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Distribution, Frequency, and Diversity of *Bacillus thuringiensis* in an Animal Feed Mill

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Bacillus thuringiensis was isolated from 36 of 50 residue samples obtained from an animal feed mill (a stored-product environment). Of 710 selected colonies having _Bacillus cereus-B. thuringiensis_ morphology isolated from the samples, 477 were classified as _B. thuringiensis_ because of production of parasporal δ-endotoxin crystals. There was a diverse population of _B. thuringiensis_, as revealed by differentiation of the isolates into 36 subgroups by using (i) their spectra of toxicity to the lepidopterans _Heliothis virescens_, _Pieris brassicae_, and _Spodoptera littoralis_ and the dipteran _Aedes aegypti_ and (ii) their parasporal crystal morphology. A total of 55% of the isolates were not toxic to any of these insects at the concentrations used in the bioassays; 40% of all isolates were toxic to one or more of the Lepidoptera; and 20, 1, and 1% of the isolates were toxic to only _P. brassicae_, _H. virescens_, and _S. littoralis_, respectively. The most frequent toxicity was toxicity to _P. brassicae_ (36% of all isolates); 18% of the isolates were toxic to _A. aegypti_ (5% exclusively), 10% were toxic to _H. virescens_, and 4% were toxic to _S. littoralis_. Toxicity to _P. brassicae_ was more often linked with toxicity to _H. virescens_ than with toxicity to _S. littoralis_. The frequency of toxicity was significantly greater in isolates that produced bipyramidal crystals than in isolates that produced irregular pointed, irregular spherical, rectangular, or spherical crystals.

Bacillus thuringiensis (Berliner) is widely distributed in the environment and can be isolated from soil (9, 25, 29–31), insects (3, 12, 14), sericulture (silk farm) environments (3, 29), and leaves of certain deciduous and coniferous trees (35). *B. thuringiensis* is a spore-forming, gram-positive bacterium that is indistinguishable from the more common (9, 29) soil bacterium *Bacillus cereus* by most phenotypic characteristics (1, 34); it can be distinguished by production of one or more proteinaceous parasporal crystals (δ-endotoxin) during sporulation. In certain strains, the δ-endotoxin proteins are toxic to members of specific insect genera, and this has led to commercial development and use of some strains as microbial insecticides.

B. thuringiensis is also frequently isolated from stored products (3, 4, 7, 11, 27). DeLucca et al. (8) described a new serotype, *B. thuringiensis* subsp. *colmeri*, that was isolated from grain dusts, and Donovan et al. (11) isolated a novel strain of *B. thuringiensis* that is toxic to coleopteran larvae from grain dust. Norris (27) traced the transport of one *B. thuringiensis* variety for great distances across world trade routes in stored products. Serological (2) and biochemical techniques have been used to differentiate newly isolated *B. thuringiensis* strains obtained from a variety of sources (28, 36); however, these techniques do not relate directly to the δ-endotoxins. Dulmage et al. (14) studied the toxicity of approximately 320 strains of *B. thuringiensis* in 23 species of insects. The strains came from many different locations and were mainly isolated from insects, stored-product residues, and sericulture environments. In this study Dulmage et al. used the specific spectrum of toxicity of each isolate to classify the strains according to their insecticidal activities. They found that there was a fairly good but limited correlation between insecticidal activity and variety, as defined by serotyping. In some cases, different isolates of the same variety produced different δ-endotoxins, revealing a diversity in the strains that was not revealed by serotyping alone. Krieg and Langenbruch (23) and Ellar et al. (15) have suggested that the toxicity of *B. thuringiensis* to different insect orders could be used for grouping strains into pathotypes.

B. thuringiensis has been applied to the environment in increasing quantities as a microbial insecticide with no reports of adverse effects. However, relatively little is known about the ecology of *B. thuringiensis*, in particular the reason for its widespread distribution. In this study we assessed the distribution, frequency, and diversity of *B. thuringiensis* in one particular environment, a mill that manufactures animal feedstuff from grain that originates in the United Kingdom and elsewhere. Such a mill is a special, relatively closed environment into which material is regularly introduced. The mill chosen for this study has never been treated with commercial formulations of *B. thuringiensis* and, as *B. thuringiensis* is rarely applied as an insecticide on cereal crops, it is unlikely that commercial strains of *B. thuringiensis* would predominate in the mill. *B. thuringiensis* isolates were selected from samples of stored-product residues and were differentiated primarily on the basis of toxicity to four insect species and also on the basis of crystal morphology.

MATERIALS AND METHODS

Sample collection. Samples of residue were collected from an animal feed mill in East Sussex, United Kingdom. The area of the site is approximately 10,000 m^2, much of which is used for storage of raw materials and finished products; the site occupies three stories, and the mill processes approximately 200 tons (ca. 204,000 kg) of animal feedstuff per month. The materials stored in the site include wheat, barley, maize gluten, and wheatfeed that originate in the United Kingdom and elsewhere. Samples (approximately 1

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g) of residue materials were collected from 50 separate locations within the mill by scooping the materials directly into sterile, disposable test tubes. The samples collected reflected the variety of residue materials present within the site (e.g., settled grain dust, parts of insect cadavers, insect webbing, whole grains, and mammal and bird feces).

