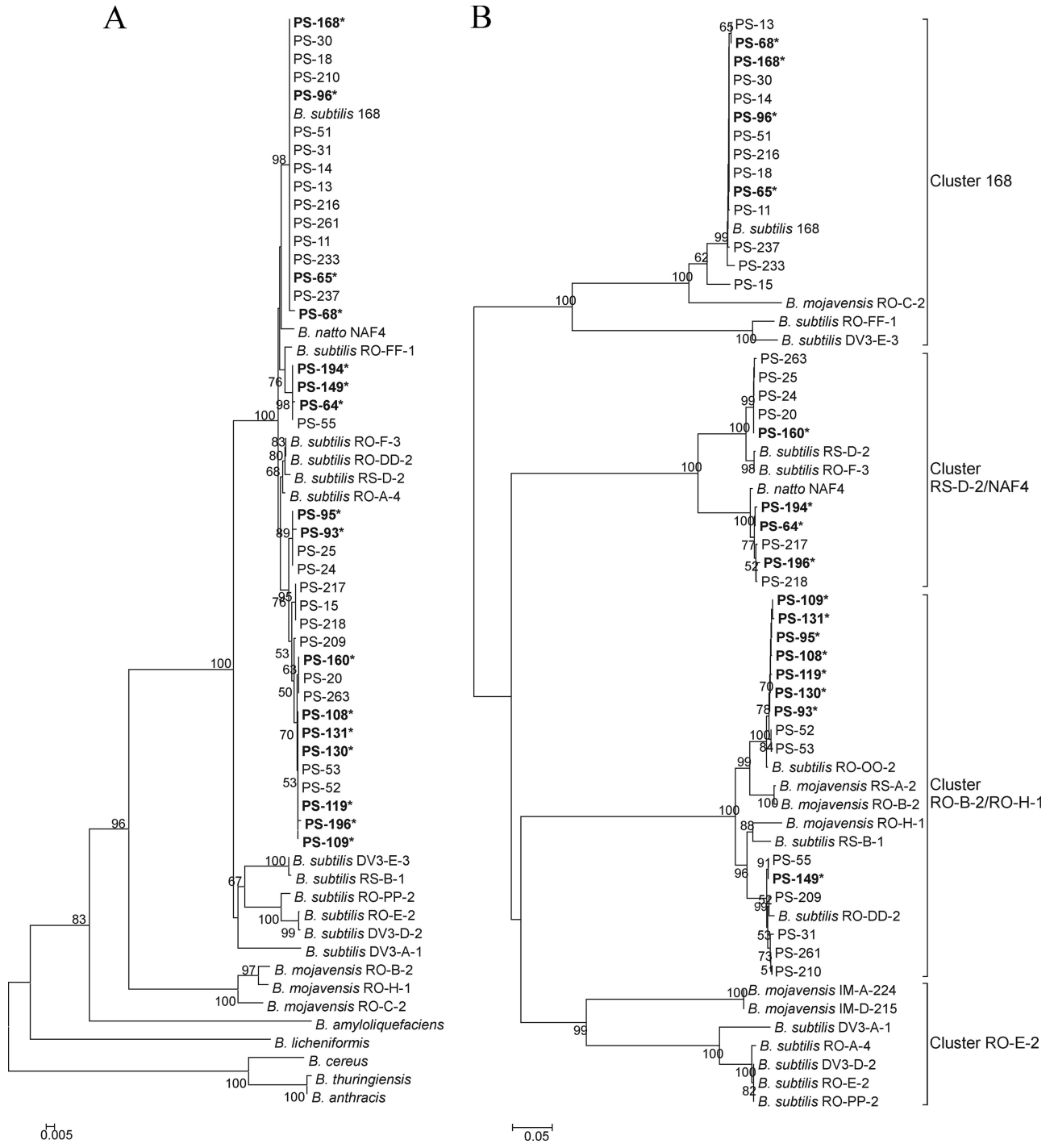

FIG. 1. Minimum-evolution trees based on partial *gyrA* nucleotide sequences (610 bp) (A) and partial *comQ* sequences (701 bp) (B). Trees were drawn using the minimum-evolution method after multiple alignment in MEGA 4 software (50). The neighbor-joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the data set. The numbers at internal branches represent the bootstrap values estimated from 1,150 resamplings. PS indicates newly acquired sequences of *B. subtilis* strains isolated from the riverbank soil, where sequences with and without asterisks originated from soil samples 2 and 1, respectively. In addition, *gyrA* and *comQ* sequences of desert *B. subtilis* strains (RO, RS, and DV) and other *Bacillus* strains obtained from the database were also included in the analyses. For clarity, clusters in panel B are named according to the *B. subtilis* tester strains used to identify their communication specificity in vivo (2). The top cluster in the similarity tree is referred as cluster 168, the second cluster as NAF4/RS-D-2, the third cluster as RO-H-1/RO-B-2, and the fourth cluster as RO-E-2.

