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# The Phylloplane as a Source of *Bacillus thuringiensis* Variants

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**Novel variants of *Bacillus thuringiensis* were isolated from the phylloplane of deciduous and conifer trees as well as of other plants. These isolates displayed a range of toxicity towards *Trichoplusia ni*. Immunoblot and toxin protein analysis indicate that these strains included representatives of the three principal *B. thuringiensis* pathotypes active against larvae of the orders Lepidoptera, Diptera, and Coleoptera. We propose that *B. thuringiensis* be considered part of the common leaf microflora of many plants.**

*Bacillus thuringiensis* is a gram-positive, spore-forming bacillus that produces a proteinaceous parasporal inclusion in association with sporulation. *B. thuringiensis* spores and/or inclusion bodies typically express insecticidal activity, and several strains are used on a global basis for control of larval forms of agriculturally important insect pests. In recent years, the need for environmentally safe pesticides has encouraged the search for new strains of *B. thuringiensis* with different target spectra. These novel isolates will augment the current commercial *B. thuringiensis* strains that exhibit various degrees of activity toward larvae of the insects of the orders Lepidoptera, Diptera, and Coleoptera.

More than 1,000 isolates of *B. thuringiensis* are categorized in two major collections (3, 12). These isolates have been recovered from numerous sources, including soils, grain dust, diseased insect larvae from insectaries, and sericulture environments. The principal source of novel *B. thuringiensis* isolates has been soil. DeLucca et al. (4) reported that *B. thuringiensis* made up less than 0.5% of more than 46,000 bacterial isolates recovered from various soils in the United States. Travers et al. (21) described a technique which increased the frequency at which *B. thuringiensis* could be recovered from soils. Subsequently, Martin and Travers (11) and workers in our laboratory (unpublished results) have recovered *B. thuringiensis* from numerous soils obtained from around the world. Ohba and Aizawa (13) have reported the recovery of over 300 *B. thuringiensis* isolates from Japanese soils and sericulture areas.

During the study of *B. thuringiensis* strains from the environment, the contradiction between the commercial use of this organism for the biological control of leaf-feeding insects and the tenet that *B. thuringiensis* is a soil microorganism became apparent. We proposed that infection of insect larvae by this organism is a natural event and that *B. thuringiensis* is present on the phylloplane, where it has access to leaf-feeding insect larvae. Literature to support our hypothesis has not been found. The majority of published studies about leaf surface microflora are concerned with fungal hyphae and spores (5, 17). Bacteria are present, often in high numbers, but little is known about their biology on leaf surfaces (5). Several reports describe *B. thuringiensis* residues from spray programs (10, 16, 18), but in only two instances have data that might indicate a natural presence of this organism on the phylloplane been reported. The first instance was an article by Grison et al. (7), who reported that *B. thuringiensis* spores could persist for several months in shaded pine forests. The second report attributed the presence of *B. thuringiensis* on balsam fir (*Abies balsamea*) to residue from a spray the previous year (18). In neither case were prespray data available to provide a baseline *B. thuringiensis* count.

The objective of this study was to survey the phylloplanes of temperate-climate trees to determine whether *B. thuringiensis* could be recovered. If *B. thuringiensis* was present on leaf surfaces, the finding would suggest an ecological connection between *B. thuringiensis* and susceptible insect larvae.

Sampling sites for leaf samples were chosen that minimized the potential for contamination by soil splash or wind-borne inoculum and that provided maximum protection of the phylloplane microbial population from UV light. Leaf samples were typically obtained 2.0 to 2.5 m above the ground, 0.3 m inside the outer leaf canopy, and from the east side of each tree or shrub. Cross contamination between samples was prevented by detaching leaves or needles while they were enclosed in standard plastic sandwich bags, which were immediately sealed for storage. This procedure was carried out by inverting the bag over the collector's hand, grasping the leaves or needles to be sampled, and removing them from the branch.

