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Natural Isolates of *Bacillus thuringiensis:* Worldwide Distribution, Characterization, and Activity against Insect Pests

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Bacillus thuringiensis **was isolated from natural samples collected from 80 countries. The majority, 45% of the 5303 isolates, originated from stored products, with 25% originating from soil. The materials richest in isolates active in insects were mushroom compost and stored products. The proportion with bipyramidal-shaped crystals was 46%, while among the range of other shapes 14% were spherical and 4% rectangular. The largest proportion of isolates, 45%, was found in the United Kingdom with the majority originating from stored mills, which import produce from all over the world; 22% came from North America. Using single-dose assays, 44% of the isolates killed less than 25% of larvae of all four Lepidoptera tested, *Heliothis virescens, Pieris brassicae, Spodoptera littoralis,* and *Agrotis ypsilon.* Among isolates killing more than 25% of the larvae, every combination of activities of the 16 possible against these 4 insects was found, in proportions ranging from 0.6 to 14.5%, suggesting considerable diversity of insect activity. The 44% of strains with little or no activity to Lepidoptera (<25% mortality) included strains selectively active against Diptera and Coleoptera. An analysis of activity using one representative species from each of the three orders of insects *H. virescens, Aedes aegypti,* and *Phaedon cochleariae* showed 48.8% of strains to be inactive (<25% mortality) in all three, with 1.2 to 14.6% active in the other 7 possible combinations. For an analysis of geographical origins of insect activity, 3 species (*P. cochleariae, S. littoralis,* and *H. virescens*) were chosen with susceptibility to different δ-endotoxins. With one exception, activity did not appear to be correlated with origin, indicating a relatively ubiquitous distribution of the selected activities and of *B. thuringiensis* in general. The worldwide spread and evolution of this insect pathogen and its different types are discussed in relation to geographical origin and source materials, as well as possible interaction with other bacteria.**

KEY WORDS: ***Bacillus thuringiensis*** **natural isolates; characterization; activity spectrum; distribution;** ***Pieris brassicae; Heliothis virescens; Spodoptera littoralis; Agrotis ypsilon; Aedes aegypti; Culex pipiens; Phaedon cochleariae; Leptinotarsa decemlineata;*** **susceptibility to *B. thuringiensis;* toxin crystal.**

INTRODUCTION

The Gram-positive spore-forming bacterium *Bacillus thuringiensis* produces distinctive parasporal inclusion bodies, which are frequently of bipyramidal shape and commonly referred to as parasporal crystals. Most known isolates cause diseases in insect larvae initially described as *sotto* (Ishiwata, 1901) or Schlaffsucht disease (Berliner, 1915). The parasporal crystals consist predominantly of proteins, called δ-endotoxins, of molecular masses ranging between 25 and 140 kDa. Pathogenicity is due to disruption of the intestinal gut lining of insect larvae by endotoxins, activated by solubilization and proteolytic cleavage of ingested parasporal crystals in the alkaline gut juice (Angus, 1954).

Since its discovery, *B. thuringiensis* has received considerable attention in the research community. Insecticidal preparations based upon various strains of *B. thuringiensis* currently account for 80–90% of all biological pest control agents sold worldwide, making it the most successful type of bioinsecticide. Dulmage and collaborators (1981) demonstrated that *B. thuringiensis* strains active against lepidopteran larvae differ considerably in potency and insecticidal spectra. Discovery of strains potent in Diptera (Goldberg and Margalit, 1977) and Coleoptera (Krieg *et al.,* 1983) demonstrated that the spectrum of potential uses is even wider than initially believed. Gonzalez *et al.* (1982) demonstrated that most δ-endotoxin genes are located on large plasmids, which either are self-transmissible

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or can be co-transferred from a donor to a receptor strain in a conjugation-like process. Since most strains of *B. thuringiensis* carry and express more than one δ-endotoxin gene, their spectra of insecticidal activity depend upon the combination of individual δ-endotoxins present in their parasporal crystals. Although in some crop/pest situations *B. thuringiensis* insecticides are as efficacious as conventional chemical insecticides, the usefulness of preparations based upon natural isolates is limited because their spectra of insecticidal activity do not always match the pest complexes which attack crops. Jarrett and Burges (1986) demonstrated that the insecticidal spectra of strains can be tailored to fit the pest complexes found on specific crops. One example is strain GC-91 (Burges and Jarrett, 1988), which was developed into a commercial insecticide (Bernhard, 1993). Further success of such strategies to improve the usefulness of *B. thuringiensis* insecticides depends upon the availability of suitable δ-endotoxin genes.

We therefore conducted a joint project to isolate and characterize naturally occurring *B. thuringiensis* strains from materials collected worldwide at Horticulture Research International (HRI) in Littlehampton and Ciba-Geigy (CG) in Basel. This project yielded valuable information on the worldwide distribution of *B. thuringiensis,* as well as its classification and activities in eight insects, which is presented in this paper.