comQ genes in the 168 cluster. However, riverbank soil isolates belonging to group 168 originating from two soil samples, separated by 5 m, were more similar, showing 94 to 100% identity in *comQ*, with 8 out of 14 isolates having 100% identical sequences, suggesting a possible clonal origin. The highest divergence was found for *comQ* genes from the RO-FF-1 and DV3-E-3 desert soil isolates, which showed only 67 to 68% identity to other *comQ* genes from the 168 group, and no

TABLE 2. Identity between *comQ* similarity clusters[a]

Cluster	% nucleotide identity within and between clusters			
	168	RS-D-2/NAF4	RO-B-2/RO-H-1	RO-E-2
168	67–100			
RS-D-2/NAF4	53–57	87–100		
RO-B-2/RO-H-1	54–57	57–60	87–100	
RO-E-2	55–59	58–60	58–62	71–100

[a] Identity of *comQ* genes from riverbank and desert isolates was determined by ClustalX identity matrix. The percent nucleotide identity was determined within and between clusters. Clusters were named according to the tester strains used for analysis of specificity of QS response (Table 3).

riverbank soil *comQ* genes clustered close to these two desert *comQ* genes. This suggested that strains in the 168 cluster might be split into two clusters.

RS-D-2/NAF4 *comQ* genes showed 87 to 100% identity within the cluster and formed two distinct subclusters, NAF4 and RS-D-2, which shared only 87 to 88% identity, with 99 to 100% and 98 to 100% identities within subclusters NAF4 and RS-D-2, respectively. The NAF4 cluster contained *comQ* from riverbank soil isolates and from *B. subtilis* natto NAF4, a starter strain for the manufacture of natto (fermented soybeans) (54). The RS-D-2 cluster contained *comQ* sequences from two desert isolates and from riverbank isolates originating from mostly one of the two samples analyzed.

comQ sequences within the RO-B-2/RO-H-1 cluster showed 87 to 100% identity and included two distinct subclusters containing desert and riverbank isolates, with identities of 93 to 100% (RO-H-1 subgroup) and 94 to 100% (RO-B-2). The identity among desert *comQ* sequences was lower: 94 to 95% within the RO-H-1 subgroup and 95 to 100% within the RO-B-2 subgroup, compared to riverbank soil *comQ* genes (99 to 100% for both subclusters). No *comQ* sequence from riverbank isolates clustered inside the RO-E-2 group.

Specificity of the *comQXP* QS loci. The 42 riverbank soil isolates were tested for their specificity in activating the QS response in six tester strains, representing four (currently recognized) pherotypes (languages). The QS response was measured by measuring expression of the *srfA-lacZ* gene, which is positively controlled by the *comQXPA* QS system. On the basis of strong and moderate activation responses, the 39 strains could be placed within three pherotypes or language groups, among which 14, 10, 15, and 0 belonged to the 168, RS-D-2/NAF4, RO-B-2/RO-H-1, and RO-E-2 tester pherotypes, respectively (Table 3). Testing of additional riverbank soil isolates whose 16S rRNA gene sequences placed them within the *B. subtilis*/*amyloliquefaciens* group indicated three strains that induced the RO-E-2 QS response. These three strains were classified according to the *rpoB* partial sequence as *B. amyloliquefaciens*.

An asymmetric response was observed in two pherotypes. Five isolates within the RS-D-2/NAF4 group induced both testers used, while five were able to induce only the RS-D-2 tester strain. Similarly, 9 of 15 isolates within the RO-B-2/RO-H-1 group activated only the RO-B-2 tester while the remainder activated both tester strains. Some isolates showed cross talk with testers outside their pherotype, but this nonspecific response (indicated in Table 3 by +/−) was never as strong as the response within the pherotype.

