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Fate of *Bacillus thuringiensis* subsp. *israelensis* under Simulated Field Conditions

BELLA OHANA, JOEL MARGALIT, AND ZE'EV BARAK*
Department of Biology, Ben-Gurion University, Beer Sheva, Israel

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The fate of *Bacillus thuringiensis* subsp. *israelensis* in a natural aquatic habitat was studied in a model system by using laboratory-simulated field waters and a mutant of the bacterium resistant to three antibiotics. Contact with mud of a sporal culture of the mutant resulted in an immediate disappearance of the larvicidal activity but had no influence on viability. The cessation of toxicity was caused by bacterial adsorption on soil particles, since 99.8% of the bacteria was found in the mud fraction within 45 min, with concurrent disappearance from the supernatant. When the mud was stirred, the bacteria could be redetected. The viability count of the mud suspension remained practically constant for at least 22 days, indicating that the spores were still fully viable but were incapable of germinating and multiplying in the mud under our experimental conditions. Approximately 8% of the colony forming ability of the bacteria could be separated from the mud by vigorous mixing followed by immediate filtration. The filtrated spores retained their toxicity, killing 90% of the larval populations even after 22 days incubation in the soil. The inactivation of the toxic activity of *B. thuringiensis* subsp. *israelensis* in the mud was therefore a reversible process and was probably due to masking of the bacteria, thus making the bacteria and their toxin inaccessible to the larvae. In the simulated field waters without mud, we observed only a very slow inhibition of the larvicidal activity. In contrast to the activity in the mud suspension, this activity could not be restored.

Bacillus thuringiensis subsp. *israelensis* shows high larvicidal activity against mosquito and blackfly larvae, without any effect on most other organisms. Unfortunately, one of its major drawbacks is its short (less than 24 h) residual effect in the field after application (4). The major reason for the rapid disappearance of the larvicidal effects of *B. thuringiensis* subsp. *israelensis* seems to be its adsorption on soil particles in the water (3, 6, 7). Nevertheless, other factors may also play a role in the discontinuation of bacterial toxicity in nature. In the present study, we investigated what happens to *B. thuringiensis* subsp. *israelensis* bacteria from the moment they are broadcast over bodies of water. Do they remain alive and multiply? Does a connection exist between the viability and the location of the bacteria after their application and the disappearance of their larvicidal effects? To answer these questions, we studied the fate of *B. thuringiensis* subsp. *israelensis* in the laboratory under simulated field conditions, i.e., water pools with or without the addition of mud, by utilizing an antibiotic-resistant mutant developed by us to enable detection of the bacteria under septic aquatic conditions.

MATERIALS AND METHODS

The strain of *B. thuringiensis* subsp. *israelensis* serotype H-14 was kindly supplied by H. de Barjac, Institut Pasteur, Paris, France. PST, a spontaneous mutant of *B. thuringiensis* subsp. *israelensis* resistant to the antibiotics penicillin, streptomycin, and tetracycline, was developed in this study.

The selection of spontaneous mutants resistant to antibiotics was carried out by placing mid-log-phase cells of *B. thuringiensis* subsp. *israelensis* on LB solid medium (5) containing the antibiotics at the following concentrations (per ml) and cell numbers (per plate): penicillin, 150 μg at 10^7 cells; streptomycin and tetracycline, 100 and 20 μg, respectively, both at 5×10^9 cells. Colonies were then reisolated on LB medium without antibiotics. Mutants were isolated after sequential selections, beginning with penicillin and ending with tetracycline. The frequencies of the resistant mutants in the populations were approximately 10^{-6} for penicillin, 10^{-8} for streptomycin, and 10^{-9} for tetracycline. The final strain, resistant to all three antibiotics, was designated PST. Mosquito larvicidal activity of PST in tap water was 210 CFU/ml, compared with 100 CFU/ml of *B. thuringiensis* subsp. *israelensis*, its parental strain.

Sporal cultures of PST active against mosquito larvae were prepared by the following procedure. The original logarithmic culture was grown with shaking (200 rpm) at 30°C in an LB medium containing 20 μg of tetracycline and 100 μg of streptomycin per ml. When the turbidity reached 40 to 50 Klett units (660-nm filter), 0.5 ml of the culture was transferred to an Erlenmeyer flask (125 ml) containing 15 ml of LB medium; the culture was then grown under the same conditions for 28 to 30 h, a period sufficient for both sporulation and crystallization (sporulation always exceeded 90%).

Larvicidal tests were carried out with third instar *Aedes aegypti* larvae in 100 ml of the water sample kept in 150-ml disposable plastic cups at 28°C. Twenty larvae were used for each experiment, and each experiment was duplicated. Mortality was counted after 24 h.