MATERIALS AND METHODS

Culture Media

Isolates were cultured either in Proflo B4 broth, containing per liter: 10.0 g cottonseed flour, 15.0 g glucose, 2.0 g yeast extract, 2.0 g peptone, 0.3 g $MgSO_4 \cdot 7\ H_2O$, 0.02 g $FeSO_4 \cdot 7\ H_2O$, 0.02 g $ZnSO_4 \cdot 7\ H_2O$, 0.02 g $MnSO_4 \cdot 7\ H_2O$, and 1.0 g $CaCO_3$, or in HGP-medium, containing per liter: 10.0 g casein peptone, 5.0 g glucose, 0.5 g yeast extract, 0.5 g NaCl, and 10 ml trace element stock solution. Trace element stock solution contained per liter: 80.0 g $CaCl_2$. $6\ H_2O$, 50.0 g $MnCl_2 \cdot 4\ H_2O$, 5.0 g $ZnCl_2$, 5.0 g $CuCl_2 \cdot 2\ H_2O$, 5.0 g $CoCI_2 \cdot 6\ H_2O$, 5.0 g $FeCI_3 \cdot 6\ H_2O$, and 100.0 g citric acid.

Sample Collection

Soil sample collection kits, consisting of numbered 10-ml plastic screw-cap tubes, forms for recording the type and origin of the samples, and self-addressed envelopes and an accompanying letter, were sent to various contacts around the world. Many soil and stored product samples were also received in larger containers provided by the senders. All samples were dried if necessary, sealed, and stored at 2°C until required.

Isolation Methods

Initially, the selection method described by Travers *et al.* (1987) was used. By trial and error it was replaced by the following procedures. Suspensions were made in various ways in 10 ml sterile distilled water. For stored product samples, 0.5 g of material was suspended in the water and mixed for 60 sec on a vortex mixer. After mixing, the solid material 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 non-spore-forming organisms. Samples of soil and mushroom compost were treated similarly, with the only difference being that 0.1 g of sample was used. Insect samples were crushed in a Griffiths tube and the water was added or, if desiccated, the crushed insect cadaver was transferred directly into the water. Phylloplane samples were cut into 1-cm pieces and placed into universal tubes which contained 10 ml of water and a layer of 3-mm glass balls. The suspension was mixed on a vortex mixer for 60 sec. Following pasteurization, for all samples, 0.1-ml aliquots of undiluted and 10^{-2} dilutions of the suspensions were plated onto nutrient agar (Oxoid, UK). After 24–48 hr incubation at 30°C, colonies having a *B. thuringiensis* or *Bacillus cereus* morphology were inspected by phase-contrast microscopy. The number of colonies which grew up from any one sample varied from zero to many hundreds. From each sample, up to 30 colonies were microscopically examined and those having visible parasporal inclusions were classified as *B. thuringiensis*. Only colonies from each sample showing a different morphology, crystal shape, or size were stored in 20% glycerol at −20°C for further study.

Crystal Characterization

The parasporal inclusions were examined before cell lysis by phase-contrast microscopy for each isolate after growth in B4 broth. Four characteristics were recorded: crystal morphology, crystal size relative to the spore, the number of crystals in each cell, and whether each cell produced a crystal. Crystal morphology was classified as one of five types: B, bipyramidal; S, spherical; R, rectangular or cuboid; I, irregularly spherical; or P, irregularly pointed.

Culture Conditions and Strain Maintenance

To provide material for single-dose bioassays, isolates were produced as broth cultures. At HRI isolates were inoculated into 50 ml Proflo B4 broth and incubated at 30°C in an incubator rotating at 250 rpm. Each culture was examined periodically by phase-contrast microscopy until at least 95% of the population had lysed, releasing spores and crystals; then glycerol was added to a concentration of 15%, and the culture was divided into aliquots and stored at −70°C.

At CG, isolates were inoculated into 50 ml HGP broth and incubated at 24°C in an incubator rotating at 125 rpm. Each culture was examined periodically by phase-contrast microscopy and harvested when sporulation and cell autolysis were greater than 95%, usually after 48 hr. For long-term storage of the isolates, 0.5-ml aliquots of the sporulated cultures were dried on 1.0 g sterile quartz sand contained in 50-ml brown glass screw-cap bottles. After air drying under a laminar flow hood, samples were taken with an inoculating loop from each bottle and streaked onto HGP agar to test for viability and uniformity of the spores. The bottles containing the dried spore preparations were kept in the dark in a cabinet at room temperature. For bioassay the broth cultures were stored at −20°C until use. Since not enough broth was left for bioassays against *Leptinotarsa decemlineata,* each strain selected for testing was grown on three HGP agar plates and the combined lawns were suspended in 30 ml distilled water and kept frozen at −20°C until use.

Insect Bioassays

Pathogenicity of *B. thuringiensis* isolates was initially assessed at a single dose of broth against larvae of representatives of the orders Lepidoptera, Diptera, and Coleoptera. Larvae for bioassays were obtained from colonies reared in the insectaries at HRI and CG. With each insect species *B. thuringiensis* isolates were normally tested in batches of 30. Each batch of tests included controls of sterile culture medium and a sporulated culture of a standard strain. The concentrations used were broth dilutions which gave about 50% mortality with the standard strains. Standard strains were GC-91 (Burges and Jarrett, 1988) for tests against lepidopteran larvae, *B. thuringiensis* subsp. *tenebrionis* for coleopteran larvae, and the *B. thuringiensis* subsp. *kyushuensis* strain HD-571 for dipteran larvae.