Isolation of *B. thuringiensis*. Approximately 0.5 g of each sample was suspended in 10 ml of sterile distilled water, and the preparations were mixed vigorously by vortexing for 1 min. After mixing, the solid matter was allowed to settle out for 2 min, and then 1 ml of the supernatant was pasteurized at 80°C for 3 min in prewarmed 20-ml glass universal bottles to kill most non-spore-forming organisms. Insect cadavers were first homogenized in 1 ml of sterile distilled water in a sterile glass tissue grinder (Quickfit), and this was followed by pasteurization. Samples were plated at two concentrations (undiluted and 10^{-1} dilution) onto nutrient agar (Oxoid) containing extra Technical No. 3 agar (Oxoid) so that the final agar concentration was 2%. The plates were incubated at 30°C for 48 h and examined for colonies having *B. cereus-B. thuringiensis* morphology. The numbers of such colonies that grew from any one sample varied from zero to many hundreds, and it was not considered feasible to examine every *B. cereus-B. thuringiensis* colony obtained from every sample. Therefore, routinely, a maximum of 30 *B. cereus-B. thuringiensis* colonies from any one sample were selected and subcultured on sporulation agar, which contained (per liter) 3.0 g of tryptone, 2.0 g of tryptose, 5.0 g of NaCl, 1.5 g of yeast extract, 7.8 g of Na_2HPO_4 (pH 7.6), and 1.0 g of $MnCl \cdot 4H_2O$ (36). Following incubation for 48 h at 30°C, the isolates were examined for the presence of parasporal crystals by phase-contrast microscopy. Isolates which had typical *B. cereus* cell morphology and contained parasporal crystals were classified as *B. thuringiensis*; isolates that did not produce parasporal crystals were classified as *B. cereus*. A random selection of isolates classified as *B. thuringiensis* were treated with Sudan Black (10), a stain that is specific for poly-β-hydroxybutyrate granules (a common storage polymer that is not specific to *B. thuringiensis*), in order to ensure that isolates which produced poly-β-hydroxybutyrate crystals but not δ-endotoxin crystals were unlikely to have been classified as *B. thuringiensis*. If necessary, following the first subculture, isolates were purified by further subculturing on nutrient agar. To minimize cross-contamination of *B. thuringiensis* from different samples, careful aseptic techniques were used throughout. In addition, the laboratory in which the isolations were performed is located in a separate building that is some distance from other laboratories in which *B. thuringiensis* is routinely handled.

Growth of isolates for bioassays. Isolates were cultured at 30°C in Proflo B4 broth (13) (10.0 g of Proflo cottonseed per liter, 2.0 g of peptone [Difco] per liter, 15.0 g of glucose per liter, 2.0 g of yeast extract [Difco] per liter, 1.0 g of $CaCO_3$ per liter, 0.3 g of $MgSO_4 \cdot 7H_2O$ per liter, 0.02 g of $FeSO_4 \cdot 7H_2O$ per liter, 0.02 g of $ZnSO_4 \cdot 7H_2O$ per liter, 0.02 g of $MnSO_4 \cdot 7H_2O$ per liter) in 250-ml fluted Erlenmeyer flasks shaken at 250 rpm. During growth, the cultures were periodically examined by phase-contrast microscopy, and the crystal morphology of each culture was determined. Cultures were harvested either when at least 95% of the population had lysed, releasing spores and crystals, or, in rare cases, when maximum lysis (less than 95%) had been reached, as determined by at least three consecutive microscopic examinations. Cultures were then stored in a 15% (vol/vol) glycerol (AnalaR) solution at −70°C.

Crystal morphology. During growth in Proflo B4 broth and prior to cell lysis, the isolates were examined by phase-contrast microscopy and, on the basis of parasporal crystal morphology, were placed in five classes (Table 1).

Bioassay. The toxicity of *B. thuringiensis* isolates for members of the Lepidoptera was determined by using neonatal larvae of *Heliothis virescens*, *Pieris brassicae*, and *Spodoptera littoralis* that were given a treated artificial diet. The toxicity for members of the Diptera was determined by using third-instar larvae of *Aedes aegypti* in a water assay. Because of the large number of isolates involved, toxicity was determined in assays in which *B. thuringiensis* broth cultures were added at a single concentration.

The three members of the Lepidoptera were chosen because they are good indicator species for a number of the lepidopteran-active genes of *B. thuringiensis*. *Pieris brassicae* is susceptible to toxins that are expressed by a wide range of *B. thuringiensis* toxin genes, whereas *Spodoptera littoralis* is susceptible to only a few toxins, which are different than the toxins that are toxic to *Pieris brassicae*. *Heliothis virescens* differs from the other two insect species in its susceptibility to *B. thuringiensis* toxins (19). In addition, there is a good relationship between the susceptibility of these insects and a number of lepidopterans that infest stored products (14).

For *Pieris brassicae*, broth was mixed thoroughly into a molten-agar-based artificial diet (6) (which included 100 μg of cefataxime [Roussel Laboratories, Ltd.] per g, 200 μg of streptomycin [Sigma Chemical Co., Ltd.] per g, and 100 μg of tetracycline [Sigma] per g to inhibit bacterial contamination) at a rate of 0.1 $\mu l\ g^{-1}$. The diet was then poured into petri dishes (diameter, 9.5 cm) and allowed to set and surface dry, after which 20 neonatal larvae were added. Replicate plates were prepared for each bioassay.

