

Genetic Differentiation between Sympatric Populations of *Bacillus cereus* and *Bacillus thuringiensis*

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Little is known about genetic exchanges in natural populations of bacteria of the spore-forming *Bacillus cereus* group, because no population genetics studies have been performed with local sympatric populations. We isolated strains of *Bacillus thuringiensis* and *B. cereus* from small samples of soil collected at the same time from two separate geographical sites, one within the forest and the other at the edge of the forest. A total of 100 *B. cereus* and 98 *B. thuringiensis* strains were isolated and characterized by electrophoresis to determine allelic composition at nine enzymatic loci. We observed genetic differentiation between populations of *B. cereus* and *B. thuringiensis*. Populations of a given *Bacillus* species—*B. thuringiensis* or *B. cereus*—were genetically more similar to each other than to populations of the other *Bacillus* species. Hemolytic activity provided further evidence of this genetic divergence, which remained evident even if putative clones were removed from the data set. Our results suggest that the rate of gene flow was higher between strains of the same species, but that exchanges between *B. cereus* and *B. thuringiensis* were nonetheless possible. Linkage disequilibrium analysis revealed sufficient recombination for *B. cereus* populations to be considered panmictic units. In *B. thuringiensis*, the balance between clonal proliferation and recombination seemed to depend on location. Overall, our data indicate that it is not important for risk assessment purposes to determine whether *B. cereus* and *B. thuringiensis* belong to a single or two species. Assessment of the biosafety of pest control based on *B. thuringiensis* requires evaluation of the extent of genetic exchange between strains in realistic natural conditions.

Genetic recombination between strains of the same species or between related or unrelated species is a key issue in bacteriological studies (5, 16, 18, 24). The evaluation of gene transfer is of critical importance not only for theoretical considerations, such as assessment of the potential of a bacterium to adapt to new environments, but also from an economic point of view. Indeed, the rates of intraspecific and horizontal gene transfer are key parameters for managing the spread of antibiotic resistance and for improving the efficacy and safety of genetically engineered microorganisms intended for environmental applications.

Fortunately, this task is facilitated by the extensive genetic variation displayed by almost all species of bacteria (32). Hence, during the last 3 decades, the methods, theory, and statistics of population genetics have been applied to bacteria, notably through the application of multilocus enzyme electrophoresis, which indexes the allelic variation in multiple chromosomal genes (18). Such studies were initially undertaken with pathogenic bacteria, such as *Escherichia coli* (see references 23 and 29), *Neisseria meningitidis* (3), *Bordetella bronchiseptica* (19), and *Haemophilus influenzae* (20), but have since been extended to a small number of free-living bacteria, such as *Bacillus subtilis* (8, 15) and *Rhizobium leguminosarum* (33). The following picture has emerged from these studies. Whereas some bacteria, such as *E. coli*, display a highly clonal pop-

ulation structure, many bacteria have genetic structures indicative of genetic exchange within local populations. Thus, there is evidence for sexual activity resulting in gene transfer between strains (17, 34). Although bacteria reproduce asexually by binary fission, the processes of conjugation, transduction, and transformation facilitate the transfer of chromosomal genes in otherwise clonal lineages.

Genetic exchange has been little studied in natural populations of bacteria of the spore-forming *Bacillus cereus* group. Such data are required, notably because several bacteria from this group are involved in human pathogenicity, whereas others are extensively used in agricultural pest control. The *Bacillus cereus* group includes three closely related species: *B. cereus*, *Bacillus thuringiensis*, and *Bacillus anthracis*. *B. cereus* is a common soil bacterium often implicated in food poisoning—causing diarrhea in particular—that is responsible for contamination problems in the dairy industry (7). *B. thuringiensis* contains large plasmids carrying various *cry* genes encoding insecticidal crystal proteins that are synthesized during the stationary phase of the growth cycle (6, 31). Finally, *B. anthracis* contains two large plasmids, pX01 and pX02, one encoding genes for the synthesis of a protective capsule and the other encoding the toxin responsible for the acute, often fatal, disease anthrax (26). Thus, *B. cereus*, *B. thuringiensis*, and *B. anthracis* are distinguished principally by the genes carried on their plasmids.

The genetic similarity between these three members of the *B. cereus* group has been extensively studied by investigating genomic variation and genetic diversity by means of DNA-DNA reassociation (21) and multilocus enzyme electrophore-

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sis (2, 12, 13). Helgason et al. (14) obtained DNA sequence data from nine chromosomal genes of *B. cereus*, *B. thuringiensis*, and *B. anthracis*. In separate examinations of each gene, they found that DNA sequences were highly conserved, with between 92.2 and 99.6% identity observed between pairs of genes. A tree taking all of these sequence comparisons into account shows that *B. anthracis* is very closely related to a periodontitis-causing *B. cereus* strain, and *B. thuringiensis* is closely related to another reference strain of *B. cereus*. Further evidence for the close relationship between these three species was provided by analysis of polymorphism at 13 allozyme loci from 239 strains of *B. cereus*, *B. thuringiensis*, and *B. anthracis*. The unweighted pair-group method for arithmetic averages (UPGMA) dendrogram based on the complete allozyme data set shows strains from the three *Bacillus* groups intermingled. Based on these results, Helgason et al. (14) suggested that these three taxa actually constitute a single species and pointed out the potential for the horizontal gene transfer of *B. thuringiensis* and *B. anthracis* plasmids to *B. cereus* strains. This conclusion was surprising because (i) the plasmids bearing *cry* genes have never been found in *B. anthracis*, and (ii) the pX01 and pX02 have never been found in *B. thuringiensis*. Furthermore, plasmid transfers have never been documented in natural populations of these different bacteria.

More generally, these genetic analyses of the *B. cereus* group must be interpreted with caution, because they used pools of strains collected at different times and from various geographical locations. Adaptation and speciation result from successive genetic recombinations and transformations within populations and geographical arrays of populations connected by the exchange of organisms and genes. Thus, local populations must be studied to determine the extent of genetic exchange within and between species in natural populations of bacteria. Istock et al. (15) pointed out that the analysis of isolates from small samples from single microsites provides the strongest test for natural recombination among strains. Thus, whereas all studies performed to date have clearly demonstrated that *B. cereus* and *B. thuringiensis* are almost indistinguishable, none have clearly evaluated relative gene flow within and between these two species.

In this study, we isolated sympatric strains of *B. thuringiensis* and *B. cereus* from small samples of soil collected at the same time from two separate geographical sites. We used these samples (i) to investigate the natural genetic structure of *B. cereus* and *B. thuringiensis* populations, focusing on fine-scale and internal population genetic structure; (ii) to explore the relative importance of intra- and interspecific gene exchange, to test the distinctness of these two closely related species; and (iii) to assess the contribution of binary fission relative to that of recombination for determining the genetic structure of each *Bacillus* species.

MATERIALS AND METHODS

Sampling and strain isolation. Strains were isolated from 10 samples spread over two restricted sites (about 100 m²) at La Minière (Guyancourt, Ile-de-France region, France). One site was located within the forest (referred as to site F), and the other was on the edge of this forest (referred as to site S). The two sites were located 2 km apart. At each site, five samples of soil (samples F1 to F5 and S1 to S5 at sites F and S, respectively) were collected on 6 December 2000. *B. cereus* and *B. thuringiensis* were isolated as described by Ohba and Aizawa (25). We examined colonies that resembled *B. cereus* in terms of morphology

under a light microscope, to check for the presence of parasporal crystals. Crystal-forming colonies were classified as *B. thuringiensis*, and the others were classified as *B. cereus*. We isolated 10 strains of each *Bacillus* sp. from each of the 10 samples of soil, except for F3 and F5, from which only 9 *B. thuringiensis* strains were recovered. In total, 198 strains were isolated: 100 were *B. cereus* strains, and 98 were *B. thuringiensis* strains.