For bioassays against *Pieris brassicae,* broths at the rate of 0.1 μl/g were mixed thoroughly into molten agar-based artificial diet (David and Gardiner, 1965), which included 100 μg cefataxime, 200 μg streptomycin, and 100 μg tetracycline per gram, to inhibit bacterial contamination. The diet was then poured into 9.5-cm-diameter petri dishes and allowed to set and surface dry, after which 20 neonate larvae were applied to the diet. Replicate plates were prepared for each broth. Bioassays were evaluated after 5 days.

For bioassays against *Heliothis virescens, Spodoptera littoralis,* and *Agrotis ypsilon,* broths at the rates of 0.5, 1.0, and 100 μl per gram, respectively, were incorporated into the agar-based diet described by Payne (1981). To avoid cannibalism, 25 or 30 neonate larvae per broth for *H. virescens* and *S. littoralis* or second-instar larvae for *A. ypsilon* were placed individually into 2-ml polystyrene dishes containing approximately 0.5 ml of diet. Bioassays were incubated for 6 days at 25°C.

For bioassays with *Aedes aegypti* and *Culex pipiens,* broths were suspended in 50 ml distilled water (10 μl/ml) contained in plastic cups to which 25 third-instar larvae were added. Assays were incubated at 25°C and the numbers of live larvae assessed after 1 and 24 hr.

For bioassays against *Phaedon cochleariae,* Chinese cabbage leaf discs 5 cm in diameter were dipped into diluted broth (100 μl/ml) and placed in a petri dish on top of three layers of circular filter paper moistened with 2.5 ml distilled water. After the surfaces of the leaf discs had dried, 10 third-instar larvae were placed onto each leaf disc. Each petri dish was covered with a circular piece of cotton cloth squeezed between petri dish and lid to prevent escape of the larvae. Three replicates were prepared for each broth. Larval mortality was evaluated after 5 days of incubation at 24°C.

For bioassays against *L. decemlineata* the same method was used, except that first-instar larvae and young potato leaves, about 5 cm long, were used.

Data Processing

In order to compare the bioassay results between different assays for each species, mortalities were corrected for 50% mortality of the standard and 0% for the control according to the following formula: $M = ((T\text{-}C)/(S\text{-}C)) \times 50$, where M is the corrected mortality, T is the mortality with the isolate tested, C is the control mortality, and S is the mortality with the standard broth, all expressed as percentages.

RESULTS

Samples Obtained

A total of 2363 samples were received from 80 countries, representing all continents except Antarctica. To compare sample types all were initially classified into one of the following five groups.

1. Stored product samples. Most came from residues in UK mills, importing produce from many areas of the world. Many of the U.S. samples were dust from maize grinders and so provided a different environment for insects and *B. thuringiensis* to the predominantly wheat grain type samples from the United Kingdom.

2. Soil samples. Although most are soil, samples of sand, mud, and peat have also been included. Also included are samples of mushroom compost taken from mushroom cropping houses on six different sites in the south of England.

3. Insect residues. These came from a variety of sources. All were cadavers of either larvae or adults from the following orders: Odonata, Orthoptera, Dermaptera, Dictyoptera, Hemiptera, Lepidoptera, Diptera,

Hymenoptera, and Coleoptera. These cadavers were found in insectaries, in stored product or agricultural environments, in or on soil, as well as in domestic environments.

4. Plant material. This included leaves of wheat, cotton, and rice. Further plant material was obtained from various sources such as fruit and vegetables imported from around the world. Fibrous material from plants such as coconuts proved to be a source for *B. thuringiensis* as did onion skins. Other plant material was obtained in the form of leaf litter from forest floors, root material, and tree bark.

5. Unusual materials. These comprise sample materials which did not conveniently fit into the categories described above. These materials include bird nests, bat nests, bird feces, bat feces, debris from animal barns, insect nests, pond water, meltwater, and unstored grain.

These groups were also further divided into seven categories for the study of crystal types.

Bacterial Isolates Obtained

A total of 5303 crystal-forming isolates were obtained. The majority of the isolates, 45.4%, originated from stored products, 25.3% from soil, 10.6% from insect residues, 3.4% from plant material, and the remaining 15.3% from unusual materials.

The proportion of isolates active in insects varied greatly in the different materials. The mean percentages of isolates active in the eight test insect species were mushroom compost, 30.7%; stored product, 29.6%; phylloplane, 24.5%; insect, 23.3%; unusual sample types, 17.5%; soil, 15.5%; other plant material; 7.6%.

The isolates were also analyzed with regard to the geographical origins of the sample materials from which they were isolated. The results are shown in Table 1.

TABLE 1
Distribution of Isolates According to Geographical Origin of Samples

Geographical origin of samples	Percentage of isolates
UK stored products	29.0
UK soil	1.1
Other UK materials	14.6
Total UK	44.7
Other European countries	8.3
South and Central America	4.8
North America	22.0
Africa	10.5
Asia	5.8
Australia and New Zealand	3.9

Distribution of Crystal Morphologies

Attempts to mitigate any subjectivity in the assessment of crystal morphology by phase-contrast microscopy were made by ensuring that growth conditions were kept constant and by having the same person classify isolates. The present analysis is therefore confined to the 2793 isolates characterized by the same person.