TABLE 3. Pherotype groups of 42 riverbank soil isolates[a]

Pherotype group and isolate from which conditioned medium was obtained[b]	β-Galactosidase activity in tester strain[c]:					
	168	RS-D-2	NAF4	RO-B-2	RO-H-1	RO-E-2
168						
PS-11	++	+/−	−	−	−	−
PS-13	++	+/−	−	−	−	−
PS-14	++	+/−	−	−	−	−
PS-15	++	−	−	−	−	−
PS-18	++	+/−	−	−	−	−
PS-30	++	+/−	−	−	−	−
PS-51	++	−	−	−	−	−
PS-216	++	+/−	−	−	−	−
PS-233	++	+/−	−	−	−	−
PS-237	++	+/−	−	−	−	−
PS-168	++	+/−	−	−	−	−
PS-96	++	+/−	−	−	−	−
PS-65	++	+/−	−	−	+/−	−
PS-68	++	+/−	−	−	−	−
RS-D-2/NAF4						
PS-217	−	++	++	−	−	−
PS-218	−	++	++	−	+/−	−
PS-64	−	++	++	−	−	−
PS-194	−	+	++	−	−	−
PS-196	−	+	++	−	−	−
PS-20	−	++	−	−	−	−
PS-24	−	++	−	−	−	−
PS-25	−	++	−	−	−	−
PS-263	−	+	−	−	−	−
PS-160	−	+	−	−	−	−
RO-B-2/RO-H-1						
PS-52	−	−	−	++	+/−	−
PS-53	−	−	−	++	−	−
PS-93	−	+/−	−	++	−	+/−
PS-95	−	+/−	−	+	+	−
PS-108	−	+	−	++	+/−	−
PS-109	−	+	−	++	−	−
PS-119	−	−	−	++	−	−
PS-130	−	+/−	−	++	+/−	−
PS-131	−	+	−	++	−	−
PS-31	−	−	−	++	++	−
PS-55	−	−	−	+	++	−
PS-209	−	+/−	−	++	++	−
PS-210	−	−	−	++	++	−
PS-261	−	+/−	−	++	++	−
PS-149	−	−	−	+	++	−
RO-E-2						
PS-207	−	−	−	−	+/−	++
PS-122	−	−	−	−	+/−	++
PS-188	−	+	−	−	−	++

[a] Specific activation of the QS response indicates a specific pherotype (2) and was measured using tester strains able to detect one of the four previously determined pherotypes through activation of the *srfA-lacZ* reporter gene. The *B. subtilis* 168 tester strain was used for detection of the first pherotype, and two tester strains, *B. subtilis* RS-D-2 and *B. subtilis* natto NAF4, were used for the second pherotype. The third pherotype was investigated using the tester strains *B. mojavensis* RO-B-2 and *B. mojavensis* RO-H-1, and the fourth pherotype was investigated using the *B. subtilis* RO-E-2 tester strain.

[b] Conditioned media were prepared from riverbank isolates as described previously (53). Isolates indicated by normal type and by boldface were obtained from soil samples 1 and 2, respectively.

[c] Tester strains were inoculated (1:50) into conditioned medium mixed with an equal volume of fresh competence medium. Samples were collected 2 hours after entry into stationary phase and were assayed for β-galactosidase activity as indicated in Materials and Methods. Symbols: ++, strong response, similar to positive control; +, moderate response, approximately 50% of the positive-control response; +/−, weak but reproducible response; −, no activation.