Allozyme electrophoresis. Samples for enzyme electrophoresis were prepared as described by Carlson et al. (2) with modifications. Each strain was cultured overnight at 30°C in 50 ml of tryptic soy broth (Difco). The overnight culture was centrifuged at 7,000 × *g* for 10 min, and the bacterial pellets were suspended in 0.6 ml of buffer (10 mM Tris-HCl, 1 mM EDTA [pH 6.8]). Cells were disrupted by sonication for 2 min with cooling. They were then centrifuged at 20,000 × *g* for 20 min at 4°C, and the supernatants were stored at -80°C.

The homogenates were subjected to horizontal starch gel electrophoresis in a Tris-borate-EDTA (pH 8.6) buffer system (28). We assayed 25 enzymes by methods described by Selander et al. (32) and Pasteur et al. (28). Nine enzymes giving unequivocal genetic interpretations were retained. These enzyme systems were as follows: malate dehydrogenase (MDH; EC 1.1.1.37), catalase (CAT; EC 1.11.1.6), glucose phosphate isomerase (GPI; EC 5.3.1.9), aspartate aminotransferase (AAT; EC 2.6.1.1), diaphorase (DIA; EC 1.6.4.3), malic enzyme (ME; EC 1.1.1.40), superoxide dismutase (SOD; EC 1.15.1.1), purine nucleoside phosphorylase (NP; EC 2.4.2.1), and alcohol dehydrogenase (ADH; EC 1.1.1.1). The loci were not selected because they were polymorphic; they were the first nine loci to resolve well enough. Electromorphs (EMs) of each enzyme, numbered in order of decreasing anodal mobility, were associated with alleles at the corresponding structural gene locus. The absence of enzyme activity was scored as a null character state and allele as suggested by Selander et al. (32). The lack of enzyme activity may reflect low enzyme concentration in the lysate or denaturation caused by storage. We therefore verified the absence of enzyme activity by running replicates of separate preparations from cultures with a higher cell density. In addition, some strains with null alleles for a particular locus displayed uniformly high activity for other enzymes. Each isolate was characterized by determining the combination of alleles at the nine loci, and distinctive multilocus genotypes were designated as electrophoretic types (ETs).

Assays for hemolytic activity. Hemolytic activity was determined at 30°C on sheep blood agar plates (BioMérieux, Marcy l'Etoile, France). The hemolytic halos generated were compared and classified into hemolytic types (HTs). Halos may vary in intensity and shape over time. Thus, to avoid potential misclassification, all 198 strains were plated on blood agar at the same time and compared after 15 h. The hemolytic activity of each strain was estimated twice, and each replicate was classified "blind" with respect to the previous one.

Data analysis. For each locus, the genetic diversity (*h*) among strains or ETs was calculated by the formula $h = 1 - \sum x_i^2 / [n(n-1)]$, where x_i is the frequency of the *i*th allele at the locus, *n* is the number of isolates or single ETs in the sample, and $n/(n-1)$ is a correction for bias in small samples (32). The mean genetic diversity (*H*) was calculated as the arithmetic mean of the *h* values of the overall loci. The clustering pattern of the ETs was determined with a matrix of coefficients of genetic distances by UPGMA implemented with PHYLIP V 3.5c (9). The genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred (mismatches) (32).

We estimated allelic frequencies for each sample by using GENEPOP 3.2a (30). The genetic differentiation (G_{ST}) between populations of *B. thuringiensis* and *B. cereus* was calculated with FSTAT 2.8 (11) and tested by using exact tests for allelic differentiation implemented by GENEPOP 3.2a (30). The unrooted tree was produced by the neighbor-joining method based on Nei's distances (22). Calculations were made, and the tree was drawn with PHYLIP V 3.5c (9) and TREEVIEW (27) software, respectively.

Two-locus linkage disequilibrium analysis was performed with GENEPOP 3.2a (30). Loci with one or more alleles with a frequency greater than 0.9 were ignored (invalid comparisons), because such loci give arithmetic results that artificially approach zero linkage disequilibrium, as if one locus was monomorphic. The "index of association" (I_A) used to test for multilocus linkage disequilibrium (clonality) was calculated as described by Maynard Smith et al. (17). $I_A = (V_O/V_E) - 1$, with V_O the observed variance in the distribution of allelic mismatches in all pairwise comparisons of the allelic profiles and V_E the expected variance in a freely recombining population (linkage equilibrium). The computer program ETLINK (35) was used to calculate V_O , V_E , the 95% confidence limits of V_E , and the standard error (SE) of I_A . For populations at linkage equilibrium, V_O equals V_E , and I_A has an expected value of zero (1, 17). To test whether I_A differed significantly from zero (i.e., whether the ratio V_O/V_E is significantly different from 1), we calculated the maximum variance obtained with 20, 100, or 1,000 randomizations of the data set by using the statistical tools provided at the Multi Locus Sequence Typing web site (<http://mlst.zoo.ox.ac.uk>). Significant link-

TABLE 1. Genetic diversity of *B. cereus* and *B. thuringiensis* at the two sampling sites used in this study

Sample	No. of:		Mean no. of alleles per locus	Genetic diversity (H) \pm SE	
	Isolates	ETs		All isolates	ETs
<i>B. thuringiensis</i> + <i>B. cereus</i>	198	71	3.44	0.350 \pm 0.109	0.373 \pm 0.113
<i>B. thuringiensis</i>	98	27	2.89	0.283 \pm 0.097	0.353 \pm 0.108
Site F	48	13	2.56	0.196 \pm 0.088	0.315 \pm 0.099
BtF1	10	3	1.22	0.042 \pm 0.042	0.111 \pm 0.111
BtF2	9	6	1.89	0.231 \pm 0.077	0.296 \pm 0.088
BtF3	10	4	1.67	0.153 \pm 0.072	0.296 \pm 0.124
BtF4	10	3	1.33	0.067 \pm 0.033	0.222 \pm 0.111
BtF5	9	3	1.22	0.068 \pm 0.047	0.148 \pm 0.098
Site S	50	15	2.33	0.311 \pm 0.100	0.368 \pm 0.117
BtS1	10	4	1.44	0.148 \pm 0.075	0.222 \pm 0.115
BtS2	10	5	2.00	0.291 \pm 0.095	0.411 \pm 0.132
BtS3	10	1	1.00	0	0
BtS4	10	3	1.56	0.163 \pm 0.055	0.370 \pm 0.117
BtS5	10	5	1.56	0.168 \pm 0.080	0.244 \pm 0.104
<i>B. cereus</i>	100	47	3.22	0.310 \pm 0.104	0.358 \pm 0.110
Site F	50	24	2.57	0.267 \pm 0.100	0.342 \pm 0.107
BcF1	10	9	2.11	0.279 \pm 0.113	0.293 \pm 0.117
BcF2	10	7	1.67	0.193 \pm 0.103	0.222 \pm 0.115
BcF3	10	7	2.00	0.316 \pm 0.100	0.354 \pm 0.107
BcF4	10	7	1.78	0.220 \pm 0.096	0.270 \pm 0.112
BcF5	10	5	1.67	0.200 \pm 0.090	0.256 \pm 0.109
Site S	50	30	3.67	0.355 \pm 0.099	0.363 \pm 0.108
BcS1	10	6	1.89	0.284 \pm 0.101	0.333 \pm 0.116
BcS2	10	7	1.89	0.262 \pm 0.091	0.312 \pm 0.109
BcS3	10	9	2.22	0.338 \pm 0.114	0.352 \pm 0.118
BcS4	10	10	2.33	0.378 \pm 0.094	0.378 \pm 0.094
BcS5	10	8	2.22	0.348 \pm 0.115	0.364 \pm 0.119

age disequilibrium was established if the observed variance, obtained with the real data set, was greater than the maximum variance observed with any of the randomized data sets (with P values of 0.05, 0.01, or 0.001 when the observed variance was greater than that found in any of 20, 100, or 1,000 randomized data sets, respectively). If the observed variance was less than that obtained with some of the randomized data sets, then there was no evidence of a deviation from linkage equilibrium.