The distribution of crystal morphologies (Table 2) was as follows: 45.9% were classified as B; 14.2% as S; 4.4% as R; 16.4% as I, and 19.1% as P. Of the 2793 isolates, 22.8% were observed to produce a small (<1.0 μm diameter), invariably spherical, secondary crystal. Isolates producing bipyramidal crystals were most likely to produce a secondary crystal.

The distribution of crystal morphologies among the sample types from which the strains were isolated was analyzed as shown in Table 2. In this analysis, isolates from leaf material and mushroom compost were analyzed separately. Strains with B crystals predominated, together with those having I and P crystals, particularly the 61.1% of strains with B crystals in stored products. The relatively large proportion of strains with S crystals from soil is also noteworthy. The proportions in other plant material and mushroom compost mean little because of the small number of samples involved, but are included for completeness.

Activity against Lepidoptera

The numbers of isolates tested with single-dose assays on artificial diet were 5303 against *A. ypsilon,* 5136 against *H. virescens,* 3077 against *P. brassicae,* and 3028 against *S. littoralis.* The distribution of isolates according to the corrected mortalities they caused to each of the test insects is shown in Fig. 1.

The 2789 isolates which had been tested against all four lepidopteran species were grouped according to their spectra of insecticidal activity using the following

TABLE 2
Distribution of Morphological Types of Parasporal Inclusions by Sample Type

Sample type	Morphological group[a] (% of morphological types in each sample type)					Number of isolates
	B	S	R	I	P	
Stored product	61.1	8.9	4.2	7.2	18.6	1537
Soil	26.0	20.8	3.6	27.5	22.0	830
Insect	38.8	16.3	7.0	14.7	23.3	129
Phylloplane	47.8	11.1	1.1	32.2	7.8	90
Other plant material	5.1	49.1	8.5	22.0	15.2	59
Mushroom compost	20.1	14.6	6.3	50.0	8.3	48
Unusual sample type	22.0	20.0	9.0	34.0	15.0	100
Total	45.9	14.2	4.4	16.4	19.1	2793

[a] Classification of morphological types of parasporal inclusions: B, bipyramidal; S, spherical; R, rectangular or cuboid; I, irregularly spherical; and P, irregularly pointed crystals.

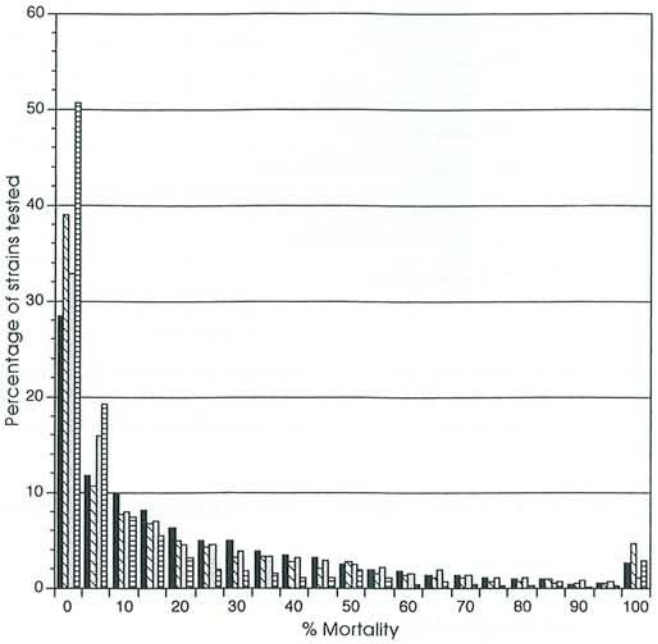

FIG. 1. Distribution of insecticidal activities with single-dose bioassays against lepidopteran larvae on artificial diet. Black bars, *Agrotis ypsilon;* diagonally striped bars, *Heliothis virescens;* dotted bars, *Pieris brassicae;* horizontally striped bars, *Spodoptera littoralis.*

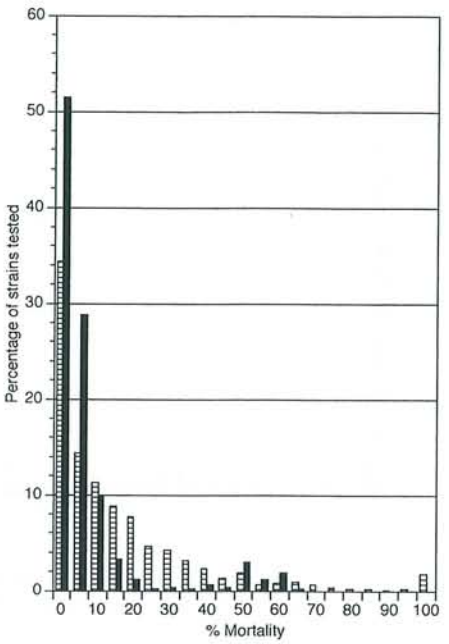

FIG. 2. Distribution of insecticidal activity in single-dose bioassays against coleopteran larvae in leaf dip assays. Striped bars, *Phaedon cochleariae;* black bars, *Leptinotarsa decemlineata.*

principle. All strains were divided into two groups with regard to their activity against one test insect. Strains with 25% or less corrected mortality were rated inactive, whereas strains giving more than 25% mortality were rated active. With four test species this resulted in 16 possible combinations of activities. The results shown in Table 3 give for each group the percentage based on the total number of strains tested in all four strains.