Three methods were used to recover *B. thuringiensis* from leaf surfaces: (i) the shaken-flask technique, (ii) the leaf lift technique, and (iii) the leaf scrub technique. For the shaken-flask technique, 5- to 6-mm sections were cut from three different leaves per sample for a total tested leaf area of approximately 120 $cm^2$. The leaf sections were placed in an Erlenmeyer flask (250 ml) containing 10 ml of Luria broth and shaken at 250 rpm for 4 h at 28 to 30°C. After incubation, 200-μl aliquots of the resulting suspensions were placed in glass test tubes (10 by 70 mm) and incubated in an 80°C water bath for 3 min. The heat-treated suspension was cooled on ice, and 100-μl aliquots were spread on plates of nutrient agar supplemented with yeast extract medium (NYSM agar). After a 72-h incubation at 28 to 30°C, colonies were examined microscopically for the presence of crystalliferous organisms. Three aliquots from the flask suspension were processed for each leaf sample.

Leaves sampled by the leaf lift technique were trimmed to fit inside a 100-mm petri dish. The abaxial leaf surface was placed in contact with NSYM agar, and a sterile, perforated stainless steel disk was placed on the leaf section to ensure maximum contact with the agar. The lid was replaced, and the sample was incubated at 28 to 30°C overnight.

The leaf scrub technique was performed as follows. Two

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TABLE 1. Estimated recovery of *B. thuringiensis* from leaf samples with highest total bacterial count from specimens observed

| Sample source | Recovery temp (°C) | Bacterial CFU/cm² ($10^1$)[a] | |
|---|---|---|---|
| | | Total | *B. thuringiensis* |
| *Tilia americana* (basswood) | 22 | 3,000 | 0 |
| | 65 | 72 | 0 |
| | 80 | 80 | 40 |
| *Tilia americana* (basswood) | 22 | 4,000 | 28 |
| | 65 | 80 | 40 |
| | 80 | 80 | 28 |
| *Acer platanoides* (Norway maple) | 22 | 1,000 | 952 |
| | 65 | 800 | 672 |
| | 80 | 1,000 | 980 |
| *Quercus macrocarpa* (bur oak) | 22 | 800 | 300 |
| | 65 | 600 | 400 |
| | 80 | 500 | 300 |
| *Ulmus serotina* (red elm) | 22 | 1,000 | 228 |
| | 65 | 1,000 | 752 |
| | 80 | 1,000 | 952 |
| *Ulmus thomasii* (rock elm) | 22 | 1,500 | 56 |
| | 65 | 200 | 32 |
| | 80 | 180 | 60 |
| *Prunus serotina* (black cherry) | 22 | >5,000[b] | 56 |
| | 65 | 112 | 32 |
| | 80 | 120 | 48 |
| *Salix fragilis* (brittle willow) | 22 | >5,000 | 32 |
| | 65 | 100 | 56 |
| | 80 | 60 | 32 |

[a] Based on ca. 120 cm² sampled per specimen.
[b] Maximum valid estimate possible from plate counts.

leaves from each sample were swabbed vigorously with a sterile cotton swab dipped in sterile Triton X-100 (50 ppm). Approximately 60 cm² of abaxial surface was swabbed, yielding 3 ml of washings. This volume was centrifuged at 15,000 × *g* for 6 min. The pellet was resuspended to 0.5 ml and subjected to the following treatments: no treatment (to identify spores and vegetative or heat-sensitive cells), 65°C for 30 min (to identify heat-sensitive spores), and 80°C for 30 min (to identify heat-resistant spores). Triplicate samples (100 μl) of each suspension were plated as described previously.

Numbers of *B. thuringiensis* CFU per leaf surface area (Table 1) were derived on the basis of a sampled leaf area of ca. 120 cm², a sample volume of 3 ml, and a plated volume of 0.1 ml in triplicate. The equation for quantifying numbers of *B. thuringiensis* CFU per sample was Bt/cm² = Bto × (Bct/Bco) × 100, where Bto is the number of *B. thuringiensis* CFU observed microscopically, Bct is the total number of *Bacillus cereus*-like CFU from the plate count, and Bco is the number of *B. cereus* CFU observed microscopically. The number 100 is a correction factor accounting for numbers of bacteria from intercellular spaces (9) and for the comparison between plate count estimates and estimates from microscopic examination (14).

