Comparative Studies of Growth Characteristic and Competitive Ability in *Bacillus thuringiensis* and *Bacillus cereus* in Soil

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We examined the growth characteristics and competitive abilities of *Bacillus thuringiensis* and *Bacillus cereus* in soil. The growth and persistence of the two bacilli, added to sterile soil with or without soil bacteria, were monitored under aerobic conditions. When vegetative cells or spores of *B. thuringiensis* and/or *B. cereus* were added to sterile soil, both bacilli increased their numbers. The final levels of population were greater for *B. cereus* than for *B. thuringiensis*. In most of the cases when vegetative cells of *B. thuringiensis* were added at the same time as indigenous soil bacteria to sterile soil, *B. thuringiensis* sporulated immediately without any growth. *B. thuringiensis* grew only when both *B. thuringiensis* and indigenous soil bacteria were inoculated at low densities. On the other hand, when vegetative cells or spores of *B. cereus* were added at the same time as indigenous soil bacteria to sterile soil, *B. cereus* grew even when indigenous soil bacteria were inoculated at high density. These data suggest that *B. thuringiensis* is less adaptive to soil habitats than *B. cereus*.

**Key words**: *Bacillus thuringiensis*, *Bacillus cereus*, competition, soil, interaction, endotoxin

**INTRODUCTION**

*Bacillus thuringiensis* is a Gram-positive bacterium that upon sporulation produces a proteinaceous crystal that is toxic to certain insects. Strains of *B. thuringiensis* that affect the larvae of insects in Lepidoptera, Diptera, Coleoptera, and other orders have been identified (Federici, 1994). Accordingly, *B. thuringiensis* has been used as a microbial pest control agent throughout the world. There have also been considerable efforts made to isolate new *B. thuringiensis* strains that have increased potency against target insect species and/or wider host range. Further, there is the potential to apply DNA technology to create new strains of *B. thuringiensis* and to transfer *B. thuringiensis* toxins to create alternative expression systems in transgenic plants, or other bacteria and baculoviruses (Carlton, 1993). In short, applications of *B. thuringiensis* seem likely to increase into the foreseeable future.

While many studies have investigated the molecular biology and biochemistry of *B. thuringiensis*, there has been only limited research on the ecology and behavior of *B. thuringiensis* in the environment. *B. thuringiensis* can be readily isolated from insects (Dulmage, 1970; Burges, 1973; Varkova and Purkini, 1979; Chilcott and Wigley, 1993), sericultural farms (Aizawa et al., 1961; Ohba et al., 1979, 1981), soil (DeLuca et al., 1981; Ohba and Aizawa, 1986 a; b; Travers et al., 1987; Kikuta et al., 1989; Martin and Travers, 1989; Meadows et al., 1990; Hasto et al., 1992; Chilcott and Wigley, 1993), stored product dusts (Burges and Hurst, 1977; DeLuca et al., 1982; Meadows et al., 1990,

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1992), deciduous and conifer leaves (Smith and Couche, 1991; Ohra, 1996), and aquatic environments (Goldberg and Margalit, 1977; Margalit and Dean, 1985). It would appear, then, that *B. thuringiensis* is an indigenous bacterium in each of these environments (Meadows, 1993). However, when viable spores of *B. thuringiensis* were added to soil in the field, they could neither germinate nor multiply but rather decreased immediately by approximately 1 log, after which their number remained constant for a long period (Petras and Casida, 1985). The reasons why *B. thuringiensis* cannot multiply in a soil environment seem to include the effect of soil pH (Saleh et al., 1970), moisture content (West et al., 1985; Petras and Casida, 1985), nutrient availability (Saleh et al., 1970; Akiba et al., 1979), and presence of indigenous microorganisms (West et al., 1985).

In environment, *B. thuringiensis* seems to exist most commonly in a spore-state. However, germination of the spore, vegetative growth, and subsequent sporulation are required for it to complete its life cycle and persist permanently in the ecosystem. There are three hypotheses concerning the possible role of *B. thuringiensis* in the environment: that *B. thuringiensis* is an entomopathogen, a phylloplane epiphyte, or a soil bacterium (Meadows, 1993).