For *Heliothis virescens* and *Spodoptera littoralis*, broth was incorporated at rates of 0.5 and 1.0 $\mu l\ g^{-1}$, respectively, in a different agar-based artificial diet (18). Because of the cannibalistic nature of *Heliothis* spp., 25 neonatal larvae were separated individually into 2-ml polystyrene autoanalyzer cups containing approximately 0.5 ml of diet. The bioassay preparations were incubated for 5 days (*Pieris brassicae*) or 7 days (*Spodoptera littoralis* and *Heliothis virescens*) at 25°C, and the numbers of live larvae were counted.

For the members of the Lepidoptera, groups of 30 isolates were tested in each assay together with a standard strain, strain GC91 (5), and a control containing no bacteria. The standard strain was chosen because it had a high level of toxicity for all of the Lepidoptera species tested; it provided a means for compensating for variations in the susceptibility of the insects in each bioassay group. For each assay (Lepidoptera and Diptera) the concentration of the broth preparation added was equivalent to the concentration of the standard strain that produced 50% mortality in the insect (i.e., the 50% lethal concentration of the standard strain).

For *Aedes aegypti*, whole broth preparations were suspended in distilled water (10 $\mu l\ ml^{-1}$) in plastic cups to which 25 third-instar larvae were added. The standard strain used was strain GC327 (33), a strain which is toxic to dipterans and is moderately toxic to *Aedes aegypti*. Assay preparations were incubated at 25°C, and the numbers of live larvae were determined after 1 and 24 h.

Because of the relatively low concentrations of the broth preparations used in the bioassays, it is unlikely that factors other than δ-endotoxin (e.g., nonspecific β-exotoxins, which

TABLE 1. Spectrum of toxicity of *B. thuringiensis* isolates obtained from an animal feed mill: isolates grouped by toxicity and crystal morphology[a]

Group	Toxicity to:				No. of isolates[b]	No. of isolates with the following crystal morphology				
	Pieris brassicae	*Aedes aegypti*	*Heliothis virescens*	*Spodoptera littoralis*		BP	IP	IS	R	S
1	–[c]	–	–	–	263*	106	74	27	20	36
2	+	–		–	96**	72	16	1	3	4
3	–	+	–	–	22***	7	9	1	3	2
4	–	–	+	–	7****	4	2	0	1	0
5	–	–	–	+	7****	5	0	0	0	2
6	+	+	–	–	33***	22	8	2	1	0
7	+	–	+	–	7****	7	0	0	0	0
8	+	–	–	+	7****	6	1	0	0	0
9	–	+	+	–	6****	4	1	1	0	0
10	–	+	–	+	1****	1	0	0	0	0
11	–	–	+	+	0–	0	0	0	0	0
12	+	+	+	–	23***	15	0	8	0	0
13	+	+	–	+	1****	0	1	0	0	0
14	+	–	+	+	2****	2	0	0	0	0
15	–	+	+	+	0–	0	0	0	0	0
16	+	+	+	+	2****	2	0	0	0	0
Total					477	253	112	40	28	44
Group 1 (as % of total)					55.1	41.9	66.1	67.5	71.4	81.8
Binomial SE						3.10	4.47	7.40	8.53	5.81

[a] The total number of isolates was 477. The number of subgroups differentiated was 36.
[b] Values with different numbers of asterisks are significantly different at the 1% level; tests were based on a *t* test in which binomial standard errors were used.
[c] –, not toxic; +, toxic.

usually must be present at much higher concentrations to be fatal) (24) were the cause of mortality.

For all of the assays, the rates of mortality for the standard strain and the isolates were corrected for the control rate of mortality in each assay group by using Abbott's formula (16), $p = c + (1 - c)p'$, where c is the control rate of mortality and p and p' are the corrected and observed rates of mortality, respectively.

Simply determining the difference between the corrected standard and test rates of mortality gave a measure of the efficacy of a test isolate and further compensated for any variation in the susceptibility of the larvae. However, proportions (like percentages) are not additive, and it was better to translate these values into an empirical logit scale (26), i.e., logit $(r/n) = \log_e [r/(n - r)]$, the logarithm of the ratio of dead larvae (r) to survivors ($n - r$). In this way we arrived at the following simple measurement: logit (corrected mortality, test isolate) = logit (50% mortality, standard) – logit (single assay, standard) + logit (single assay, new isolate), which was positive if the test isolate caused higher mortality than the standard strain. Since the concentration for each lepidopteran assay was set at the average 50% lethal concentration obtained from a series of strain GC91 assays, the measurement described above could be considered a mortality rate for the new isolate that compensated for day-to-day variation in the susceptibility of larvae. These calculations were performed by using a computer program that was written in Genstat (32), although they could just as easily have been made by using other statistical packages, such as MINITAB, or indeed by using a simple calculator. If we made the additional assumption that multiconcentration assays for test isolates and for standards would give log concentration-logit mortality curves with the same slope, then the statistic described above expressed the potency of a test isolate relative to the potency of the standard, based on single-concentration assays performed with the isolate and the standard (16).