Three standard *B. thuringiensis* strains were used for a comparison of toxin protein patterns and for dot blot analysis of phylloplane isolates. *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* are the strains used in the commercial products DiPel and Vectobac, respectively; both products are manufactured by Abbott Laboratories. The *B. thuringiensis* subsp. *tenebrionis* strain was obtained from A. Kreig, University of Heidelberg. These strains are representative of pathotype organisms that exhibit toxicity toward the larvae of three economically important insect orders: Lepidoptera, Diptera, and Coleoptera.

Crystal protein analysis was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by dot blot enzyme immunoassay, as described previously (2). Strains used for analysis were grown to sporulation in 50 ml of nutrient broth per 250-ml flask on a rotary shaker (250 rpm) at 28°C. Particulates from 100 μl of sporulated culture were washed first with 1 M NaCl containing 5 mM EDTA and then with 5 mM EDTA. Washed crystals and spores were extracted for 5 min at 100°C in 100 μl of 1× sample buffer containing 5 mM EDTA. Insoluble material was removed by centrifugation. Aliquots (5 μl each) were loaded onto 10% acrylamide gels.

Immunogens for the preparation of antisera were derived from purified *B. thuringiensis* crystals. Crystals were purified from sporulated cultures by isopycnic centrifugation in 20 to 35% (wt/wt) linear gradients of sodium bromide (*B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis*) or 40 to 65% Renografin (Squibb) gradients (*B. thuringiensis* subsp. *tenebrionis*). Purified crystals were dialyzed against deionized water and lyophilized. Antisera were raised in New Zealand White rabbits as described previously (2). The immunogen consisted of macerated acrylamide gel strips containing approximately 200 μg of protoxin emulsified in Freund's incomplete adjuvant. The protoxins were the 130/133-kDa toxin complex from *B. thuringiensis* subsp. *kurstaki*, the 70-kDa toxin band from the *B. thuringiensis* subsp. *israelensis* crystal protein complex (15), and the 65-kDa band from *B. thuringiensis* subsp. *tenebrionis* crystal proteins. Procedures for the affinity purification of immunoglobulin G with protein A and for an enzyme immunoassay with dot blots were as described by Couche and Gregson (2), except that the concentration of the primary antibody reagent was 10 μg/ml for *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* toxin immunoglobulin G and 20 μg/ml for *B. thuringiensis* subsp. *tenebrionis* toxin immunoglobulin G. The antibodies used in these studies displayed no cross-reaction with nonhomologous toxin proteins.

Samples prepared for SDS-PAGE were also used for immunodot blots. Aliquots containing approximately 1 μg of protein were processed as described by Couche and Gregson (2). Samples were applied to nitrocellulose filters by using a Biodot microfiltration apparatus (Bio-Rad) as described in the manufacturer's instructions. Immunological reactions were quantified with a Bio-Rad model 620 video densitometer in reflectance mode. Data were corrected for background. A reaction less than 25% of the appropriate standard was considered negative.

The biological activities of *B. thuringiensis* strains isolated from leaf surfaces against third-instar cabbage loopers (*Trichoplusia ni*) were determined. Lyophilized plate cultures were incorporated into an artificial diet at six dose levels and were assayed in duplicate on two successive days. Mortality was assessed after 4 days, and biological activity units were calculated by comparing this mortality with the mortality from a standardized preparation of *B. thuringiensis* strain HD-1.

Our initial survey of leaf surfaces for the presence of *B. thuringiensis* was conducted in Lake County, Illinois, during the summer and autumn of 1987. All samples were subjected to shaken-flask recovery and heat treatment at 80°C. *B. thuringiensis* was recovered from the phylloplanes of 10 of 12 common species of deciduous trees or shrubs. These included *Prunus serotina* (black cherry), *Morus alba* (mulberry), *Quercus* sp. (oak), *Acer nigrum* (black maple), *Acer saccharinum* (silver maple), *Castanea dentata* (American chestnut), *Cornus stolonifera* (red dogwood), *Parthenocissus tricuspidata* (Boston ivy), *Malus* sp. (apple), and *Tilia americana* (basswood). No *B. thuringiensis* was recovered from *Fraxinus americana* (white ash) or *Betula papyrifera* (canoe birch) during this phase of the study.