Differences in HT frequency between *Bacillus* spp. were assessed by means of a two-sided Fisher's exact test on 4×8 contingency tables. Fisher's exact tests were performed with the STRUC program implemented in GENEPOP 3.2a (30).

RESULTS

Overall genetic and hemolytic diversities. (i) Multilocus genotypes. In the collection of 198 strains from two geographical sites in France, seven loci (*Sod*, *Me*, *Cat*, *Aat*, *Gpi*, *Mdh*, and *Adh*) of the nine tested were polymorphic, and the remaining two loci, *Np* and *Dia*, had only one allele. At polymorphic loci, the number of alleles ranged from two for *Gpi* and *Mdh* to six alleles for *Aat* and *Cat*, with a mean of 3.44 alleles per locus (Table 1). In total, 71 different ETs were distinguished (Table 2). The overall mean genetic diversity (H) for these ETs was 0.373 ± 0.113 , with *Aat* (0.743), *Sod* (0.736) and *Cat* (0.733) being the most diverse loci. Genetic relationships between the 71 ETs are shown in the dendrogram in Fig. 1. Most genetic distances between ETs were small. At a genetic distance of 0.50, two clusters of ETs were identified, cluster I and cluster

II, which included 61 and 10 isolates, respectively (Fig. 1). Each cluster contained at least one ET from each site and for each *Bacillus* group.

(ii) Hemolytic activity. Nine different hemolytic types were identified in the 198 strains studied (Fig. 2). HTF was nonhemolytic, whereas HTD and HTG displayed strong hemolytic activities resulting in the complete lysis of sheep erythrocytes. The other HTs (i.e., A, C, E, B, H, and I) had activities intermediate between these two extremes. HTI was the main HT recovered in our sample, with 38% of the isolates displaying this HT (Table 3). The frequencies of the other HTs were between 0.020 (HTA) and 0.121 (HTB).

Genetic diversity within and between populations of each *Bacillus* species. Each soil sample harbored *B. cereus* and *B. thuringiensis* strains of various multilocus genotypes, with the exception of BtS3, from which only one ET was recovered. In *B. thuringiensis*, the genetic diversity (H) per sample varied from 0 (BtS3) to 0.291 (BtS2) and from 0.042 (BtF1) to 0.231 (BtF2) at sites S and F, respectively (Table 1). In *B. cereus*, the genetic diversity per sample was higher, varying from 0.262 (BcS2) to 0.378 (BcS4) and from 0.193 (BcF2) to 0.316 (BcF3) at sites S and F, respectively. At each site, overall genetic diversity was significantly higher in *B. cereus* (0.267 and 0.355 at sites F and S, respectively) than in *B. thuringiensis* (0.196 and 0.311 at sites F and S, respectively). However, if duplicated ETs were eliminated from the analysis, genetic diversities were found to be almost identical in the two *Bacillus* species (over the two sites, $H = 0.353$ and 0.358 for *B. thuringiensis* and *B. cereus*, respectively). In both species, the genetic diversity, calculated by using all isolates or all ETs, was higher at the edge of the forest (site S) than within the forest (site F).

In *Bacillus cereus*, seven ETs (ET9, ET28, ET29, ET31, ET40, ET49, and ET61) were shared between sites S and F. Four of these ETs displayed the same HT at both sites: ET29, ET49, and ET9 were always associated with HTI, and ET61 was associated with HTH at both sites. Conversely, ET28, ET31 and ET40 were associated with different HTs at the two sites. In *B. thuringiensis*, only one ET (ET66) was found at both sampling sites. This ET was associated with different HTs at the two sites. Finally, three ETs were shared by the two *Bacillus* species (ET12, ET13, and ET17), but these ETs were also associated with different HTs.

Genetic differentiation between *B. thuringiensis* and *B. cereus*. We analyzed genetic differentiation between *B. thuringiensis* and *B. cereus* at two levels. We first considered all isolates of *B. thuringiensis* and *B. cereus* found at each site (i.e., 198 strains). Because strains displaying the same ET and HT may be clones, we performed a second analysis in which we retained, for each *Bacillus* species and at each site, only those strains with different ETs/HTs.

(i) All isolates. The genetic differentiation between pairs of soil samples indicated that *B. thuringiensis* populations had differentiated from *B. cereus* populations. This result is illustrated in Fig. 3 with an unrooted tree based on Nei's distance (based on allelic frequencies) in which the 10 soil samples of *B. cereus* are clustered separately from 9 of the 10 soil samples of *B. thuringiensis*. The position of the two clusters is supported by a bootstrap value of 68% (1,000 resamplings). Hence, samples of a given *Bacillus* species (*B. cereus* or *B. thuringiensis*) are

TABLE 2. ETs found in the 198 strains isolated from two sites at La Minière (Ile-de-France region, France)^a