One of the reasons for studying *B. thuringiensis* in soil was that the closest relative of *B. thuringiensis* is *B. cereus*, a common soil bacterium. The two bacteria share similar morphologies and nutritional requirements, and also have a high degree of DNA homology and genetic relatedness (Fergus et al., 1988). Despite these similarities, *B. cereus* is commonly isolated, whereas *B. thuringiensis* is rarely isolated from soil (DeLucca et al., 1981). Furthermore, *B. cereus* may be able to grow under conditions that do not permit the growth of *B. thuringiensis* (West et al., 1985). To date, the only consistent characteristic in which *B. thuringiensis* and *B. cereus* have been found to differ taxonomically is the production by *B. thuringiensis*, of parasporal crystals endotoxin (Baumann et al., 1984; Priest et al., 1988; Zahnner et al., 1989).

In this study we investigated how the presence of indigenous soil bacteria affects the ability of introduced bacilli to germinate and grow in soil. We compared the competitive ability of *B. thuringiensis* against indigenous soil bacteria with that of *B. cereus*, and we evaluated the possibility of *B. thuringiensis* as a soil bacterium.

**MATERIALS AND METHODS**

*Soil.* The soils used in this experiment were collected from an experimental farm of the Tokyo University of Agriculture and Technology, Tokyo, Japan. Before use, the soil was air-dried, sieved <2 mm, and stored at 5°C. The soil was a silt loam of pH 5.8, and with a C:N ratio of 15.0.

*Bacteria.* *Bacillus thuringiensis* serovar *aizawai* KH was originally isolated from the cadaver of the Japanese silk moth, *Antheraea yamamai*, and continuously maintained in our laboratory. *Bacillus cereus* K213, which was originally isolated from soil, was kindly supplied by Dr. K. Kato of the National Institute of Agrobiological Resources, Japan.

To obtain a mutant of each bacillus resistant to two antibiotics, streptomycin and rifampicin, streptomycin-resistant mutant was first isolated by spreading a culture of the parent strain onto Lennox L agar (Lennox, 1955) plates containing 0.01% streptomycin. Isolated streptomycin-resistant mutant was cultured in Lennox L broth containing 0.01% NNNG (mutagen, N-methyl-N'-nitro-N-nitrosoguanidine) for 1 h. Then, cultures were washed by sterile distilled water (SDW) by centrifugation for 20 min at 3,000 rpm, and
plated on Lennox L agar containing 0.01% streptomycin and 0.005% rifampicin. Colonies were re-isolated on Lennox L agar without antibiotics.

For inoculants of the spores, each of the bacilli was cultured with shaking in Lennox L broth at 30°C for 20 h. The cultures were plated on Lennox L agar and incubated at 30°C for 7 days. Cells on the plates were scratched and suspended in SDW, washed three times with SDW by centrifugation for 20 min at 3,000 rpm, and suspended again in SDW. The suspension was heated at 65°C for 15 min to kill any vegetative cells in the suspension. The obtained suspension, designated as spore suspension, was stored at 5°C until use.

To inoculate vegetative cells of bacilli, the spore suspension was cultured in Lennox L broth at 30°C for 20 h. During this period, the bacilli were in log phase. Cells were harvested, washed three times with SDW by centrifugation, and suspended in SDW. These suspensions were made immediately before use.

Indigenous soil bacteria were collected from sampled soil as follows. Ten grams of sampled soil was shaken strongly with 50 ml of SDW for 2 min. Serial dilutions of soil extract were spread onto Lennox L agar plates, and incubated at 30°C for 48 h. Approximately ten colonies were selected at random and were cultured in Lennox L broth at 30°C for 20 h. Cells were harvested by centrifugation for 20 min at 3,000 rpm, washed three times with SDW, and resuspended in SDW.

Experimental procedure. Five grams of dried soil were placed in 100 ml Erlenmeyer flasks, and the flasks were plugged with silicon caps and autoclaved (120°C, 20 min). The soil moisture was adjusted to 70% by bacteria inoculants. B. thuringiensis or B. cereus and indigenous soil bacteria were inoculated simultaneously into the autoclaved soil. The inoculated flasks were then incubated at 25°C in darkness. The concentration of B. thuringiensis, B. cereus or indigenous soil bacteria varied by 10^3, 10^4 and 10^5 colony forming unit (CFU)/g of dry soil. Separate competitive trials were run for both the bacilli spore and bacilli vegetative cell inoculations.