Isolates were considered toxic if they caused 20% or more mortality in a bioassay, after compensation for control and standard mortality. Thus, under the test conditions which we used, isolates were considered nontoxic if they caused 19% or less mortality. Sorting of the bioassay data for analysis of results was performed with the aid of dBase IV (Ashton Tate).

RESULTS

Distribution of *B. thuringiensis* in the mill. We collected samples which represented a variety of residues found in the animal feed mill. Some samples were from very dry places, while others were dampened by proximity to the outside brick walls of the old building. Some samples consisted primarily of settled dust, and many consisted of a variety of grains, either whole or in various degrees of insect attack and decay. Samples were collected from spillages of grain in isolated corners or in dead spaces in machinery runs. Settled dust samples were collected from the tops of girders, from around and on top of machinery, and from under conveyer belts. In many places, rodent droppings were found, and in the upper stories, bird droppings were found; on window sills, there were aggregations of diverse insects and spiders and their frass shrouded in dust. Most of the residue grain was infested with beetles, and frequently there were tangled knots of silk, cocoons, and frass of lepidopterous larvae. Most species of stored-product insects that are likely to be found in United Kingdom mills (e.g., the lepidopterans *Anagasta kuehniella*, *Hofmannophila pseudospretella*, *Endrosis sarcitrella* and the coleopterans *Laemophloeus minuta* and *Tribolium* spp.), grain stores (e.g., *Sitophilus* spp. and *Rhizopertha dominica*), and farm stores (e.g.,

TABLE 2. Sample materials collected from the animal feed mill

Sample material	No. of samples collected	No. of samples yielding *B. thuringiensis*
Settled dust	18	14
Debris	9	6
Insects	7	4
Grain	6	3
Insect webbing	3	3
Guano	3	3
Mammalian fecal pellets	2	2
Bird nests	2	1

Oryzaephilus surinamensis) were observed in various parts of the mill.

A total of 36 of the 50 samples collected, including at least one-half of the samples in all categories, yielded *B. thuringiensis* (Table 2); all samples of guano, mammalian fecal pellets, and insect webbing contained *B. thuringiensis*. In general, the numbers of colonies of both *B. cereus* and *B. thuringiensis* that were isolated from the settled-dust and webbing samples were greater than the numbers of colonies that were isolated from the other sample materials. Samples that were noticeably damp yielded very few *B. cereus-B. thuringiensis* colonies. All of the samples that yielded *B. thuringiensis* also contained *B. cereus*, but *B. cereus* was also present in a number of samples that did not yield *B. thuringiensis*. We examined a representative cross-section of all bacterial colonies and found no isolates that had colony morphology different from the *B. cereus-B. thuringiensis* type and produced parasporal inclusion bodies.

Overall, *B. thuringiensis* was isolated more frequently than *B. cereus*. We selected 710 colonies of *B. cereus-B. thuringiensis* from all of the samples, and 67% of these colonies were classified as *B. thuringiensis* because of production of parasporal crystals; these colonies yielded 477 *B. thuringiensis* isolates.

Differentiation of *B. thuringiensis* isolates on the basis of toxicity. There were 16 possible combinations of toxicity to the four insect species which we used, ranging from nontoxic to any of the insects under the conditions used to toxic to all four insects (Table 1), and each isolate was placed in 1 of the 16 groups. In addition, the isolates were further differentiated into subgroups on the basis of crystal morphology (Table 1).

There were 14 groups of *B. thuringiensis* that were differentiated on the basis of toxicity alone. The members of the largest group appeared to be nontoxic to any of the insects at the concentrations used; this group (group 1) contained 263 isolates (55% of the total). The second largest group, group 2, contained those isolates that were selectively toxic to *Pieris brassicae*; this group contained 96 isolates (20% of the total). Several groups contained only a few isolates, and two groups (groups 11 and 15) (Table 1) contained no isolates; these groups were defined as toxic to *Heliothis virescens* and *Spodoptera littoralis* but nontoxic to *Pieris brassicae*. In all of the groups, only 20 isolates (4%) were defined as toxic to *Spodoptera littoralis*. Overall, 40% of the isolates were toxic to members of the Lepidoptera at the concentrations used, with 20, 1, and 1% being toxic exclusively to *Pieris brassicae*, *Heliothis virescens*, and *Spodoptera littoralis*, respectively. A total of 18% of isolates were toxic to *Aedes aegypti* (5% exclusively), 10% were toxic to *Heliothis virescens*, and 4% were toxic to *Spodoptera littoralis*. The most frequent insect toxicity was toxicity to *Pieris brassicae* (36% of all

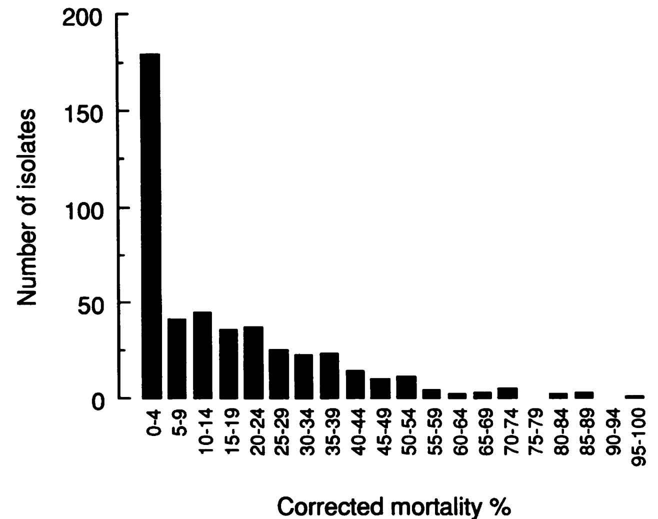

FIG. 1. Distribution of corrected mortality of *Pieris brassicae* caused by *B. thuringiensis* isolates.

isolates; 64% of these isolates were also toxic to *Heliothis virescens*, and 40% were toxic to *Spodoptera littoralis*).