ET	Locus:										ET	Locus:									
	<i>Adh</i>	<i>Mdh</i>	<i>Sod</i>	<i>Gpi</i>	<i>Np</i>	<i>Aat</i>	<i>Me</i>	<i>Cat</i>	<i>Dia</i>	<i>Adh</i>		<i>Mdh</i>	<i>Sod</i>	<i>Gpi</i>	<i>Np</i>	<i>Aat</i>	<i>Me</i>	<i>Cat</i>	<i>Dia</i>		
1	0	2	3	2	1	0	2	0	1	37	3	2	4	2	1	2	1	3	1		
2	0	2	3	2	1	4	2	3	1	38	3	2	4	2	1	2	1	1	1		
3	0	2	4	2	1	0	0	3	1	39	3	2	5	2	1	3	1	3	1		
4	0	2	4	2	1	4	1	3	1	40	3	2	5	2	1	3	1	5	1		
5	3	2	3	2	1	0	1	3	1	41	3	2	5	2	1	4	1	0	1		
6	3	2	3	2	1	0	2	0	1	42	3	2	5	2	1	4	1	5	1		
7	3	2	3	2	1	0	2	3	1	43	3	2	5	2	1	4	2	0	1		
8	3	2	3	2	1	3	1	0	1	44	3	2	1	2	1	3	1	5	1		
9	3	2	3	2	1	3	1	3	1	45	3	2	1	2	1	3	2	3	1		
10	3	2	3	2	1	4	1	3	1	46	3	2	2	2	1	0	0	0	1		
11	3	2	3	2	1	4	1	2	1	47	3	2	2	2	1	0	1	1	1		
12	3	2	3	2	1	4	2	0	1	48	3	2	2	2	1	3	1	3	1		
13	3	2	3	2	1	4	2	3	1	49	3	2	2	2	1	3	1	5	1		
14	3	2	3	2	1	4	2	4	1	50	3	2	2	2	1	4	1	3	1		
15	3	2	3	2	1	4	2	5	1	51	3	2	2	2	1	4	1	1	1		
16	3	2	3	2	1	5	2	0	1	52	3	2	2	2	1	4	2	3	1		
17	3	2	3	2	1	5	2	3	1	53	3	2	2	2	1	5	2	3	1		
18	3	2	3	2	1	1	1	3	1	54	3	1	5	2	1	0	1	3	1		
19	3	2	3	2	1	1	1	5	1	55	4	2	3	2	1	4	2	5	1		
20	3	2	3	1	1	3	2	2	1	56	4	2	3	2	1	5	2	3	1		
21	3	2	3	1	1	5	2	3	1	57	4	2	4	2	1	4	1	5	1		
22	3	2	4	2	1	0	1	3	1	58	4	2	4	2	1	4	2	5	1		
23	3	2	4	2	1	0	1	1	1	59	4	2	4	2	1	5	2	3	1		
24	3	2	4	2	1	0	2	0	1	60	4	2	5	2	1	4	1	4	1		
25	3	2	4	2	1	3	0	5	1	61	4	2	5	2	1	4	1	5	1		
26	3	2	4	2	1	3	0	3	1	62	4	2	1	2	1	3	1	3	1		
27	3	2	4	2	1	3	1	0	1	63	1	2	1	2	1	5	2	5	1		
28	3	2	4	2	1	3	1	3	1	64	2	2	3	2	1	4	1	0	1		
29	3	2	4	2	1	3	1	5	1	65	2	2	3	2	1	4	2	0	1		
30	3	2	4	2	1	4	1	0	1	66	2	2	3	2	1	4	2	3	1		
31	3	2	4	2	1	4	1	3	1	67	2	2	3	2	1	5	2	0	1		
32	3	2	4	2	1	4	1	5	1	68	2	2	3	2	1	5	2	3	1		
33	3	2	4	2	1	4	2	0	1	69	2	2	4	2	1	3	1	5	1		
34	3	2	4	2	1	4	2	5	1	70	2	2	2	2	1	4	1	1	1		
35	3	2	4	2	1	4	2	1	1	71	2	2	2	1	1	5	2	3	1		
36	3	2	4	2	1	5	2	0	1												

^a The designations of the ETs are the same as those in subsequent dendrograms and Figures.

genetically more similar to each other than to samples of the other species, even if isolated from the same soil sample.

For each *Bacillus* species, strains from the same site can be pooled, resulting in two populations per species: BcS (BcS1 to BcS5) and BcF (BcF1 to BcF5) for *B. cereus* and BtS (BtS1 to BtS5) and BtF (BtF1 to BtF5) for *B. thuringiensis*. All populations differed significantly from each other (Table 4). However, pairs of populations from the same species displayed a much lower level of differentiation, as measured by the G_{st} (BcS versus BcF, $G_{st} = 0.040, P < 10^{-5}$; BtS versus BtF, $G_{st} = 0.099, P < 10^{-5}$) than did interspecies comparisons (G_{st} ranged from 0.130 to 0.229, with $P < 10^{-5}$ for all comparisons [see details in Table 4]).

(ii) **ETs/HTs.** Clones may artificially increase the genetic differentiation between populations. For each *Bacillus* species and at each site, we recovered strains with identical ETs and HTs, which may be considered to be putative clones. We re-analyzed the data set, retaining, for each *Bacillus* species and for each site, only one strain for each combination of ET and HT. BcS, BcF, BtS, and BtF contained 30, 29, 17, and 18 different ET/HT strains, respectively. The allelic frequencies at each locus are given in Table 5. As expected, the level of genetic differentiation was significantly lower than that calcu-

lated for the whole data set. The level of genetic differentiation between populations of the same *Bacillus* species did not differ significantly from zero (BcS versus BcF, $G_{st} = 0.011, P = 0.130$; BtS versus BtF, $G_{st} = 0.030, P = 0.063$). Conversely, all comparisons between *B. cereus* and *B. thuringiensis* strains showed highly significant differentiation (G_{st} ranged from 0.040 to 0.137, with $P < 10^{-3}$ for all comparisons [see details in Table 4]). Therefore, populations of *B. cereus* and *B. thuringiensis* collected at the same site differed from each other to a greater extent than from their corresponding populations collected ~2 km away.

HT diversity within and between *Bacillus* species. For each *Bacillus* species and at each site, relative HT frequencies did not differ according to whether the analysis was performed with all isolates or with only unique ETs/HTs (two-tailed Fisher exact tests: $P > 0.20$ for all comparisons). At each site and between the two sites, the distribution of HT frequencies differed significantly for the two *Bacillus* species (two-tailed Fisher's exact tests, $P < 0.001$ for all comparisons considering either all isolates or unique ETs/HTs only). One reason for this is that three of the nine HTs recovered (HTA, HTB, and HTC) were restricted to *B. thuringiensis* (Table 3). In addition, even those HTs identified in both species displayed very different