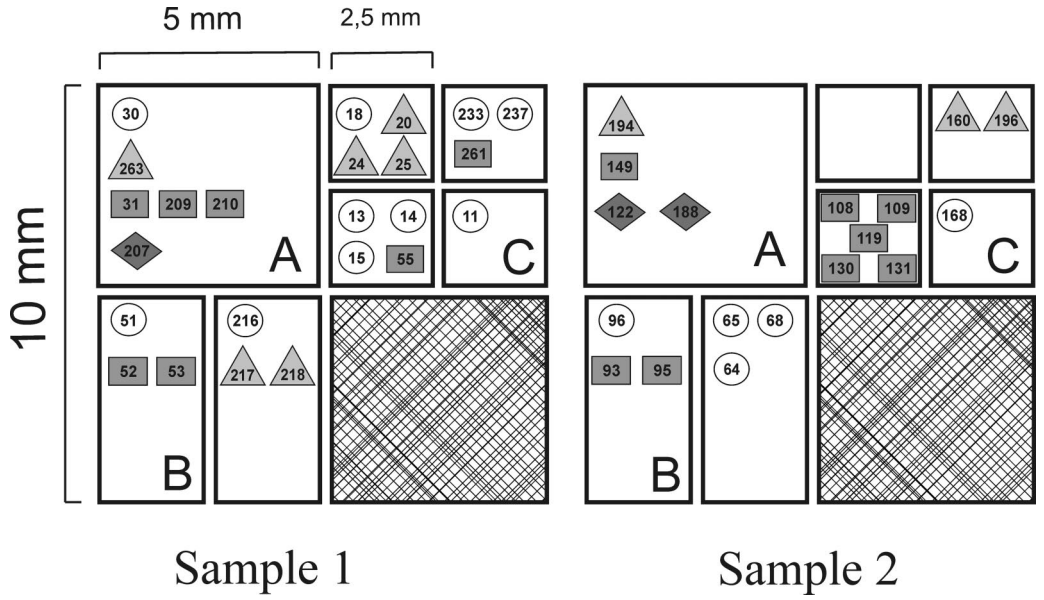

FIG. 2. Distribution of isolates in two 1-cm^3 samples (1 and 2) of riverbank soil. Letters A, B, and C indicate the one-fourth, one-eighth, and 1/16 sample sections, respectively. Specific PS isolates are indicated as a number, and the pherotype of the isolate is shown by a geometric shape. Isolates belonging to pherotypes 168, RS-D-2/NAF4, RO-B-2/RO-H-1, and RO-E-2 are represented by circles, triangles, rectangles, and diamonds, respectively.

Distribution of isolates in soil aggregates of different sizes. The presence of different pherotypes was determined in the two 1-cm^3 soil samples as they were progressively subdivided into aggregates that were 2.5 mm in diameter (Fig. 2). All four pherotypes were isolated from both 1-cm^3 samples, and four and three pherotypes were present in section A (one-fourth) of samples 1 and 2, respectively (Fig. 2). All four section B (one-eighth) aggregates contained two pherotypes, although the combinations of pherotypes varied between samples. Of the eight smallest aggregates (section C, 1/16), three contained two pherotypes, four contained one pherotype, and one contained no obtainable isolates (Fig. 2). There was a slight difference in prevailing pherotypes in the two samples, with the 168 and RO-H-1/RO-B-2 pherotypes more frequently isolated from samples 1 and 2, respectively.

DISCUSSION

In this study genomic and functional diversification of the *B. subtilis* QS system, encoded by the *comQXP* locus, was addressed in relation to spatial scale. Previous studies indicated a high level of polymorphism of the *comQXP′* QS locus, identifying at least four different languages (pherotypes) within this species (2) in isolates from various desert locations separated by meters or kilometers. A similar degree of polymorphism was observed in the present study through analysis of isolates from two 1-cm^3 soil samples separated by 5 m.

Polymorphism and specificity. The *comQXPA* QS locus of *Bacillus* consists of three genes, *comQ*, *comX*, and *comP*, which are highly polymorphic, and the conserved *comA* gene. The first two genes and the N-terminal region of *comP* are subject to coevolution and determine the specificity of the QS response (53). The average GC content reported for the polymorphic QS genes (29.5%) is lower than that of housekeeping genes *gyrA* and *rpoB* (41.1%) or the entire *B. subtilis* 168 genome (43.5%). (1, 2). The average GC contents of *comQ* and *gyrA* gene in soil isolates obtained in this study were 32.6% and 42.4%, respectively, agreeing with previous findings and supporting the proposed acquisition of the *comQXP′* loci by horizontal gene transfer (1, 2).

The similarity tree obtained using *comQ* sequences from 39 soil isolates was congruent with trees constructed for *comQ*, *comX*, and the N-terminal region of *comP* obtained in previous studies (1), justifying only partial sequencing of the soil *comQ* genes in this study. Soil isolate *comQ* sequences fell within three of four previously identified similarity clusters (2), and no sequence clustered with the RO-E-2-related desert *comQ* genes. This suggests its lower frequency in the riverbank soil or bias in the methodology for *B. subtilis* strain identification toward the other three pherotypes. Indeed, it was not possible to amplify *gyrA* in 28 of 67 potential *B. subtilis* strains identified phenotypically (11), while 25% of the remaining 28 strains had 16S rRNA gene sequences homologous to *B. subtilis* or *B. amyloliquefaciens* genes and were subsequently, based on the *rpoB* partial sequence, classified as *B. amyloliquefaciens* strains.