Distribution of toxicity to *Pieris brassicae*. Figure 1 shows the distribution of corrected mortality for the isolates separated into groups (as suggested by K. Bernhard [1a]); in Fig. 1, each value represents a 5% step in mortality to *Pieris brassicae*. *Pieris brassicae* was chosen because it was susceptible to the greatest number of isolates. A large number of isolates with a particular toxicity would have produced a large peak (or peaks). The only large peak is in the 0 to 4% corrected mortality group, which contained most of the isolates. However, within this group, isolate diversity was evident, as many isolates could be differentiated on the basis of toxicity to other insects and on the basis of crystal morphology (Table 1, groups 1, 3, 4, 5, and 9). There are no other large peaks in Fig. 1, suggesting that repeated isolation of one strain or of a small number of strains that caused substantially different mortality levels did not occur.

Differentiation of *B. thuringiensis* isolates on the basis of toxicity and crystal morphology. The following five major classes of crystal morphology were represented in our isolates: class 1, bipyramidal (BP) or near BP, such as the morphology observed in *B. thuringiensis* subsp. *kurstaki* (although the class 1 isolates could contain crystals having other morphology in addition to BP); class 2, irregularly pointed (IP); class 3, spherical (S); class 4, irregularly spherical (IS), as in *B. thuringiensis* subsp. *israelensis*; and class 5, rectangular (R), as in *B. thuringiensis* subsp. *tenebrionis*. Isolates that produced BP crystals were the most common (53% of the isolates) (Fig. 2). Of the remaining isolates, 24% produced IP crystals, 9% produced S crystals, 8% produced IS crystals, and 6% produced R crystals. Isolates containing combinations of IP, S, IS, and R crystals were rare and were classified on the basis of the larger crystals or the predominant crystals. The frequency of toxicity was significantly greater (significance was assessed by using binomial standard errors) in isolates that produced BP crystals than in isolates that produced IP, IS, R, or S crystals (Fig. 2 and Table 1); also, the frequency of toxicity was greater in isolates that produced IP or IS crystals than in isolates that produced S crystals. A total of 34% of the isolates that produced IP crystals were toxic to one or more

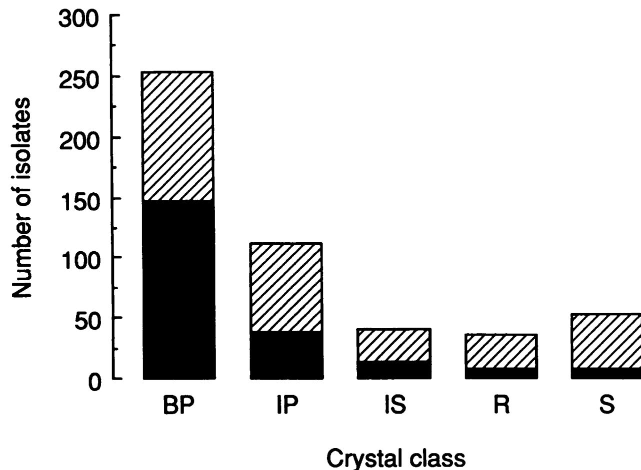

FIG. 2. Frequency of classes of parasporal crystal morphology in isolates. Toxic isolates caused 20% or more corrected mortality in one or more of the test insect species. Solid bars, toxic isolates; cross-hatched bars, nontoxic isolates.

insects, and 33, 29, and 18% of the isolates that produced IS, R, and S crystals, respectively, were also toxic to one or more insects. Of the 192 isolates that were toxic to members of the Lepidoptera, 73% produced BP crystals, 15% produced IP crystals, 3% produced S crystals, 6% produced IS crystals, and 3% produced R crystals. In contrast, two-thirds of the 22 isolates that were exclusively toxic to *Aedes aegypti* had non-BP types of crystals.

Of the 14 groups that were differentiated on the basis of toxicity (Table 1), groups 1 through 6, 8, 9, and 12 had more than one crystal class represented. Since all isolates were grown in the same medium under very similar conditions (within the limits of batch culture techniques) and crystal morphology was always determined by the same person, it was decided for the purposes of this study only that crystal morphology could be used as a secondary characteristic, after toxicity spectrum, to further characterize isolates. Assuming that different crystal morphologies within a given toxicity group represented additional subgroups of *B. thuringiensis*, 36 subgroups were differentiated.