In 1988, leaf samples were taken from the time of leaf emergence to October. During this period, *B. thuringiensis* was regularly recovered from the phylloplanes of *Acer* sp., *Quercus* sp., and *Ulmus* sp. In addition to being recovered from deciduous trees, *B. thuringiensis* was also recovered from the leaves of *Podophyllum peltatum* (mayapple), *Typha latifolia* (cattail), and various turf and prairie grasses.

Recovery techniques were refined during these studies. The refinements included the introduction of leaf lift and leaf swab recovery techniques, an assessment of the effect of isolation temperature on the recovery of *B. thuringiensis* from leaves of selected trees, and a quantitative estimation of *B. thuringiensis* numbers per unit of leaf area (Table 1). While *B. thuringiensis* could be recovered under ambient conditions, such recovery was not desirable because of extensive contamination by phylloplane bacteria and fungi. Asporogenous bacteria such as *Pseudomonas* sp. and *Flavobacterium* sp. were most common between May and July. Fungi were more prominent from August to October. Acrystalliferous, sporeforming bacteria were infrequent, thus making recovery of *B. thuringiensis* from leaves simpler than recovery from soil, where the sporeformer background is significantly higher. A moderate heat treatment at 65°C eliminated contamination by fungi and asporogenous bacteria and appeared to promote the germination of spores from *B. thuringiensis* and other sporogenous bacteria. *B. thuringiensis* spores from leaves of *Rhus copallina* and *P. serotina* were sensitive to the more typical 80°C heat treatment.

The recovery frequency of *B. thuringiensis* from leaf surfaces was high, 50 to 70% of trees being positive for this organism. Numbers of *B. thuringiensis* CFU relative to other sporeforming bacteria were calculated for plant species that yielded the highest numbers of *B. thuringiensis* CFU during the 1988 surveys (Table 1). *B. thuringiensis* accounted for 30 to 100% of the sporeformer population detectable on leaves from these plants. The density of *B. thuringiensis* recovered from leaf surfaces varied over 2 orders of magnitude, from 3 CFU per $cm^2$ from leaves of *Salix fragilis* to approximately 100 CFU per $cm^2$ from leaves of *Acer platanoides* and *Ulmus serotina* (Table 1). The numbers of *B. thuringiensis* CFU obtained from the phylloplane and the recovery frequency were higher than would be expected with casual isolates (19). Although the literature addresses nonsporeformers more commonly than it does sporeformers, some insights into expected sporeformer populations can be found. At peak infection periods, the nonsporeformer *Pseudomonas savastanoi* has been reported to be present on the phylloplane of *Olea europaea* (olive) at a density of $10^4$ bacteria per $cm^2$ (6). The remainder of the year, *P. savastanoi* was present as an epiphyte at densities of $10^2$ to $10^3$ bacteria per $cm^2$. Sporeforming bacilli have been identified on the phylloplane at densities of 6 to $10^3$ bacteria per $cm^2$ (8, 19). Organisms recovered from the phylloplane at densities of less than 10 bacteria per $cm^2$ are generally regarded as casual isolates (19). On the basis of these criteria, *B. thuringiensis* should be considered a phylloplane epiphyte on many of the plants sampled in the current survey.

From September 1988 to March 1989, we extended our phylloplane studies to include conifer trees. *B. thuringiensis* was recovered from the phylloplanes of conifers in the north, west, and central United States throughout the winter. Trees yielding *B. thuringiensis* and their locations were as follows: *Pinus strobus* (white pine), Illinois and Michigan; *Pinus resinosa* (red pine), Illinois; *Pinus banksiana* (jack pine), Illinois; *A. balsamea* (balsam fir), Illinois, Wisconsin, and Michigan; *Pinus nigra* (Austrian pine), Illinois; *Thuja occidentalis* (northern white cedar), Wisconsin; *Pinus contorta* (lodgepole pine), Colorado; *Picea* sp. (spruce), Colorado; and *Pinus sylvestris* (scotch pine), Nebraska. *B. cereus* has previously been reported to be present on Japanese red pine (*Pinus densiflora*) throughout the year (22), and bacilli have been reported to be a prominent segment of the microflora isolated from Norway spruce (*Pinus abies*) in the United Kingdom (1). Recovery of *B. thuringiensis* from conifers in these studies is the first evidence that the spore form of this organism is present on the phylloplane for an extended period of time.