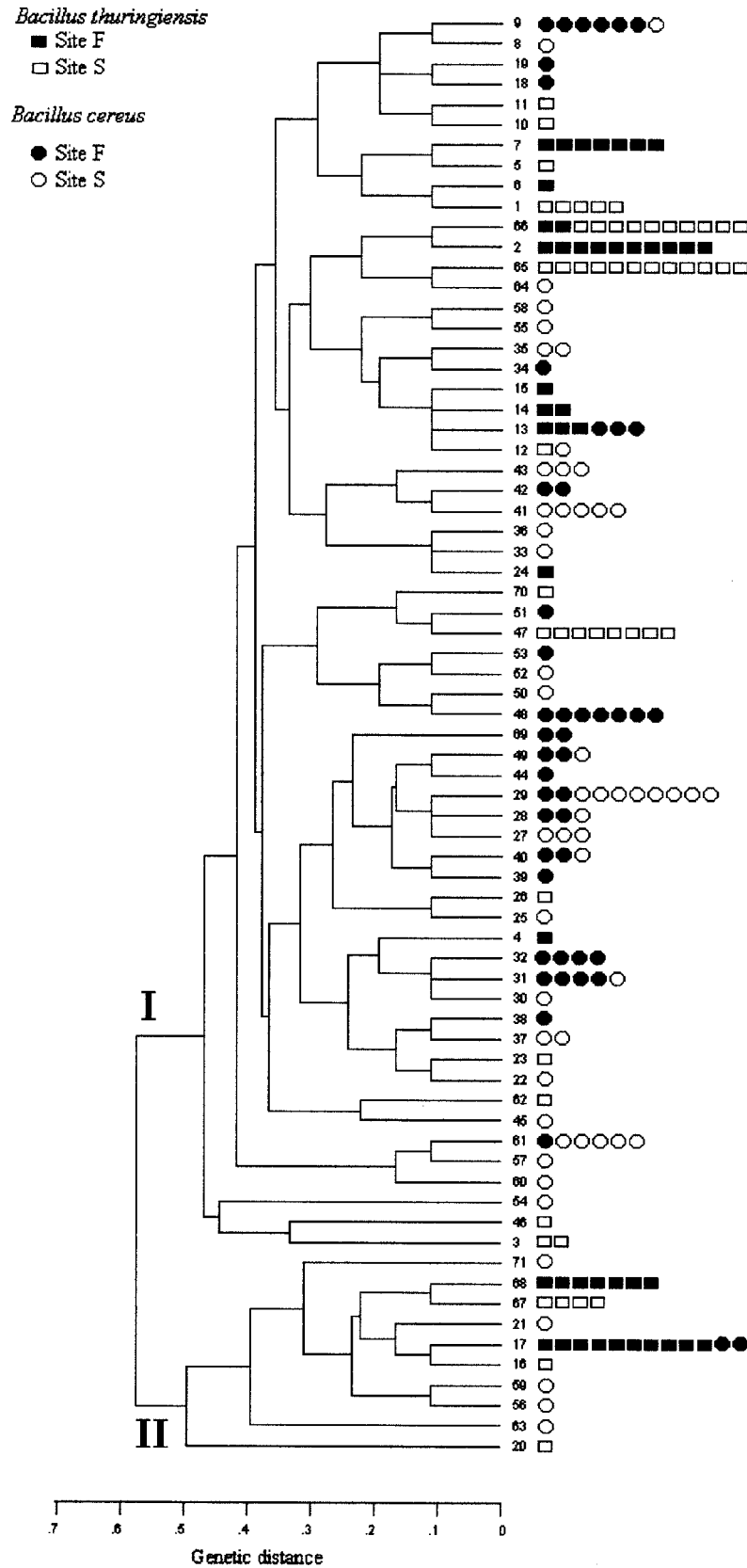


FIG. 1. Genetic relationships between the 71 ETs of the *B. cereus* and *B. thuringiensis* strains isolated. Clustering of the ETs was determined with a matrix of coefficients of genetic distances by the UPGMA method by using PHYLIP V 3.5c (9). ETs are numbered as described in Table 2. Symbols indicate the isolates from the two *Bacillus* taxa at the two sampling sites: ●, populations BcF1 to BcF5; ○, populations BcS1 to BcS5; ■, populations BtF1 to BtF5; □, populations BtS1 to BtS5.

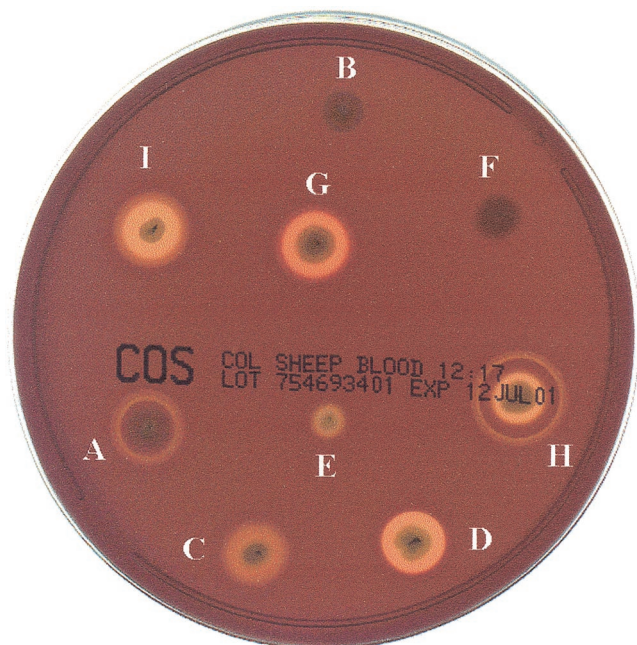


FIG. 2. HTs found in 198 strains of *B. cereus* and *B. thuringiensis*.

frequencies in the two species. For example, when considering all isolates, HTI had frequencies of 0.720 and 0.031 among *B. cereus* and *B. thuringiensis* strains, respectively.

In *B. cereus*, relative HT frequencies did not differ significantly between the two sites (two-tailed Fisher's exact tests: $P = 0.551$ and 0.685 when considering all isolates and unique ETs/HTs, respectively). In contrast, *B. thuringiensis* strains collected from sites F and S had significantly different HT relative frequencies (two-tailed Fisher's exact tests: $P < 0.0001$ when considering either all isolates or unique ETs/HTs). The two

sites shared only four HTs (HTB, HTC, HTD, and HTI), three (HTA, HTE, and HTG) of which were restricted to site S and two (HTF and HTH) of which were restricted to site F.

Linkage disequilibrium analysis. Analyses of linkage disequilibrium were also carried out at two levels: all isolates and unique ETs/HTs only. Two-locus analysis of all pairwise comparisons and the approach using an I_A index (17) differ in sensitivity to linkage disequilibrium. Because I_A is a summary of the data on several loci, the calculation of this index leads to a considerable loss of information. As pointed out by Duncan et al. (8), multilocus analysis provides a stringent criterion for the approach of a population to panmixia, but cannot completely exclude the possibility of recombination. Hence, two-locus analysis provides a more detailed assessment and identifies the loci statistically in and out of equilibrium.

When considering all isolates of the two *Bacillus* species, we observed highly significant ($P < 10^{-5}$) linkage disequilibrium for all valid two-locus comparisons and significant multilocus disequilibrium, as measured by the index of association ($I_A = 0.437 \pm 0.092$, $P < 0.001$) (Table 6). All valid two-locus comparisons were also significant if we considered all *B. thuringiensis* strains, with a significant ($P < 0.001$) value of $I_A = 0.771 \pm 0.131$. Similarly the $I_A = 0.286 \pm 0.131$ calculated for the 100 strains of *B. cereus* was significant ($P < 0.05$), and only 2 of the 10 associations tested were not significant.

The extent of linkage disequilibrium was very different if the sites were analyzed separately. The indices of association calculated for BcF ($I_A = 0.265 \pm 0.180$), BcS ($I_A = 0.351 \pm 0.180$), and BtF ($I_A = 0.157 \pm 0.188$) were not significantly different from zero (Table 6). Conversely, strains of *B. thuringiensis* isolated at the edge of the forest (i.e., population BtS) showed a high degree of clonality: all pairs of loci were in strong linkage disequilibrium ($P < 10^{-5}$), and an I_A value of 1.610 ± 0.180 ($P < 0.001$) was obtained.

If we considered unique ETs/HTs only, the index of association calculated for the pool of *B. cereus* and *B. thuringiensis*

TABLE 3. Relative frequency of HTs in natural populations of *B. thuringiensis* and *B. cereus*

Isolate	No. of isolates	Relative frequency of HT:								
		A	B	C	D	E	F	G	H	I
All isolates										
<i>B. thuringiensis</i> + <i>B. cereus</i>	198	0.020	0.121	0.050	0.101	0.040	0.121	0.050	0.116	0.379
<i>B. thuringiensis</i>	98	0.041	0.245	0.102	0.194	0.061	0.235	0.010	0.082	0.031
Site F	48	0	0.271	0.021	0.021	0	0.479	0	0.167	0.042
Site S	50	0.080	0.220	0.180	0.360	0.120	0	0.020	0	0.020
<i>B. cereus</i>	100	0	0	0	0.010	0.020	0.010	0.090	0.150	0.720
Site F	50	0	0	0	0.020	0.040	0	0.100	0.120	0.720
Site S	50	0	0	0	0	0	0.020	0.080	0.180	0.720
Unique ETs/HTs										
<i>B. thuringiensis</i> + <i>B. cereus</i>	91	0.022	0.077	0.022	0.055	0.077	0.088	0.077	0.132	0.451
<i>B. thuringiensis</i>	35	0.057	0.200	0.057	0.114	0.143	0.200	0.029	0.114	0.086
Site F	18	0	0.167	0.056	0.056	0	0.389	0	0.222	0.111
Site S	17	0.118	0.235	0.059	0.176	0.294	0	0.059	0	0.059
<i>B. cereus</i>	56	0	0	0	0.018	0.036	0.018	0.107	0.143	0.679
Site F	29	0	0	0	0.035	0.069	0	0.103	0.138	0.656
Site S	30	0	0	0	0	0	0.033	0.100	0.133	0.733