Analysis of the data revealed a diverse *B. thuringiensis* population at the site, and in order to establish whether such diversity also existed within a single residue sample, all 68 *B. thuringiensis* isolates that were obtained from one particular sample were differentiated into subgroups on the basis of toxicity and crystal morphology (Table 3) in the same way as the total isolate collection. In this analysis, 21 subgroups were differentiated, indicating that there was also a diverse population of *B. thuringiensis* within one sample. The most frequent isolates were selectively toxic to *Pieris brassicae* (group 2 contained 20 isolates, 29% of the total). Isolates that appeared to be not toxic (group 1, containing 15 isolates [22% of the total]) were the second most frequent isolates. This contrasts with our results for the isolates obtained from all of the samples (Table 1), in which nontoxic isolates were the most frequent isolates (group 1, containing 263 isolates [55% of the total]), indicating that there was a difference in the distribution and frequency of *B. thuringiensis* strains between the single sample and all samples.

The diversity of the isolates from the single sample was supported by the results of a plasmid profile analysis performed by using the method of Jarrett (20). Many isolates that were differentiated on the basis of toxicity spectrum and crystal morphology (Table 3) also had different plasmid profiles. In particular, isolates belonging to the same toxicity groups (Table 3) that were differentiated on the basis of crystal morphology were found to have much different plasmid profiles. This confirmed that for the purposes of this study, crystal morphology was a useful secondary characteristic by which isolates could be differentiated.

TABLE 3. Spectrum of toxicity of *B. thuringiensis* isolates obtained from a single residue sample: isolates grouped by toxicity and crystal morphology[a]

Group	Toxicity to:				No. of isolates	No. of isolates with the following crystal morphology:				
	Pieris brassicae	*Aedes aegypti*	*Heliothis virescens*	*Spodoptera littoralis*		BP	IP	IS	R	S
1	–[b]	–	–	–	15	8	4	1	1	1
2	+	–	–	–	20	19	0	0	1	0
3	–	+	–	–	3	2	1	0	0	0
4	–	–	+	–	0	0	0	0	0	0
5	–	–	–	+	0	0	0	0	0	0
6	+	+	–	–	4	3	1	0	0	0
7	+	–	+	–	4	4	0	0	0	0
8	+	–	–	+	3	2	0	1	0	0
9	–	+	+	–	3	2	1	0	0	0
10	–	+	–	+	0	0	0	0	0	0
11	–	–	+	+	0	0	0	0	0	0
12	+	+	+	–	14	8	1	5	0	0
13	+	+	–	+	0	0	0	0	0	0
14	+	–	+	+	1	1	0	0	0	0
15	–	+	+	+	0	0	0	0	0	0
16	+	+	+	+	1	1	0	0	0	0
Total					68	50	8	7	2	1

[a] The total number of isolates was 68. The number of subgroups differentiated was 21.
[b] –, not toxic; +, toxic.

DISCUSSION

In a survey of grain residue samples obtained from a number of different sites, DeLucca et al. (7) found that *B. thuringiensis* was present in 55% of their settled-dust samples. This compares well with the findings of our study, in which *B. thuringiensis* was isolated from 78% of the settled-dust samples. However, in this study the overall rate of isolation of *B. thuringiensis* was higher; *B. thuringiensis* made up 67% of the 710 colonies examined, compared with 31% of the 255 colonies examined by DeLucca et al. (7). This may reflect natural variations in bacterial populations in different locations and sample materials. In addition, DeLucca et al. observed no insect cadavers, webbing, or fecal material (7) and examined only settled dust.

In this study, in the residue samples that contained *B. thuringiensis*, this species substantially outnumbered *B. cereus*. This is in contrast to the findings in studies in which workers have isolated *B. thuringiensis* from soil, where *B. cereus* greatly outnumbers *B. thuringiensis*. For example, in a study of 136 soil samples from Japan, in which pasteurization was used as the only selection procedure, only 189 of 6,910 *B. cereus-B. thuringiensis* group colonies contained parasporal crystals (29). In a study of United States soils in which antibiotic selection was used, DeLucca et al. (9) found that only 0.5% of 46,373 isolates belonging to the *B. cereus-B. thuringiensis* group were *B. thuringiensis* isolates. Using acetate broth selection, Martin and Travers (25) found that *B. thuringiensis* was a widespread soil bacterium, although no comparison with the occurrence of *B. cereus* was made.

The reason for the higher frequency of *B. thuringiensis* than *B. cereus* in the mill environment compared with soil is not known, but there may be opportunities for enrichment of *B. thuringiensis* within a stored-product environment. In some cases, *B. thuringiensis* multiplies in the cadavers of insects that have been killed by the *B. thuringiensis* toxin (4). These cadavers may themselves be ingested by Lepidoptera larvae (4), scavenging insects, birds, and mammals, who thus spread spores in their feces. Indeed, in this present study, *B. thuringiensis* was isolated from samples of bird and mammal feces. There was evidence of much lepidopterous and coleopterous activity in the mill. There was insect webbing on the walls and beams, and there were portions of insect cadavers, as well as whole cadavers, in residues; this ecological situation fits the concept of possible spread and enrichment of *B. thuringiensis*.