Activity against Coleoptera

Five thousand three hundred two isolates were tested against larvae of the chrysomelid beetle *P. cochleariae* in leaf dip assays on Chinese cabbage leaf discs. The 579 most active isolates were also tested in leaf dip assays on potato leaves against first-instar larvae of *L. decemlineata.* The distribution of biological activities against these two species is shown in Fig. 2.

TABLE 3

Classification of Isolates Based upon Their Spectra of Activity against Lepidopterous Larvae[a]

	Spodoptera+		*Spodoptera*−	
	Pieris+	*Pieris*−	*Pieris*+	*Pieris*−
Heliothis+				
Agrotis+	3.6	0.6	4.7	2.7
Agrotis−	1.5	0.9	5.1	3.8
Heliothis−				
Agrotis+	1.3	1.7	4.3	14.5
Agrotis−	1.5	3.4	6.1	44.1

[a] The numbers represent the percentage within each group based upon the 2789 isolates tested against all four species. "+" means more than 25% corrected mortality; "−" means 25% or less corrected mortality.

Activity against Diptera

Two thousand five hundred seventy nine isolates were tested against *A. aegypti* and 1945 against *C. pipiens.* The distribution of activities observed against these species among the isolates tested is shown in Fig. 3.

Activity against Lepidoptera, Coleoptera, and Diptera

In another study, broad spectrum activity was analyzed. Two thousand four hundred eighteen isolates have been tested against *H. virescens, P. cochleariae,* and *A. aegypti,* representatives of the orders Lepidoptera, Coleoptera, and Diptera, respectively. According to the same principles as those outlined in the lepidopteran study, these three species allow eight different combinations of insecticidal activities. The results are shown in Table 4.

Correlation between Geographical Origin of Strains and Their Biological Activity

Correlation between biological activity of strains against *P. cochleariae, S. littoralis,* and *H. virescens* and their geographical origins was analyzed. The species was chosen because, in strains analyzed up until now,

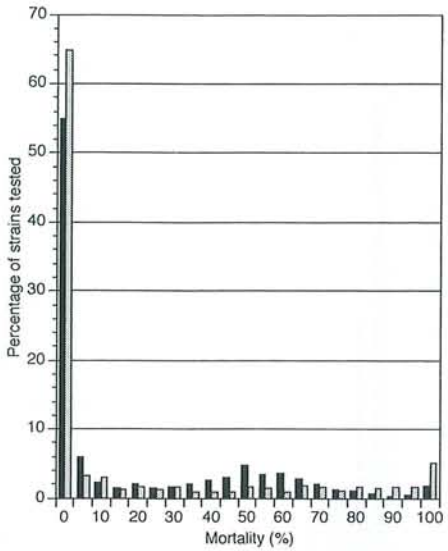

FIG. 3. Distribution of insecticidal activity after 24 hr in single-dose bioassays against dipteran larvae. Black bars, *Aedes aegypti;* dotted bars, *Culex pipiens.*

activities against these three test species have been due to different δ-endotoxins, which are not closely linked genetically with one another. As with the previous studies, strains causing more than 25% corrected mortality were rated active. The results are shown in Fig. 4. Since the population sizes were different, the results are given as percentage of active strains in each population analyzed. The only obvious correlation is the high frequency of strains active in *H. virescens* in North America.

Correlation between Type of Material and Biological Activity

Correlations were also analyzed between biological activity and materials from which strains had been isolated, as shown in Fig. 5. Isolates with all types of biological activity were found in all types of materials. There was no clear correlation between the materials and the frequency of any type of isolate, with the exception of stored products, where strains active against *H. virescens* appear more abundant than average.

TABLE 4

Classification of Isolates Based upon Their Spectra of Activity against the Three Orders of Insects[a]

	Phaedon+		*Phaedon*−	
	Aedes+	*Aedes*−	*Aedes*+	*Aedes*−
Heliothis+	2.3	1.2	11.2	7.2
Heliothis−	3.2	11.2	14.6	48.8

[a] The numbers represent the percentage within each group based upon the 2418 isolates tested against all three species. "+" means more than 25% corrected mortality; "−" means 25% or less corrected mortality.