For the purpose of the leaf surveys, *B. thuringiensis* was defined as any sporeforming bacillus that made a parasporal body in association with sporulation. Isolates obtained during these surveys were further characterized by analysis of crystal proteins and by their biological activity against *T. ni* (cabbage looper). Crystal proteins were analyzed by SDS-PAGE and immunological techniques to determine the similarity of these strains to known *B. thuringiensis* pathotypes and to assess the degree of diversity among them. Protein patterns from 21 of these strains are shown in Fig. 1. Some of the isolates exhibited protein patterns similar to those of *B. thuringiensis* subsp. *kurstaki* (Fig. 1, lanes 5, 11, 17, and 20), *B. thuringiensis* subsp. *israelensis* (lanes 9 and 10), and *B. thuringiensis* subsp. *tenebrionis* (lane 19). Other strains exhibit unique protein patterns. For instance, protein patterns in lanes 6, 7, 12, 13, 14, and 22 differ from those of the more commonly studied standard strains (lanes 2 through 4).

Antibodies to crystal proteins can be used to differentiate among isolates of the three major pathotypes (20). SDS extracts of 81 leaf isolates were analyzed by an immunoblot technique using antibodies raised against toxin proteins of *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *israelensis*, and *B. thuringiensis* subsp. *tenebrionis*. Extracts from 63% of the phylloplane isolates reacted strongly with the lepidopteran toxin antibody, 2% reacted strongly with the dipteran toxin antibody, and 16% reacted with the coleopteran toxin antibody. Five percent of the extracts reacted equally to both lepidopteran and coleopteran antibodies, and 14% of the isolates did not produce immunoreactive protein.

We further demonstrated the insecticidal activity of many of these isolates (Table 2). Eleven of sixteen isolates selected for bioassay against *T. ni* larvae were active in the same range as strain HD-1, a component of the commercial product DiPel, which is used to control lepidopteran larvae. Of five strains with minimal activity against *T. ni*, one isolate (6777) produced protein homologous to the coleopteran-specific toxin according to our immunological analysis and would not be expected to exhibit activity against lepidopterans. Finally, one biologically active isolate (6781) was immunologically unreactive in our analysis.

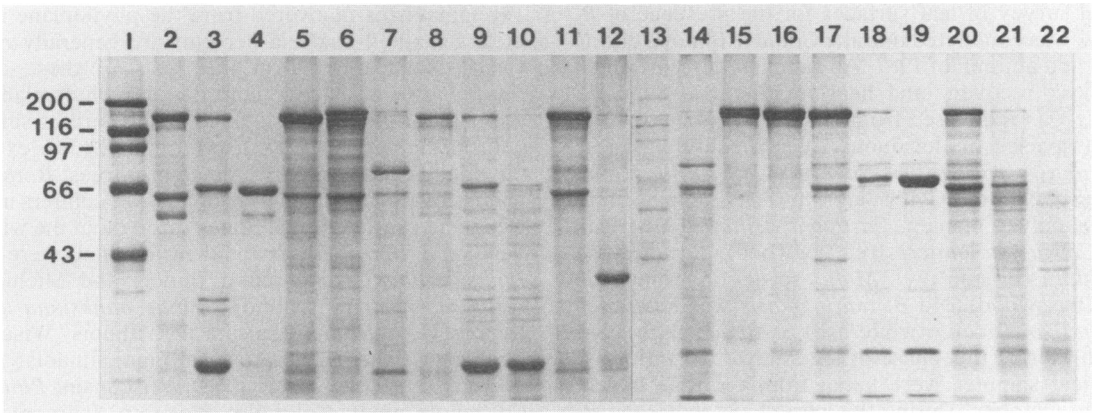


FIG. 1. SDS-PAGE analysis of selected *B. thuringiensis* strains isolated from phylloplanes. SDS-soluble toxin protein was extracted from sporulated cultures. Numbers at left indicate the molecular masses (in kilodaltons) of marker proteins (Bio-Rad). Lanes 2, 3, and 4, Standard strains *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *israelensis*, and *B. thuringiensis* subsp. *tenebrionis*, respectively.