Enumeration. The flasks were periodically removed and the soil was firmly shaken with 50 ml of SDW for 2 min. Serial dilutions of the soil were spread onto Lennox L agar to estimate the number of total viable cells in soil samples. The total viable cells of B. thuringiensis and B. cereus were estimated by the drop plate method (HOHEN and SOMASOGARAN, 1982) using Lennox L agar containing the appropriate antibiotics. To estimate the density of B. thuringiensis or B. cereus spores, serial dilutions were heated at 80°C for 5 min and used in the drop plate method. Bacterial numbers were determined by counting three plates from the appropriate dilution, each plate containing between 10 to 300 CFU. The density of indigenous soil bacteria was determined by subtracting the number of total B. thuringiensis or B. cereus (obtained from the drop plate method using non-heated suspension) from the total number of bacteria (obtained from spread method using Lennox L agar without antibiotics). When the inoculation density of indigenous soil bacteria was lower than that of B. thuringiensis or B. cereus, however, subtraction could not be used. In such cases we checked antibiotics sensitivity of all colonies onto the Lennox L agar plate by replating onto the plate containing antibiotics and the non-antibiotics-resistant bacteria were regarded as indigenous soil bacteria.

Saturation density and growth rate were estimated from a growth curve that contained all the periodical enumerations of the experiments. Saturation density was estimated as the mean density of the stationary phase period, and growth rate was estimated as a ratio of the saturation density to the initial inoculation density.
RESULTS

Growth of B. thuringiensis and B. cereus in autoclaved soil

As a control, each bacillus was inoculated into autoclaved soil alone. When B. thuringiensis vegetative cells were inoculated (Fig. 1A), total B. thuringiensis began to increase at day 1, achieved 10^6 CFU/g of dry soil by day 2, and maintained this population for 10 days during the experimental period. B. thuringiensis spores began to form on day 2, achieved 10^5 CFU/g of dry soil at day 3, and thereafter increased gradually to achieve nearly total density.

When B. thuringiensis spores were inoculated alone (Fig. 1B), total B. thuringiensis began to increase at day 2, achieved 10^6 CFU/g of dry soil by day 3, and maintained this population for 10 days. B. thuringiensis spores began to increase at day 3, achieved 10^5 CFU/g of dry soil by day 5, and maintained this density for 10 days.

When B. cereus vegetative cells were inoculated alone (Fig. 1C), total B. cereus began to increase at day 1, achieved 10^5 CFU/g of dry soil by day 3, and maintained this population for 10 days. No B. cereus spore was detectable at day 1, but spore density reached the density of total B. cereus at day 2, and after that fluctuated along with the density of total B. cereus.

When B. cereus spores were inoculated alone (Fig. 1D), total B. cereus began to increase at 1 day, achieved 10^5 CFU/g of dry soil by day 5, and maintained this population for 10 days. B. cereus spores began to increase at day 2, achieved the same density as total B. cereus at day 3, and thereafter fluctuated along with total B. cereus.

B. thuringiensis vs. indigenous soil bacteria

When vegetative cells of B. thuringiensis were added at the same time as indigenous soil bacteria to sterile soil, the competitive outcome of B. thuringiensis varied with both initial density of soil bacteria and of vegetative cells of B. thuringiensis (Fig. 2A, Fig. 3A). When soil bacteria were inoculated at high (10^6 CFU/g) and intermediate (10^4 CFU/g) density, B. thuringiensis hardly increased less than 1 order of magnitude (Fig. 2A, Fig. 4A). A single

![Graph](image-url)

Fig. 1. Viable count of total (●) and spores (○) of bacilli inoculated alone in autoclaved soil. Inoculant states were as follows: A, B. thuringiensis vegetative cell; B, B. thuringiensis spore; C, B. cereus vegetative cell; D, B. cereus spore. Arrows denote below-detectable level.
exception occurred when indigenous soil bacteria were inoculated at $10^4$ CFU/g and vegetative cells of *B. thuringiensis* were inoculated at $10^5$ CFU/g. In this case, *B. thuringiensis* spores increased for 2 days up to the initial density of vegetative cell of *B. thuringiensis*. When soil bacteria were inoculated at low ($10^5$ CFU/g) density, on the other hand, *B. thuringiensis* increased from 1 to 2 orders of magnitude. Spore density of *B. thuringiensis* began to increase at day 1 and achieved total density of *B. thuringiensis* by day 2, then maintained this spore density throughout the experimental period.