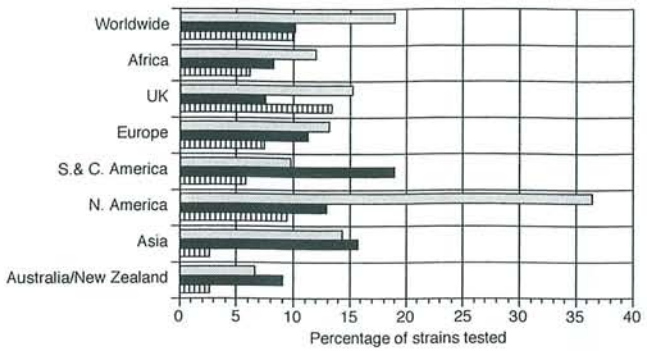

FIG. 4. Correlation between frequency of isolates biologically active and their geographical origin. Dotted bars, *Heliothis virescens;* black bars, *Spodoptera littoralis;* striped bars, *Phaedon cochleariae.*

Correlation between Biological Activity and Crystal Morphology

Correlations between biological activity and crystal morphology were analyzed. In the crystal morphology classification system used for the culture collection, GC91 is characterized as B, *B. thuringiensis* subsp. *israelensis* as I, and *B. thuringiensis* subsp. *tenebrionis* as R. Table 5 shows that for all insects, isolates with bipyramidal crystal morphology were most likely to be active at the dosages tested.

DISCUSSION

Initially strains were isolated by the acetate enrichment method described by Travers *et al.* (1987). These authors claimed that germination of *B. thuringiensis* spores is selectively inhibited in the presence of sodium acetate, whereas most other sporeformers germinated. Selection of *B. thuringiensis* was attempted by eliminating germinated cells by a 3-min heat treatment at 80°C.

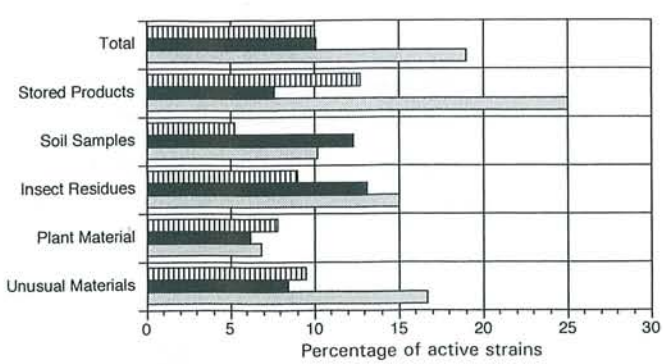

FIG. 5. Correlation between frequency of isolates biologically active and the types of material from which they were isolated. Vertically striped bars, *Phaedon cochleariae;* black bars, *Spodoptera littoralis;* dotted bars, *Heliothis virescens.*

TABLE 5

Percentage of Active[a] Strains in Each Morphological Group[b]

Species	Morphological group (% of active strains in each morphological group)				
	B	S	R	I	P
Aedes aegypti	46.4	12.1	10.7	16.2	18.7
Spodoptera littoralis	20.5	5.2	7.8	12.9	10.7
Heliothis virescens	33.1	7.8	5.7	9.2	9.6
Pieris brassicae	49.3	9.8	10.7	12.5	14.8
Agrotis ypsilon	42.5	23.5	18.1	25.8	24.0
Phaedon cochleariae	28.6	17.3	20.2	13.8	20.3

[a] More than 25% corrected mortality.

[b] Classification of morphological types of parasporal inclusions: B, bipyrarnidal; S, spherical; R, rectangular or cuboid; I, irregularly spherical; and P, irregularly pointed crystals.

We observed only marginal selection by acetate over other bacilli, as reported by Keller and Vriesen (1990). Since acetate selection was time-consuming, the results did not warrant the extra effort; thus, it was abandoned in favor of simple pasteurization.

Most strains were isolated from stored products. The residues in animal feed mills, grain storage silos/barns, and flour mills represent an environment in which isolates of *B. thuringiensis* from worldwide sources can collect and concentrate. The consequence appeared to be a wide variety of isolate types. This finding is consistent with a number of studies: the early discovery of *B. thuringiensis* by Berliner (1915) and Mattes (1927), who isolated strains from mills; the distribution of *B. thuringiensis* isolates in an animal feed mill described by Meadows *et al.* (1992); an experimental investigation into the mechanism of spread of *B. thuringiensis* in stores by Burges and Hurst (1977); and a discussion of the same by Dulmage and Aizawa (1982).

Geographically, the majority of the isolates, 44.7%, originated from materials collected in the United Kingdom (Table 2). Roughly two-thirds of them were isolated from stored product samples. Most of these samples came from produce imported into UK mills. As *B. thuringiensis* is not frequently isolated from UK soil, it can be inferred that the origins of most of the strains isolated from this source were outside the United Kingdom. The true geographic diversity of the isolates in the collection is, therefore, broader than the available data suggest. Among the strains originating from unusual sample types, the majority were isolated from UK samples (Table 2). Unusual sample types were sent only rarely by external contacts.

Thus the most rewarding sample source in which to hunt for isolates active in insects is stored products. This is because of its diversity, ready availability, and high proportion of active isolates.

Selection of isolates depended exclusively on phase-contrast microscopy. Isolates producing bipyramidal crystals were the most common morphological type in the collection (Table 2). This was mainly caused by the large number of isolates originating from stored product samples. However, spherical or irregularly shaped inclusion bodies were the most common types isolated from some of the other sample materials.