We believe that *B. thuringiensis* isolates recovered from the phylloplane are naturally occurring organisms rather than residues from agricultural sprays for the following reasons. Firstly, these organisms include crystalliferous isolates that do not respond to antibodies reactive against proteins from the three pathotypes currently formulated into commercial products. Secondly, we observed a high percentage of coleopteran-specific isolates. It seems unlikely that use of coleopteran-specific *B. thuringiensis* has been sufficient for these organisms to make up 16% of our phylloplane isolates from across the United States. Thirdly, several isolates produced proteins with immunoreactivity to both lepidopteran- and coleopteran-specific toxins, indicating that these strains produce novel combinations of toxin proteins.

The observed population densities of *B. thuringiensis* on the phylloplane suggest that this species should be considered a phylloplane epiphyte. It is also likely that *B. thuringiensis* is metabolically active on the phylloplane or in intercellular spaces. If *B. thuringiensis* does indeed proliferate to any degree on the phylloplane, one can postulate that it may contribute to a low level of insect larva mortality in the field. *B. thuringiensis* has not been reported to be the causative agent in insect epizootics. We propose that rather than cause epizootics, *B. thuringiensis* populations may be so large that they may serve as insect larval antifeedants, thereby offering a degree of plant protection from phytophagous insect larvae. This situation may be a commensal or symbiotic relationship in which *B. thuringiensis* also benefits from being a phylloplane epiphyte. For example, the plant may provide nutrients from leaf exudates and associated microflora and also provide a niche free from competition with other soil-borne sporogenous bacteria. On the phylloplane, *B. thuringiensis* is accessible to leaf-feeding insect larvae. If the larvae ingest sufficient toxin spore and/or crystals to perturb feeding, host plant protection due to reduced defoliation may result. This hypothesis does not exclude other concepts of the ecological role of *B. thuringiensis*, for example, that they may serve as antifungal or antibacterial agents in soil microcosms; rather, it accounts for the high frequency at which *B. thuringiensis* was observed on leaves of temperate-region deciduous and conifer trees.

TABLE 2. Insecticidal activity of *B. thuringiensis* phylloplane isolates as measured against *T. ni* larvae

| Isolate | Immunological response[a] | Bioactivity units/mg[b] |
|---|---|---|
| 6743 | L | <200 |
| 6755 | L | 939 |
| 6756 | L | 806 |
| 6777 | C | <200 |
| 6780 | L | <200 |
| 6781 | NR | 500 |
| 6782 | NR | <200 |
| 6800 | L | 933 |
| 6801 | L | 822 |
| 6803 | L | 500 |
| 6817 | L | 891 |
| 6818 | L | 882 |
| 6829 | L | <200 |
| 6835 | L | 903 |
| 6836 | L | 850 |
| 6838 | L | 500 |
| HD-1 | L | 792 |

[a] L, Lepidopteran-specific; C, coleopteran-specific; NR, no response to antibodies.

[b] Mean units of bioactivity per milligram of lyophilized powder for duplicate tests. Mean standard deviation was ±25%. Activity based upon a standardized strain HD-1 powder.

The results of these studies suggest that the role of *B. thuringiensis* in the environment should be reconsidered. We are extending these studies to include quantifying the succession of *B. thuringiensis* through the growing season, determining whether *B. thuringiensis* is metabolically active on the phylloplane, and examining the bark and leaf buds to determine whether they are additional sources of novel *B. thuringiensis* isolates.