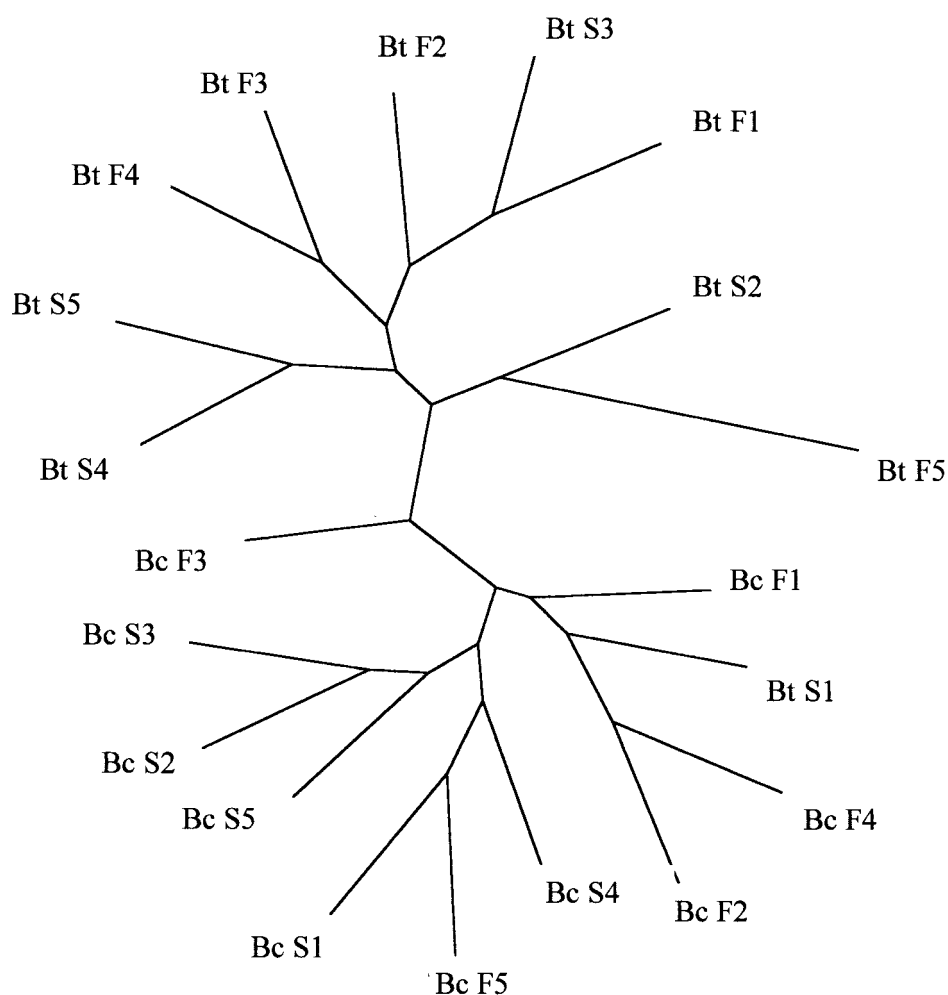


FIG. 3. Unrooted dendrogram inferred from Nei's genetic distance (neighbor-joining method). Genetic distances were calculated and the tree was drawn by using PHYLIP V 3.5c (9) and TREEVIEW software (27), respectively.

($I_A = 0.257 \pm 0.137$) was not significant (Table 6). However, analysis of associations for each pair of loci detected four significant (*Adh* and *Sod*, *Sod* and *Me*, *Aat* and *Me*, and *Sod* and *Cat*) and three marginally significant (*Sod* and *Aat*, *Aat* and *Cat*, and *Me* and *Cat*) nonrandom associations among the 10 tested. This was mainly due to several nonrandom associations (*Adh* and *Sod*, *Adh* and *Aat*, *Sod* and *Aat*, *Sod* and *Me*, and *Aat*

and *Me*) within *B. thuringiensis*, in which $I_A = 0.507 \pm 0.217$ and was significant (Table 6). Only one (*Aat* and *Me*) pair of loci displayed significant ($P < 10^{-5}$) linkage disequilibrium in *B. cereus*, resulting in a nonsignificant I_A of 0.195 ± 0.170 .

If we performed linkage analysis with unique ETs/HTs only for each site separately, BcF, BcS, and BtF displayed nonsignificant I_A s (Table 6). The index of association calculated for the unique ETs/HTs collected in BtS was lower than that for all strains— 0.785 ± 0.311 —but remained significant ($P < 0.05$).

Distribution of clone families. Duplicated ETs/HTs may be treated as clones. Among the 198 strains, 29 ETs/HTs were found in duplicate, but none was shared by *B. thuringiensis* and *B. cereus*, even in populations collected in the same soil sample. Thus, 14 and 15 different ETs/HTs were found more than once in *B. thuringiensis* and *B. cereus*, respectively. The two species did not differ in the mean number of copies per clone, with 2 to 10 copies in *B. thuringiensis* and 2 to 9 in *B. cereus*. Each ET/HT was repeated 5.50 times in *B. thuringiensis* and 3.93 times in *B. cereus*, and this difference was not significant (Wilcoxon test, $P = 0.173$).

The geographical distributions of clones were significantly

TABLE 4. Genetic differentiation between populations of the two *Bacillus* taxa

Comparison	All isolates		Unique ETs/HTs	
	G_{st}	P value	G_{st}	P value
Within species				
BtS vs BtF	0.099	$<10^{-5}$	0.030	0.063
BcS vs BcF	0.040	$<10^{-5}$	0.011	0.130
Between species				
BtF vs BcF	0.229	$<10^{-5}$	0.137	$<10^{-5}$
BtF vs BcS	0.219	$<10^{-5}$	0.091	$<10^{-5}$
BtS vs BcF	0.177	$<10^{-5}$	0.085	$<10^{-5}$
BtS vs BcS	0.130	$<10^{-5}$	0.040	$<10^{-3}$

TABLE 5. Relative allele frequencies at each allozyme locus, at each site, for the two *Bacillus* taxa^a

Locus	Relative allele frequency at locus:			
	<i>B. cereus</i>		<i>B. thuringiensis</i>	
	Site S (n = 30)	Site F (n = 29)	Site S (n = 17)	Site F (n = 18)
<i>Adh</i>				
0	0	0	0.118	0.111
1	0.033	0	0	0
2	0.033	0.069	0.353	0.278
3	0.700	0.897	0.529	0.556
4	0.233	0.034	0	0.056
<i>Mdh</i>				
1	0.033	0	0	0
2	0.967	1	1	1
<i>Sod</i>				
1	0.033	0.069	0	0.056
2	0.100	0.172	0.176	0
3	0.233	0.207	0.647	0.833
4	0.433	0.379	0.176	0.111
5	0.200	0.172	0	0
<i>Gpi</i>				
1	0.033	0.034	0	0.056
2	0.967	0.966	1	0.944
<i>Np</i>				
1	1	1	1	1
<i>Aat</i>				
0	0.033	0.034	0.353	0.167
1	0	0.069	0	0
2	0.033	0.034	0	0
3	0.267	0.379	0.059	0.111
4	0.500	0.345	0.471	0.444
5	0.167	0.138	0.118	0.278
<i>Me</i>				
0	0.033	0	0.176	0
1	0.567	0.759	0.353	0.111
2	0.400	0.241	0.471	0.889
<i>Cat</i>				
0	0.300	0	0.412	0.111
1	0.033	0.069	0.176	0
2	0	0	0.059	0.056
3	0.333	0.483	0.353	0.722
4	0.033	0	0	0.056
5	0.300	0.448	0	0.056
<i>Dia</i>				
1	1	1	1	1

^a For each site, each ET or HT was represented only once. (Duplicated ETs or HTs were eliminated from the analysis)

different in the two *Bacillus* groups (Fig. 4). Clones were more scattered in *B. thuringiensis* than in *B. cereus*. The dispersion of each clone can be evaluated by dividing the number of copies of each clone by the number of sites at which that clone was present. The mean dispersions of the various clones were 1.91 for *B. thuringiensis* and 4.00 for *B. cereus*; this difference was significant (Wilcoxon test, $P = 0.0021$). Finally, four *B. cereus* ETs/HTs (ET9/HTI, ET29/HTI, ET49/HTI, and ET61/HTH) were ob-

served at both sites, whereas all *B. thuringiensis* clones were restricted to one site only (Fig. 4).