A total of 36 subgroups of *B. thuringiensis* were differentiated on the basis of toxicity and crystal morphology, revealing a diverse *B. thuringiensis* population within the mill. There was also a disparate and diverse *B. thuringiensis* population within a single residue sample, as indicated by the fact that the distribution and frequency of subgroups in a single sample differed from the distribution and frequency of subgroups in all of the samples. In addition, if additional insects orders (e.g., Coleoptera) were used, it is possible that additional subgroups of *B. thuringiensis* would be revealed. No susceptible coleopteran insect colony was available at the time of this study, but work is currently under way to assess the distribution of coleopteran-toxic *B. thuringiensis* isolates in the mill.

There are two factors that may contribute to the diversity of isolates found in this location. Grain and other products are regularly brought into the mill after they have been held previously in farm stores and warehouses prior to shipment, allowing exposure to insects and buildup of *B. thuringiensis* from a wide variety of sources. *B. thuringiensis* may also be present naturally on the plant material (35) or from soil contamination. Thus, there is likely to be a regular input of new strains (and hence genotypes) of *B. thuringiensis* into the mill ecosystem. Also, within infected insects, there is the opportunity for transfer of plasmids that code for δ-endotoxin genes from one bacterial strain to another (22) by a conjugationlike process (17). Thus, there is a potential for emergence of diverse populations of *B. thuringiensis* brought about by natural processes of plasmid transfer in the environment. Such diversity may not be revealed by biochemical or serological tests since these methods are not directly related to δ-endotoxin genes, whereas the insect bioassay is a sensitive method for revealing the spectrum of δ-endotoxin genes present in a strain (14).

The most frequent isolates were those that appeared to be nontoxic at relatively low concentrations. It seems paradoxical that an entomopathogen should carry genes for the production of a crystal that is not toxic. However, many of the apparently nontoxic varieties isolated in this study are probably toxic at higher concentrations (21) or may affect different insect or other animal species. It is also possible that δ-endotoxins have other, as-yet-undiscovered, properties and functions that are not related to insect toxicity. Of the toxic isolates, those with activity against *Pieris brassicae* were the most common. This indicates that *Pieris brassicae* may generally be more susceptible to *B. thuringiensis* than the other insect species used in this study. Interestingly, it is unlikely that the source of the strains that were toxic to *Pieris brassicae* in the grain dust was originally this host insect, as *Pieris brassicae* is predominately a pest of plants belonging to the cabbage family and does not live on cereal crops or in stored-product warehouses. A number of isolates in this study exhibited toxicity to *Aedes aegypti* at a relatively high concentration. On further testing at a much lower concentration, however, none was found to be as active as the mosquitocidal isolate *B. thuringiensis* subsp. *israelensis* IPS 82.

In this investigation, as well as in other studies (7, 9, 24, 28, 30, 31), *B. thuringiensis* and *B. cereus* were usually isolated together. This indicates that in addition to the close taxonomic relationship of these organisms (1, 34), there may be a close ecological relationship. Additional comparisons of the distribution and frequency of *B. thuringiensis* isolates obtained from a number of different ecosystems may lead to a better understanding of the ecology of this organism and its relationship with *B. cereus*.

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REFERENCES

1. **Baumann, L., K. Okamoto, B. M. Unterman, M. J. Lynch, and P. Baumann.** 1984. Phenotypic characterisation of *Bacillus thuringiensis* and *Bacillus cereus*. J. Invertebr. Pathol. **44:**329–341.

1a. **Bernhard, K.** Personal communication.

2. **Bonnefoi, A., and H. de Barjac.** 1963. Classification des souches du groupe *Bacillus thuringiensis* par la determination de l'anti-