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## REFERENCES

1. **Collins, M. A., and A. J. Hayes.** 1976. Seasonal incidence of microbes on the surface of first year needles of Norway spruce. Trans. Br. Mycol. Soc. **66**:457–461.
2. **Couche, G. A., and R. P. Gregson.** 1987. Protein inclusions produced by the entomopathogenic bacterium *Xenorhabdus nematophilus* subsp. *nematophilus*. J. Bacteriol. **169**:5279–5288.
3. **DeBarjac, H.** 1986. Centre collaborateur O.M.S. de recherche et de reference pour les bacteries sporulees entomopathogenes.

Institut Pasteur, Paris.
4. **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.
5. **Dickenson, C. H., and T. F. Preece (ed.).** 1976. Microbiology of aerial plant surfaces. Academic Press, Inc., New York.
6. **Ercolani, G. L.** 1978. *Pseudomonas savastonoi* and other bacteria colonizing the surface of olive leaves in the field. J. Gen. Microbiol. **109**:245–257.
7. **Grison, P., D. Martouret, B. Servais, and M. Devriendt.** 1976. Pesticides microbiens et environnement. Ann. Zool. Ecol. Anim. **8**:133–160.
8. **Kangsen, I. N., and R. G. Kapooria.** 1979. Studies on the succession of phylloplane microflora of developing leaves of *Hevea* rubber. Trop. Ecol. **20**:41–47.
9. **Last, F. T., and F. C. Deighton.** 1965. The non-parasitic microflora on the surfaces of living leaves. Trans. Br. Mycol. Soc. **48**:83–89.
10. **Leong, K. L. H., R. J. Cano, and A. M. Kubinski.** 1980. Factors affecting *Bacillus thuringiensis* total field persistence. Environ. Entomol. **9**:593–599.
11. **Martin, P. A. W., and R. S. Travers.** 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. **55**:2437–2442.
12. **Nakamura, L. K., and H. T. Dulmage.** 1988. *Bacillus thuringiensis* cultures available from the U.S. Department of Agriculture. ARM-S-30. U.S. Department of Agriculture, Washington, D.C.
13. **Ohba, M., and K. Aizawa.** 1989. Distribution of the four flagellar (H) antigenic subserotypes of *Bacillus thuringiensis* H serotype 3 in Japan. J. Appl. Bacteriol. **67**:505–509.
14. **Paul, E. A., and F. E. Clark (ed.).** 1989. Soil microbiology and biochemistry. Academic Press, Inc., New York.
15. **Pfannenstiel, M. A., G. A. Couche, E. J. Ross, and K. W. Nickerson.** 1986. Immunological relationships among proteins making up the *Bacillus thuringiensis* subsp. *israelensis* crystalline toxin. Appl. Environ. Microbiol. **52**:644–649.
16. **Pinnock, D. E., R. J. Brand, and J. E. Milstead.** 1971. The field persistence of *Bacillus thuringiensis* spores. J. Invertebr. Pathol. **18**:405–411.
17. **Preece, T. F., and W. C. Wong.** 1981. Detectable attachment of bacteria to intact plant and fungal surfaces, p. 399–410. *In* J. P. Blakeman (ed.), Microbial ecology of the phylloplane. Academic Press, Inc., New York.
18. **Reardon, R. C., and K. Haissig.** 1984. Efficacy and field persistence of *Bacillus thuringiensis* after ground application to balsam fir and white spruce in Wisconsin. Can. Entomol. **116**:153–158.
19. **Singh, N., P. S. Randhawa, and B. Kumar.** 1984. Quantitative and qualitative bacterial flora of two cotton cultivars in relation to age of the crop and environmental conditions. Indian J. Ecol. **11**:127–133.
20. **Smith, R. A.** 1987. Use of crystal serology to differentiate among varieties of *Bacillus thuringiensis*. J. Invertebr. Pathol. **50**:1–8.
21. **Travers, R. S., P. A. W. Martin, and C. F. Reichelderfer.** 1987. Selective process for efficient isolation of soil *Bacillus* spp. Appl. Environ. Microbiol. **53**:1263–1266.
22. **Yoshimura, F.** 1982. Phylloplane bacteria in a pine forest. Can. J. Microbiol. **28**:580–592.