When the spores of *B. thuringiensis* were inoculated to autoclaved soil at the same time as indigenous soil bacteria, *B. thuringiensis* spores maintained almost their initial density throughout the experimental period (Fig. 2B, Fig. 3B, Fig. 4B). There were, however, two notable exceptions, the first being the simultaneous inoculation of *B. thuringiensis* at $10^5$ CFU/g and indigenous soil bacteria at $10^4$ CFU/g, and the second being the simultaneous inoculation of *B. thuringiensis* at $10^5$ CFU/g and indigenous soil bacteria at $10^4$ CFU/g. In both these cases, *B. thuringiensis* increased about 1 order of magnitude at day 10 and spores of *B. thuringiensis* maintained their total density throughout the experimental period.

*B. cereus* vs. indigenous soil bacteria

When vegetative cells of *B. cereus* were inoculated to autoclaved soil at the same time as indigenous soil bacteria, *B. cereus* increased from 1 to 3 orders of magnitude for 3 days (Fig. 2C, Fig. 4C). Saturation densities of *B. cereus* decreased as initial densities of indigenous soil bacteria increased (Fig. 3C). When initial densities of *B. cereus* were higher than those of indigenous soil bacteria, saturation densities and growth rates of *B. cereus* were not affected by the presence of indigenous soil bacteria (Fig. 2C, Fig. 3C). The density of *B. cereus* spores began to increase for 1 day, achieved the total density of *B. cereus*, and then fluctuated along with total density throughout the experimental period.

When the *B. cereus* spores were added to autoclaved soil at the same time as indigenous soil bacteria (Fig. 4D), *B. cereus* increased 1 order of magnitude on each of days 3, 5 or 7. In
Fig. 3. Saturation densities (log CFU/g dry soil) of bacilli inoculated in autoclaved soil at the same time as soil bacteria. Inoculant states were as follows: A, B. thuringiensis vegetative cell; B, B. thuringiensis spore; C, B. cereus vegetative cell; D, B. cereus spore. Inoculation densities of bacilli were as follows: closed columns, $10^6$ CFU/g soil; dotted columns, $10^4$ CFU/g soil; open columns, $10^5$ CFU/g soil.

Fig. 4. Viable count of total (●) and spores (○) of bacilli inoculated in autoclaved soil at the same time as soil bacteria (▲). Inoculant concentration of both bacilli and indigenous soil bacteria were $10^5$ CFU/g dry soil. States of inoculant were as follows: A, B. thuringiensis vegetative cell; B, B. thuringiensis spore; C, B. cereus vegetative cell; D, B. cereus spore.

the cases when B. cereus was inoculated at high ($10^5$ CFU/g) or intermediate ($10^4$ CFU/g) density and indigenous soil bacteria were inoculated at $10^5$ CFU/g, B. cereus maintained their initial densities (Fig. 2D, Fig. 3D). In some of the experimental cases, B. cereus decreased at day 10. Spore density of B. cereus fluctuated, as did total density, throughout the experimental period.

In all the competitive trials, the outcome of indigenous soil bacteria was as follows. The
density of indigenous soil bacteria increased to $10^9$ CFU/g of dry soil for 1 day, then after a few days increased gradually to $10^9$ CFU/g of dry soil and maintained this population for 10 days.

**DISCUSSION**

In most of the cases when vegetative cells of *B. thuringiensis* and indigenous soil bacteria were added to sterile soil at the same time, *B. thuringiensis* sporulated immediately without any growth. *B. thuringiensis* multiplied only when both *B. thuringiensis* and indigenous soil bacteria were inoculated at low densities. Such a situation seems to be very rare in natural soil habitats because $10^8$--$10^9$ bacteria per gram are found in soil habitats (Atlas and Bartha, 1987). Furthermore, *B. thuringiensis* vegetative cells have been shown to disappear rapidly, losing 91% of their population in the first 24 h of incubation in natural soil (West et al., 1984). Therefore, it appears that *B. thuringiensis* vegetative cells cannot persist for a long period and grow in a natural soil environment.