In order to compensate for differences in susceptibility between insect species, as well as between different batches of larvae from one species, all bioassay data were corrected for control mortality and test mortality relative to a standard strain in every assay batch. The activities reported are therefore not absolute values, but are relative to the standard strains. The distribution of insecticidal activity among the isolates tested is similar for all four lepidopterous species, with most isolates being less active than the standard, demonstrating that strain GC-91 has been a sufficiently demanding standard for all test Lepidoptera (Fig. 1).

Every possible combination of activities can be found among the strains tested against all four lepidopterous species (Table 3). If activities against the different species were distributed at random, each group would contain 6.25%. Not unexpectedly, the group comprising strains inactive against all four species is the largest. This group also comprises strains selectively active against other orders of insects. The other groups comprise between 0.6 and 14.5% of the strains tested, suggesting considerable diversity of insecticidal activity. The group comprising strains active against all four test insect species also contains an inevitable few reisolates of the GC-91 standard strain.

The percentages of strains belonging to the groups of strains active against both *H. virescens* and *S. littoralis* are very low. This is understandable, since in the standard strain, GC-91, against which they are compared, activity against these two species is high and can be attributed to two different δ-endotoxins, which are not normally found in one strain. It is interesting to note that strains selectively active against only *A. ypsilon* (14.5%) or only *P. brassicae* (6.1%) are more abundant than strains that are also active against a second or third test insect (0.6–5.1%), suggesting the presence of more selective genes against either of these two species than against the other two. In contrast, strains selectively active against *H. virescens* (3.8%) are less abundant than those which are active against *P. brassicae* alone (6.1%) or *P. brassicae* and *A. ypsilon* (4.3%), suggesting the presence of few genes selectively active against *H. virescens*.

In the tests against the coleopteran *P. cochleariae,* as with the lepidoptera, a wide spectrum of activities was observed with a small peak at 100% mortality (Fig. 2). Although only strains active against *P. cochleariae* were tested against *L. decemlineata,* the most active strains were only about as active as the standard. The tests against *P. cochleariae* revealed a wider range of

toxins in the *B. thuringiensis* culture collection than the tests against *L. decemlineata*. This difference may be explained by the fact that, as such a high concentration of broth was used against *P. cochleariae,* further material had to be produced for the tests against *L. decemlineata*. As this was produced on agar plates, it is less likely to contain soluble factors found in broth cultures. Therefore, exotoxin-producing isolates may be present among those active against *P. cochleariae*. Such exotoxins could be either β-exotoxin, effective because a high dosage of broth was used, or others, which have not yet been described. No attempt was made to further investigate this observation.

Against *A. aegypti,* a high proportion of strains were active (Fig. 3), the major reason being that the standard strain used was strain HD-571. This strain is about 1000-fold less potent against *A. aegypti* compared to *B. thuringiensis* subsp. *israelensis,* the strain used commercially against dipteran insects.

Some strains exhibit a spectrum of insecticidal activity to species belonging to the three different orders of insects. In our analysis, every possible combination of activity among these orders could be found among the strains tested (Table 4). The percentages of strains found in each group deviate considerably from the 12.5% which would be expected from random distribution. In this analysis, as well as that with the Lepidoptera (Table 3), the most abundant group (48.8%) comprises strains inactive against all three test species. Due to the selectivity even within the order Lepidoptera, this group is likely also to comprise strains which are selectively active against *P. brassicae, S. littoralis,* or *A. ypsilon* but not *H. virescens*. As in the analysis on Lepidoptera, strains with narrow spectra of insecticidal activity are more abundant (7.2–14.6%) than strains with wider ranges of activity. Accordingly, strains selectively active against *P. cochleariae* are more abundant (11.2%) than strains with additional activity against either *H. virescens* or *A. aegypti* alone or both species (1.2–3.2%). Strains active only against *H. virescens* and *P. cochleariae* (1.2%) are also much less abundant than strains selectively active against *H. virescens* (7.2%). Similar results can be observed with *A. aegypti*. One exception is with *H. virescens,* where strains active against both *H. virescens* and *A. aegypti* (11.2%) are more abundant than strains selectively active against *H. virescens* (7.2%). A likely reason is the relatively high dose rates used in the *A. aegypti* bioassays, due to the much less demanding standard strain used. Isolates producing only moderately to weakly active δ-endotoxins, like the CryII proteins (Iizuka and Yamamoto, 1983; Hofte and Whitely, 1989) found in many strains, would be rated as active.

The finding that a high proportion of the isolates were inactive in all test insects fits similar observations in the literature using different criteria for activity and different insects. Ohba and Aizawa (1986a) were the first to observe that nontoxic *B. thuringiensis* isolates predominate and are widely distributed in natural environments. Martin and Travers (1989) found 60% of isolates from soil worldwide to be inactive in Lepidoptera and 77% in mosquitoes, with 40% inactive in all the species tested. Abdel-Hameed and Landen (1994) found that 26% of isolates from Swedish soils were inactive in test Lepidoptera, Diptera, and Coleoptera.

We had expected to establish correlations between the frequency of active isolates with either geographical origin or types of the material samples from which they had been isolated. With two possible exceptions, no such correlation could be established (Figs. 4 and 5). Isolates representing each major spectrum of activity could be found in materials obtained from every geographic region (Fig. 4). This indicates that *B. thuringiensis* is relatively ubiquitous. There may be several explanations for this.