DISCUSSION

The presence of sympatric populations of *B. thuringiensis* and *B. cereus* at our study sites appeared to be common: (i) both *Bacillus* species were isolated from both sites, from all samples from less than 1 g of soil, and (ii) the relative abundances of the two species (*B. thuringiensis*/*B. cereus* ratio, ~1:10) were similar in the various samples and at both sites. Despite their coexistence, we observed genetic differentiation between these two species. Populations of a given *Bacillus* species—*B. thuringiensis* or *B. cereus*—collected at two separate sites were genetically more similar to each other than to sympatric populations of the other *Bacillus* species. Indeed, allozyme data showed that there was less differentiation within species than between species. Further evidence for the genetic divergence of populations of *B. cereus* and *B. thuringiensis* was provided by analysis of hemolytic activity. Three of the nine HTs that were recovered among the 198 strains were restricted to *B. thuringiensis*. Moreover, the six HTs present in both *Bacillus* species greatly differed in frequency between the two species. Thus, electrophoretic and hemolytic data provide evidence for a high level of genetic exchange within each of these *Bacillus* taxa, but a lower level of exchange between them. This may be due to unknown genetic differences that restrict, but do not totally eliminate, gene flow between *B. thuringiensis* and *B. cereus*. Thus, although the horizontal transfer of plasmids between *B. thuringiensis* and *B. cereus* has been demonstrated under laboratory conditions (10), our results, although limited to two populations, provide no evidence that such transfer occurs randomly in sympatric natural isolates of *B. cereus* and *B. thuringiensis*. In a remarkable hierarchical population genetics analysis, Duncan et al. (8) found that populations of *Bacillus subtilis* were genetically differentiated from sympatric populations of *B. licheniformis*, despite the known potential for genetic exchange between these species demonstrated in laboratory experiments.

These two *Bacillus* species also displayed different genetic structures. Indeed, there are several indications that *B. cereus* populations are less structured than *B. thuringiensis* populations. First, the two *B. cereus* populations displayed lower levels of genetic differentiation than did the two *B. thuringiensis* populations. Second, the distribution of HT frequencies did not differ between the two sites for *B. cereus* strains, whereas *B. thuringiensis* populations from the two sites displayed completely different HT distributions, with more than half of the HTs restricted to one of the two sites only. Third, four different ETs/HTs—putative clones—were shared by *B. cereus* strains isolated from the two sampling sites, whereas the two populations of *B. thuringiensis* had no ET/HT in common. Finally, duplicated *B. cereus* ETs/HTs were more widely dispersed than duplicated *B. thuringiensis* ETs/HTs. All of these results suggest a higher rate of dispersal, and hence of gene flow, for *B. cereus* than for *B. thuringiensis*.

Our data indicate that sexuality may be frequent enough in natural *B. cereus* populations to break down considerably the clonal population structure resulting from bacterial cell multiplication. A high level of recombination is suggested by the low

TABLE 6. Statistical analysis of multilocus linkage disequilibrium for the two *Bacillus* taxa

Isolate	No. of isolates	V_E	95% confidence limits of V_E^a	V_O	P value ^b	$I_A \pm SE$
All isolates						
<i>B. thuringiensis</i> + <i>B. cereus</i>	198	1.154	0.942–1.365	1.658	<0.001	0.437 ± 0.092
<i>B. thuringiensis</i>	98	1.132	0.835–1.428	2.004	<0.001	0.771 ± 0.131
Site F	48	0.715	0.446–0.984	0.827	NS	0.157 ± 0.188
Site S	50	1.211	0.774–1.647	3.160	<0.001	1.610 ± 0.180
<i>B. cereus</i>	100	1.096	0.809–1.382	1.408	<0.05	0.286 ± 0.131
Site F	50	0.885	0.567–1.203	1.119	NS	0.265 ± 0.180
Site S	50	1.162	0.739–1.585	1.570	NS	0.351 ± 0.182
Unique ETs/HTs						
<i>B. thuringiensis</i> + <i>B. cereus</i>	91	1.145	0.831–1.459	1.439	NS	0.257 ± 0.137
<i>B. thuringiensis</i>	35	1.184	0.671–1.697	1.785	<0.01	0.507 ± 0.217
Site F	18	1.005	0.387–1.624	1.534	NS	0.526 ± 0.308
Site S	17	1.180	0.446–1.914	2.106	<0.05	0.785 ± 0.311
<i>B. cereus</i>	56	0.891	0.589–1.194	1.065	NS	0.195 ± 0.170
Site F	29	0.772	0.392–1.153	0.960	NS	0.243 ± 0.246
Site S	30	0.933	0.597–1.359	1.113	NS	0.194 ± 0.228

^a Calculated as described by Brown et al. (1).

^b Probability of observing a V_O/V_E ratio as extreme as or more extreme than the original data, based on randomization. NS, nonsignificant ($P > 0.05$).

indices of associations and by very few loci being in linkage disequilibrium (17). Thus, conjugation and/or transduction events may result in the transfer of chromosomal genes between strains in this *Bacillus* species. Although natural competence for DNA transformation has never been demonstrated in *B. cereus* under laboratory conditions, the nearly completed *B. anthracis* genome sequence has revealed homologues of *B. subtilis* competence genes so that we may yet learn that genetic competence actually occurs within the *B. cereus* group. These genetic exchanges may result in bacterial populations behaving more like a panmictic unit than as arrays of clonal lineages. Such a genetic structure may be common in the *Bacillus* genus: at a fine-scale population level, two locus and multilocus statistical analyses have demonstrated that *B. subtilis* and *B. licheniformis* behave as truly panmictic units (8, 15). In *B. thuringiensis*, the balance between clonal proliferation and recombination seems to depend on location. In the forest, *B. thuringiensis* strains appeared to have a low level of clonality. Conversely, *B. thuringiensis* strains collected at the edge of the forest displayed a clonal genetic structure. This did not seem to be due to the presence of highly duplicated clones, which may be the case in “epidemic” bacterial populations (17), because the index of association remained significantly different from zero even if duplicated ETs/HTs were eliminated from the analysis. The ecological circumstances and evolutionary consequences of this mixed mode of propagation remain to be explored.

Although we found a higher level of recombination within each *Bacillus* species than between *B. cereus* and *B. thuringiensis*, the two species are probably not completely isolated. Instead, the high level of genetic similarity may result from genetic recombination between strains of the two taxa. Alternatively, one may argue that the low genetic differentiation between populations of *B. cereus* and *B. thuringiensis* is not the

consequence of gene flow between these two species. Instead, the two *Bacillus* species may be completely separated, but these two gene pools have not had time to diverge because the divergence is too recent.