Ansaldi et al. (2) showed that some of the pherotypes are completely closed while limited cross-communication may occur in others, but the low number of strains analyzed prevented firm conclusions on the prevalence of cross talk between soil isolates. There was, however, evidence of cross talk between RS-D-2/NAF4 and RO-B2/RO-H-1 pherotypes and asymmetric response and diversification into two sublanguages at functional and structural levels. At the sequence level, two subclusters of mixed riverbank and desert soil *comQ* sequences could be depicted inside the RO-B-2/RO-H-1 and RS-D-2/NAF4 clusters. Functionally, this clustering correlated with the specificity of the QS response of the NAF4 tester strain, where only

riverbank soil isolates with 99 to 100% identical *comQ* sequences could induce the response in this strain. In contrast, the RS-D-2 tester strain showed broader specificity and communicated with strains carrying *comQ* sequences from both subclusters and showing only 87 to 88% identity.

The evolutionary process leading to *comQXP* diversification requires coordination of mutations affecting the three determinants of this QS system: the ComX pheromone, the receptor (ComP), and the processing enzyme (ComQ). Coevolution of three genes implies that the genetic diversification found at the level of *comQ* will correlate with diversification of *comX*, which interacts directly with ComQ in its peptide form. Based on this assumption, our data suggest that the ComP receptor of RS-D-2 shows broader specificity than does the NAF4 tester strain. Similar asymmetry has been detected with RO-B-2 and the RO-H-1 tester strains, with the former showing substantially broader specificity. Separation of strains within a pherotype into two asymmetric response groups suggests continuous diversification of *comQXP* loci. RS-D-2 and RO-H-1 were also able to detect signals from strains of noncognate pherotypes, in agreement with observations by Ansaldi et al. (2). It is likely that concerted evolution in the *comQXP* locus would involve intermediary mutational stages with broadened specificity, since mutations losing activity might be an evolutionary dead end (62). This interesting hypothesis suggests that RS-D-2 and RO-H-1 may represent an intermediary evolutionary stage with broadened specificity. However, it should be noted that the observed cross talk between pherotypes was never as strong as that within a pherotype, implying that a barrier between pherotypes was preserved at some level, even in strains with promiscuous behavior. Cross talk may also result from production of another factor by our soil isolates, such as CSF, which can induce *srfA* in the absence of the specific ComX pheromone (36), although testers of other pherotypes would also be expected to respond to CSF. Besides, this peptide induces *srfA* only when at relatively low concentrations (1 to 5 nM) while higher concentrations (20 nM) inhibit expression of this target gene (23). Also, Ansaldi et al. (2) showed cross-inhibition between some pherotype pairs, implying that the lack of cross talk in some pairs might also be due to inhibition by the respective ComX peptide present in conditioned media of tested strains.

Biogeography. Environmental and genetic diversity may be correlated (27, 32, 44, 45), and adaptation and speciation of *Bacillus simplex* strains are driven by environmental forces, such as temperature stress (22, 45). *gyrA* sequences of closely related bacilli formed two clusters, one containing only sequences from desert isolates that were previously classified as *B. subtilis* subsp. *spizizenii* and the other containing the laboratory strain *B. subtilis* 168, all the riverbank *gyrA* genes, and a few desert *gyrA* genes that may be phylogenetically placed into *B. subtilis* subsp. *subtilis*. Previous studies indicated two closely related but genetically and phenotypically distinct groups within *B. subtilis* (29). The genes in the *B. subtilis* subsp. *subtilis* cluster analyzed in this study showed high identity (98 to 100%), and no higher identity of *gyrA* was found among riverbank isolates compared to all isolates in this cluster. However, between *B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii* only 92 to 95% identity of *gyrA* was detected, which is in accord with previous studies (40). The mixing of desert and riverbank strains in the riverbank cluster is in agreement with the "everything is everywhere" hypothesis, suggesting high rates of dispersal and colonization that prevent spatial differentiation (26). However, it is interesting that no riverbank isolates clustered into the *B. subtilis* subsp. *spizizenii* group, suggesting the importance of environmental factors in *gyrA* diversification, in agreement with previous studies of *Bacillus* diversification (21, 45). However, it is also possible that an enrichment of riverbank isolates in the 168 group is the consequence of insufficient sampling along the river gradient.

In contrast, diversification of *comQ* pherotypes indicated mixing of desert and riverbank strains in each of the four pherotypes, supporting the findings of Ansaldi and Dubnau (1) that diversification of QS and housekeeping genes is driven by different selective forces. The results also suggest that selective forces acting on adaptive evolution of QS loci may target a trait(s) that is important only during specific growth stages. One candidate trait (52) is development of competence for transformation, which can be predicted to increase fitness in any environment. It is interesting that a higher rate of evolution has been reported for various proteins involved in sexual reproduction in different species, from plants to mammals (5), a trend also observed in *comQXP* QS genes involved in bacterial gene exchange.