1. Distribution of B. thuringiensis by humans. B. thuringiensis is commonly found in stored agricultural products, which for many centuries have been transported between continents on a large scale. Evidence for such distribution in recent times has been documented by Norris (1969). In recent decades, insecticidal preparations based upon a few strains have found increasing use as insecticides. However, Martin and Travers (1989) did not isolate *B. thuringiensis* from the Washington area of the United States, where *B. thuringiensis* has been used extensively in agriculture, as frequently as from many other areas of the world.

2. Distribution of B. thuringiensis by natural causes. B. thuringiensis produces endospores which are highly tolerant to adverse environmental conditions. It is therefore likely to survive nonanthropogenic transport by water, wind, and migrating animals. Dulmage and Aizawa (1982) discuss its persistence and transport in the environment.

3. Molecular parasitism/symbiosis. No consistent correlation could be found between types of material and frequency of active strains (Fig. 5). In this context it is interesting to note that, at the doses tested, 15.5% of the strains isolated from soil were active although none of our test insects is a true soil insect. In comparison, the percentages of active strains in stored products and insects are only 29.6 and 23.3%, less than twice as high. These observations suggest that insecticidal activity may not be an important positive selective factor. The observation that about half the isolates are only marginally active or inactive against the test insects (Tables 3 and 4) is also consistent with this, even if one considers that some of them might be highly toxic to organisms not tested. Therefore some other factor(s) is needed which allows maintenance of *B. thuringiensis* in its environment: *B. thuringiensis* is closely related to

the common soil bacterium *B. cereus* from which it differs only by the production of parasporal crystals. As Gonzalez *et al.* (1982) and others have shown, self-transmissible plasmids can transfer δ-endotoxin genes from *B. thuringiensis* to *B. cereus* at high frequency, where they are expressed to give rise to production of parasporal inclusions. If the rate of transfer is higher than the rate of spontaneous plasmid loss, δ-endotoxin genes could be maintained and may even spread in the *B. cereus* population together with their self-transmissible plasmid vectors. Ohba and Aizawa (1986b) reported that there was a high serological cross-reactivity between *B. thuringiensis* and *B. cereus,* providing insight into the genealogical and ecological relationships between these two organisms.

Martin and Travers (1989) showed both a geographical and a climatic correlation with the number of isolates they found. Their results differed from those above. However, most correlations had notable exceptions. They also did not find a correlation between high incidence of *B. thuringiensis* and the abundance of insects in soil. This, however, would not exclude the possibility that there are instances where insecticidal activity may be a positive selective factor for *B. thuringiensis.* In one instance, correlation between origin or sample type and insecticidal activity could be found: almost twice the average of all strains isolated from samples collected in North America are active against *H. virescens* (Fig. 4). Since *Heliothis* spp. are major agricultural pests in this region, the high incidence of active strains suggests a selective advantage. The frequency of strains active against *H. virescens* is also highest among strains isolated from stored product samples (Fig. 5). Only a few lepidopterous species are stored product pests. If they happen to be susceptible to the same types of δ-endotoxins as *H. virescens,* it would also indicate natural selection due to insecticidal activity.

Strains were selected for entry into our strain collection solely on account of the production of parasporal inclusion bodies. The initial isolates recorded in the literature of *B. thuringiensis* toxic to lepidopteran larvae produced parasporal inclusion bodies of bipyramidal shape. Since the initial isolates with activity against other orders of insects exhibited other crystal shapes, and were therefore initially not classified as *B. thuringiensis,* we were anxious not to miss novel strains because of the unusual shape of their parasporal inclusion bodies. This was justified, since although strains with bipyramidal crystals were the most likely to be toxic to insect larvae, we also found many toxic strains of other crystal shapes (Table 5). Surprisingly, there is no clear correlation between the type of insecticidal activity and the crystal morphology. Considering for example that spherical or irregular crystal types are historically associated with dipteran activity, it is surprising that so many B crystal types should prove to be active against the mosquito (Table 5). Secondary crystals, as found in strain HD-1 have been shown to have dipteran activity, but there is no particular difference between the proportion of isolates with (45.6%) and without (46.9%) a secondary crystal which have *Aedes* activity. Strains with bipyramidal crystals also have the highest proportion of strains active against *P. cochleariae,* followed by strains with rectangular and with pointed crystals. Although there may be a number of reasons for this, it is obvious that coleopteran activity of strains cannot be predicted from the crystal morphology. No correlation between crystal morphology and the type of insect activity was found by Martin and Travers (1989) or by Obha and Aizawa (1986a).

Regarding soil, it can be concluded that *B. thuringiensis* is distributed sparsely but frequently and widespread, both locally and worldwide. Martin and Travers (1989) reached the same conclusion globally, as did a number of authors in regional studies, e.g., Padua *et al.* (1982) in the Philippines, Landen *et al.* (1994) in Sweden, Ohba and Aizawa (1978) in Japan, and Chilcott and Wigley (1993) in New Zealand. In the United States, DeLucca *et al.* (1981) found about two orders of magnitude fewer isolates in soil than Martin and Travers (1989). Smith and Couche (1991) found *B. thuringiensis* to be common on the phylloplane in the United States.

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