Based on the assumption that populations are at equilibrium migration, recurrent recombination events would account for the observation that none of the trees based on allozymes (2, 12, 13, 14) or on any other genetic markers (4) have been able to separate *B. cereus* and *B. thuringiensis* into distinct exclusive clusters. The results reported here reinforce this conclusion: the two clusters found in our neighbor-joining tree (Fig. 1) include both *B. thuringiensis* and *B. cereus* strains from the two sites. This pattern can be probably extended to other members of the *B. cereus* group, such as *B. anthracis*, *Bacillus mycoides*, *Bacillus pseudomycolides*, and *Bacillus weihenstephensis*. Horizontal gene transfer between strains of these different “species” makes it impossible to delineate discrete clusters. This is illustrated by the recent work of Daffonchio et al. (4): their tree, based on 16S-23S internal transcribed spacers (ITS), failed to ascribe the strains of each *B. cereus* species to a separate cluster. Most of the strains of the same species fell into the same cluster, but this only indicates a higher level of recombination within species than between species.

At first glimpse, the widespread occurrence of genetic exchanges between *Bacillus* strains may be useful for the classification of strains into species, with genes freely exchanged within a species, but with different species genetically isolated from one another. Unfortunately, the absence of genetic barriers that completely prevent gene transfer between strains of different species rules out the possibility of such a classification. The remark made by Maynard Smith et al. (18) for the classification of *Neisseria* is particularly relevant to this situation: a study of the genetic and phenotypic variation in a taxon such as *B. cereus* should be “compulsory for all philosphers

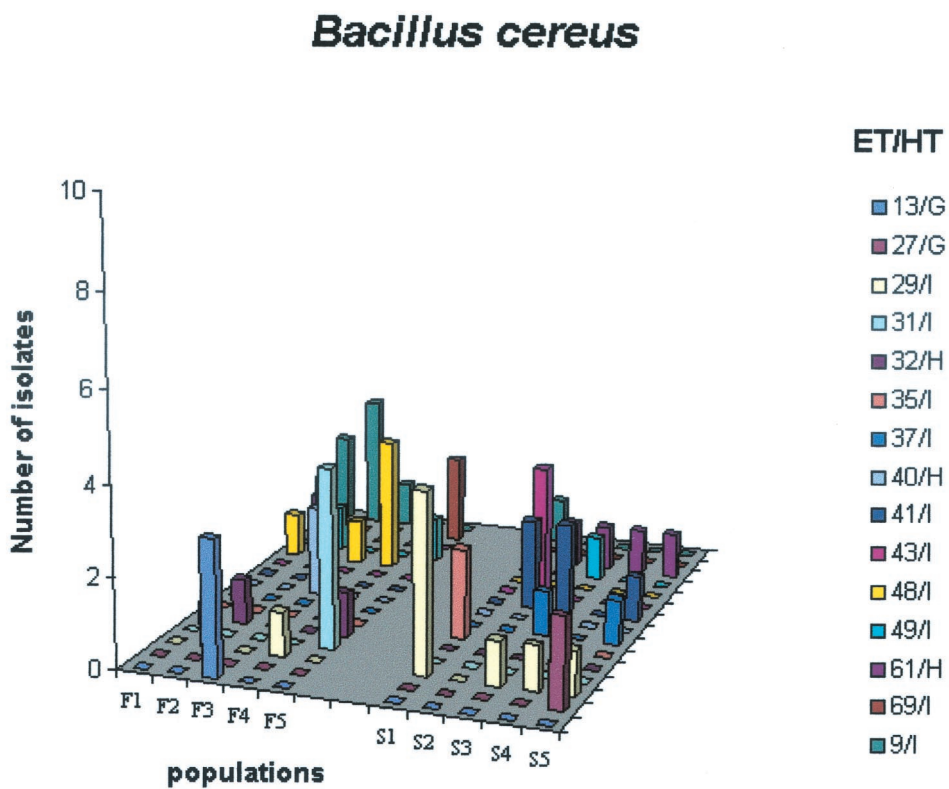
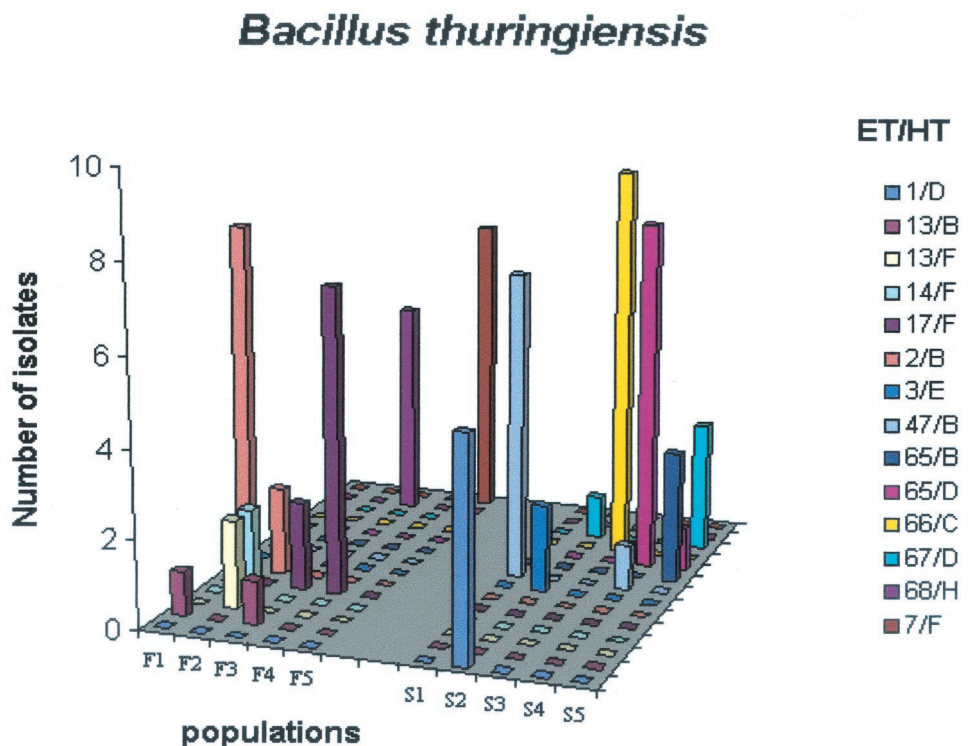


FIG. 4. Distribution of clonal families (strains displaying the same ETs/HTs) in the soil sampled at the two sampling sites for each *Bacillus* taxon.

who believe in the existence of natural kinds, for all philosophers who believe in the universal validity of phylogenetic classification, and for all pheneticists, whatever they believe.” A pragmatic approach leads us to view the *B. cereus* group as an intermingled genetic cluster partially characteristic, but also sharing some common identity with other clusters as a consequence of horizontal gene transfer. Thus, instead of falling into groups that either interbreed freely, or not at all, the frequency of recombination seems to decrease continuously with genetic distance. This is why we partly share the view of Maynard-Smith et al. (18) that there is no clear discontinuity that can be used in species delineation for bacteria.

In conclusion, the discussion concerning the classification of *B. cereus* and *B. thuringiensis* as a single species or as two species is a philosophical one. The outcome of such a discussion may have economic and political implications but is not useful in risk assessment. Only empirical studies under realistic natural conditions will bring us closer to assessing the extent of genetic exchange between members of these puzzling bacterial lineages.

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