Although the same number of pherotypes was found among desert and riverbank strains, the frequencies of occurrence of RO-E-2 and 168 groups were higher in desert and riverbank soils, respectively. Studies of microbial distribution of soil bacteria have revealed correlations between genetic diversity and distance (4, 32), and our results suggest that isolates separated by km have lower identity at the *gyrA* loci than the riverbank isolates obtained from both 1-cm^3 samples (above 82 and 98%, respectively). A similar trend is observed in the faster-evolving *comQ* loci inside each similarity cluster, with identity at desert macroscale being above 67% and at the riverbank scales being above 87%. However, the main difference observed at microscale is the number of isolates with identical *gyrA* sequences (*gyrA* clonemates) and sequences of the rapidly evolving *comQ* gene. For example, of the 14 riverbank isolates in the 168 group, 10 showed 100% identical *comQ* sequences (*comQ* clonemates), while three desert strains in this cluster showed only 67 to 94% identity. At desert macroscale identical *comQ* sequences were only rarely identified (in the cluster RO-E-2), suggesting that the frequency of clonemates decreases with distance. In several studies based on different scales, clonemates were isolated from cm to km scales (14, 18, 57, 58), and Vogel et al. (58) found that clones of *Agrobacterium* species in a 1-cm^3 cube were distributed and did not form tight microcolonies.

B. subtilis spores are easily made airborne and might migrate long distances and land in a given environment but not necessarily germinate there (9); therefore, it is hard to determine whether the organism, when isolated, was in its spore form that had landed at the site of isolation and remained for an unknown time or was a spore that derived from a vegetative cell, a form that gives us an insight into the ecology and actual distribution of the *B. subtilis* at small scale. However, a relatively high number of *comQ* clonemates among riverbank isolates suggests that they have been actively growing in this

environment and that the observed number of pherotypes is not only the consequence of spore accumulation.

In our study clonemates also originated from both 1-cm^3 samples and seem to be homogenously allocated. The presence of clonemates and all four pherotypes in both 1-cm^3 samples suggests that sample size was sufficient to observe the functional and genetic diversity of the QS system present at microscale. However, further decrease in sample size suggested a decrease in the number of pherotypes. All four pherotypes were found only in one of the largest subsamples (A), and all other subsamples, even the composite ones, contained lower numbers of pherotypes. B and C subsamples contained no more than two pherotypes, and one C subsample contained no pherotype, although five *Bacillus subtilis*-related strains were isolated from this sample. When the number of pherotypes was plotted against the size of the samples, a decrease in pherotype numbers was observed but with a rather low R^2 value (R^2 = 0.6763) (data not shown). This may suggest that samples smaller than 1 cm^3 may not contain the full pherotype richness within an environment, and competitive exclusion may operate at this scale. However, this could also be due to the sampling strategy performed. The sampling was performed so that 30 colonies were examined from each subsample, which gave from zero to six *B. subtilis* isolates per subsection. It is possible that with a more extensive sampling strategy the four pherotypes would be found even in the smaller subsections.

The ComQXPA QS system, apart from controlling genetic competence, also participates in transcriptional regulation of other traits, including swarming and production of extracellular degradative enzymes and a capsular poly-γ-glutamate (23, 28, 30), whose influence in mixed pherotype populations is unknown. Presumably it would be of a competitive advantage for different pherotype populations to have a system that could coordinate complex responses inside the pherotype population while not affecting members of other pherotypes. Little is known about ecological differentiation of *B. subtilis* isolates, but members of two pherotypes might be ecologically distinct and occupy distinct niches. Large portions of the chromosome are very variable in different *B. subtilis* strains, suggesting a vast functional diversification within species (10). Indeed, Koeppel et al. (21) found 13 ecotypes inside the *B. subtilis-B. licheniformis* clade in the "Evolutionary Canyon." Spatial heterogeneity within soil provides one explanation for the high levels of microbial diversity observed, through microniche specialization. Competition between pherotypes and potential competitive exclusion provide one mechanism driving richness within bacilli, and a reduction in pherotype richness with decreasing soil aggregate size below 5 mm provides an indication of the range of bacterial communication mechanisms and the spatial scale at which they may control prokaryote diversity.

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