In order to examine the possibility of germination, spores of *B. thuringiensis* were set in competition with indigenous soil bacteria. As a result, *B. thuringiensis* did not germinate but maintained their initial density and remained in the spore state. *B. thuringiensis* increased slightly only when indigenous soil bacteria were inoculated at low densities, so it seems that *B. thuringiensis* spore can neither germinate nor grow in natural soil environments.

In contrast to the above, the competitive trials of *B. cereus*, which was thought to be a soil bacterium, against indigenous soil bacteria suggested that *B. cereus* is more characteristic of soil bacteria than is *B. thuringiensis*. Vegetative cells of *B. cereus* grew even if indigenous soil bacteria were inoculated at high density, under which condition *B. thuringiensis* was unable to grow. When spores of *B. cereus* were set in competition with indigenous soil bacteria, *B. cereus* increased their number even if indigenous soil bacteria were inoculated at high density, under which condition *B. thuringiensis* never germinated. In these experiments, we could not confirm germination because the total and spore densities of *B. cereus* fluctuated similarly. But it would appear that *B. cereus* did germinate, since spore-forming bacteria never multiply in a spore state.

Akira (1992) reported that *B. thuringiensis* *sotto* suppressed their multiplication when *B. thuringiensis* was inoculated in sterilized soil in addition to a little nonsterilized soil. West et al. (1985) showed that *B. thuringiensis* could not grow in non-sterilized soil, whereas *B. cereus* could. Similar results were obtained in our experiments, suggesting that the presence of indigenous soil bacteria is a major factor inhibiting the growth of *B. thuringiensis* in natural soil.

In these experiments, it appears that *B. thuringiensis* has less competitive ability against indigenous soil bacteria than *B. cereus* has. Difference in competitive ability between *B. thuringiensis* and *B. cereus* seems to originate from the presence or absence of proteinaceous crystal toxin. The rate of crystal toxin synthesis is about 33 to 43% of the overall rate of total protein synthesis (Arnonson et al., 1986). Even though most crystal toxin genes are on plasmids, which are easily lost or altered, as a rule, *B. thuringiensis* tenaciously retains crystal (Martin, 1994). The crystal toxin must contribute to the survival of *B. thuringiensis* in the environment despite the obvious cost to the cell in terms of increased genome size and synthesis of a large protein body during nutrient stress (sporulation). Based on the principle of allocation (Cody and Diamond, 1975), there seems to be a trade-off between the ability to compete and the ability to produce proteinaceous crystal toxin. *B. thuringiensis* appears to
expend its energy in the production of proteinaceous crystal toxin rather than in competition against indigenous soil bacteria, and thus is less competitive than *B. cereus*. MARTIN (1994) suggested that *B. thuringiensis* is a soil bacterium, and that proteinaceous crystal toxin may be stored as a food for use in germination, as a direct antagonist against other microbes or as bait to divert predators in the soil. On the other hand, FEDERICI (1994) suggested that the natural role of proteinaceous crystal toxin is to kill host insects, providing *B. thuringiensis* with resources for growth and reproduction. If *B. thuringiensis* is taken to be an entomopathogenic bacterium, then it gains an advantage over non-toxic spore-formers in the environment of an insect gut because of the presence of crystal toxin. FEITELSON et al. (1992) have discovered *B. thuringiensis* strains that show activity against plant- and animal-parasitic nematodes, Protozoa, flatworms, and mites. Whether *B. thuringiensis* is an entomopathogenic or soil bacterium, crystal toxin must contribute to its survival in the environment.

*B. thuringiensis* seems to maintain a spore state in the soil environment, where as *B. cereus* seems to complete a full life cycle of spore germination, vegetative cell multiplication, and sporulation in order to maintain its environmental longevity. If *B. thuringiensis* is a soil bacterium, it would seem to gain fewer advantages from this classification than *B. cereus*. However, because the *B. thuringiensis* used in these experiments was isolated from insect cadaver, further experiments will be needed using various serovarieties of *B. thuringiensis*, especially *B. thuringiensis* isolated from soil environments.

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