gene flagellaire. Entomophaga **8**:223–229.
3. **Burges, H. D.** 1973. Enzootic diseases of insects. Ann. N.Y. Acad. Sci. **217**:31–49.
4. **Burges, H. D., and J. A. Hurst.** 1977. Ecology of *Bacillus thuringiensis* in storage moths. J. Invertebr. Pathol. **30**:131–139.
5. **Burges, H. D., and P. Jarrett.** June 1990. Preparation of strains of *Bacillus thuringiensis* having an improved activity against certain lepidopterous pests and novel strain produced thereby. U.S. patent 4,935,353.
6. **David, W. A. L., and B. O. C. Gardiner.** 1965. Rearing *Pieris brassicae* L. larvae on a semi-synthetic diet. Nature (London) **207**:882–883.
7. **DeLucca, A. J., M. S. Palmgren, and A. Ciegler.** 1982. *Bacillus thuringiensis* in grain elevator dusts. Can. J. Microbiol. **28**:452–456.
8. **DeLucca, A. J., M. S. Palmgren, and H. de Barjac.** 1979. A new serovar of *Bacillus thuringiensis* from grain dust: *Bacillus thuringiensis* serovar *colmeri* (serovar 21). J. Invertebr. Pathol. **43**:437–438.
9. **DeLucca, A. J., J. G. Simonson, and A. D. Larson.** 1981. *Bacillus thuringiensis* distribution in soils of the United States. Can. J. Microbiol. **27**:865–870.
10. **Doetsch, R. N.** 1981. Determinative methods of light microscopy, p. 21–37. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
11. **Donovan, W. P., J. M. Gonzalez, M. P. Gilbert, and K. Dankoscik.** 1988. Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the gene. Mol. Gen. Genet. **214**:365–372.
12. **Dulmage, H. T.** 1970. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* variety *alesti*. J. Invertebr. Pathol. **15**:232–239.
13. **Dulmage, H. T., J. A. Correa, and A. J. Martinez.** 1970. Coprecipitation with lactose as a means of recovering the spore-crystal complex of *Bacillus thuringiensis*. J. Invertebr. Pathol. **15**:15–20.
14. **Dulmage, H. T., et al.** 1981. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control, p. 193–222. *In* H. D. Burges (ed.), Microbial control of pests and plant diseases 1970–1980. Academic Press, London.
15. **Ellar, D. J., B. H. Knowles, F. A. Drobniewski, and M. Z. Haider.** 1986. The insecticidal specificity and toxicity of *Bacillus thuringiensis* δ-endotoxins may be determined respectively by an initial binding to membrane-specific receptors followed by a common mechanism of cytolysis, p. 7–10. *In* R. A. Samson, J. M. Vlak, and D. Peters (ed.), Fundamental and applied aspects of invertebrate pathology. Agricultural University, Wageningen, The Netherlands.
16. **Finney, D. J.** 1964. Probit analysis: a statistical treatment of the sigmoid response curve. Cambridge University Press, Cambridge.
17. **Gonzalez, J. M., B. J. Brown, and B. C. Carlton.** 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. Proc. Natl. Acad. Sci. USA **79**:6951–6955.
18. **Guennelon, G., H. Audenard, J. C. Fremond, and M. A. El Idrissi Ammarri.** 1981. Progrès réalisés dans l'élevage permanent du carpocapse (*Laspeyresia pomella*) sur milieu artificial. Agronomie (Paris) **1**:59–64.
19. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53**:242–255.
20. **Jarrett, P.** 1985. Potency factors in the delta-endotoxins of *Bacillus thuringiensis* var. *aizawai* and the significance of plasmids in their control. J. Appl. Bacteriol. **58**:437–448.
21. **Jarrett, P., and H. D. Burges.** 1982. Effect of bacterial varieties on the susceptibility of the greater wax moth *Galleria mellonella* to *Bacillus thuringiensis* and its significance in classification of the bacterium. Exp. Appl. Entomol. **31**:346–352.
22. **Jarrett, P., and M. Stephenson.** 1990. Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoralis*. Appl. Environ. Microbiol. **56**:1608–1614.
23. **Krieg, A., and G. A. Langenbruch.** 1981. Susceptibility of arthropod species to *Bacillus thuringiensis*, p. 193–222. *In* H. D. Burges (ed.), Microbial control of pests and plant diseases 1970–1980. Academic Press, London.
24. **Lecadet, M.-M., and H. de Barjac.** 1981. The β-exotoxin of *Bacillus thuringiensis*, p. 293–321. *In* E. W. Davidson (ed.), Pathogenesis of inveretebrate microbial diseases. Allanheld Osmun and Co., Totowa, N. J.
25. **Martin, P. A. W., and R. S. Travers.** 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. **55**:2437–2442.
26. **McCullagh, P., and J. A. Nelder.** 1989. Generalized linear models, 2nd ed. Chapman & Hall, London.
27. **Norris, J. R.** 1969. The ecology of serotype 4B of *Bacillus thuringiensis*. J. Appl. Bacteriol. **32**:261–267.
28. **Ohba, M., and K. Aizawa.** 1978. Serological identification of *Bacillus thuringiensis* and related bacteria in Japan. J. Invertebr. Pathol. **32**:303–309.
29. **Ohba, M., and K. Aizawa.** 1986. Distribution of *Bacillus thuringiensis* in soils of Japan. J. Invertebr. Pathol. **47**:277–282.
30. **Ohba, M., K. Aizawa, and S. Sudo.** 1984. Distribution of *Bacillus thuringiensis* in sericulture farms of Fukuota Prefecture, Japan. Proc. Assoc. Plant Prot. Kyushu. **30**:152–155. (In Japanese with English summary.)
31. **Padua, L. E., B. P. Gabriel, K. Aizawa, and M. Ohba.** 1982. *Bacillus thuringiensis* isolated from the Philippines. Philipp. Entomol. **5**:199–208.
32. **Payne, R. W., et al.** 1987. Genstat 5 reference manual. Clarendon Press, Oxford.
33. **Pethybridge, N. J., P. F. White, and P. Jarrett.** 1992. Laboratory and cropping chamber experiments with *Bacillus thuringiensis* for control of the mushroom sciarid pest *Lycoriella auripila* (Diptera: Sciaridae), p. 143–146. 3rd European Meeting of the IOBC Working Group on Insect Pathogens and Entomoparasitic Nematodes, Wageningen, The Netherlands.
34. **Priest, F. G., M. Goodfellow, and C. Todd.** 1988. A numerical classification of the genus *Bacillus*. J. Gen. Microbiol. **134**:1847–1882.
35. **Smith, R. A., and G. A. Couche.** 1991. The phylloplane as a source of *Bacillus thuringiensis* variants. Appl. Environ. Microbiol. **57**:311–315.
36. **Travers, R. S., P. A. W. Martin, and C. F. Reichelderfer.** 1987. Selective process for the efficient isolation of soil *Bacillus* species. Appl. Environ. Microbiol. **53**:1263–1266.