Laboratory-simulated field water was prepared as follows. Deionized water (45 liters) was kept in open asbestos containers (35 by 51 by 31 cm) for 8 weeks. During this period, the containers became contaminated with soil dust carried by wind. They were aerated for the first 5 days, after which the water became greenish and turbid. The soil dust was composed of 21% clay, 58.7% sand, 20% silt, and 0.3% organic matter. Evaporated water was replenished. Analysis of the water showed (mg/liter): K^+, 6, Na^+, 0.02; Mg^{2+}, 6.5, Cl^-, 0.08; HCO_3^-, 150; and SO_4^{2-}, 7.2. Chemical oxygen

* Corresponding author.

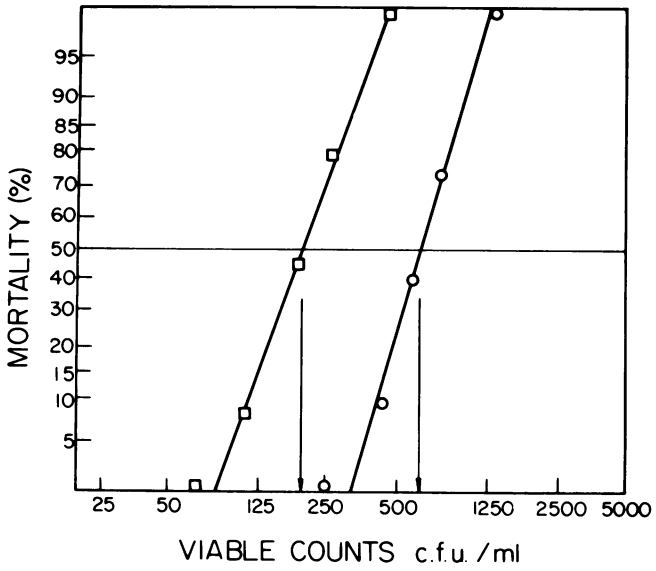

FIG. 1. Comparison of larvicidal effect of the PST mutant in tap water (□) and in stagnant water (○). Toxic sporal culture was prepared and larvicidal tests were performed as described in Materials and Methods.

demand was 4 mg of O_2 per liter. Total nitrogen was 0.6 mg/liter, and phosphorous was 2.4 mg/liter.

All experiments were carried out with 4-liter samples of this water in 5-liter glass beakers. When appropriate, mud (80 g [dry weight]) collected from the 45-liter container was added to these samples. To ensure homogenized samples, the mud and water in the asbestos container were mixed thoroughly before collection into the beakers and then separated by allowing the mud to settle. The water obtained is designated stagnant water. The mutant PST was introduced at the concentrations indicated below.

RESULTS

Tracing PST in septic field water samples was possible after eliminating background bacteria on plates containing streptomycin (100 μg/ml) and tetracycline (20 μg/ml). In the five cases examined, the addition of penicillin was not necessary for selection.

To follow the fate of *B. thuringiensis* subsp. *israelensis* in nature, a sporal culture of the PST mutant was added to the laboratory-simulated field water at a concentration of 75 times the 50% lethal concentration (the 50% lethal concentration, 600 CFU/ml, was determined in stagnant water [Fig. 1]). To test the influence of mud on bacterial activity, mud was added and mixed with the bacterial suspension. At 15-min intervals for 1 h and then at intervals of 2 h, two samples of 100 ml were taken for larvicidal tests from the upper level of the 5-liter glass beakers. Viability counts of bacteria were also carried out on portions of these samples. A parallel experiment was performed in stagnant water which did not contain mud. Immediately after adding the mud, the larvicidal efficacy of the mutant was drastically reduced (from 100 to almost 0% [Fig. 2]). No significant difference could be detected in viability counts immediately after the addition of the mud. However, after 15 min, the counts fell steeply to about 2% of the original count and to even lower levels later. Concurrently with this reduction, the top layer of the water cleared up. On the other hand, in the stagnant water no reduction in larvicidal activity or in viability counts occurred during the 6 h of the experiments.

The disappearance of the viability of PST within the first 15 min after contact with the mud by stirring is most probably the result of PST settling on the bottom of the container after being adsorbed on mud particles. Another possibility is that the PST is being killed, before or after spore germination, by a component of the mud. To determine which of these possibilities is correct, we stirred the mud every 24 h and tested the suspension (mud suspension) obtained for bacterial viability counts and for larvicidal activity. Although, after stirring, the viability count of the mud suspension remained practically constant ([5 ± 0.65] × 10^4 CFU/ml) for at least 22 days, no larvicidal activity could be detected in it (Fig. 3). On the other hand, if the stirred mud suspension was restirred and passed immediately through a Whatman no. 1 filter, the filtrate showed high larvicidal activity (90 ± 5%) and the viability count was (3.3 ± 0.94) × 10^3 CFU/ml, which constitutes about 8% of the initial bacterial concentration.

It should also be mentioned that when the biological assays were carried out with mud suspensions which were aerated by continuous mixing and pumping of air bubbles for 24 h, larvicidal activity approached 100% despite the presence of mud in the water.

The concentration of bacteria used in the previous experiments was excessively high (42 times the 95% lethal concentration). Therefore, moderate effects of inactivation which might have occurred in the stagnant water may have been undetectable. To follow such effects, we tested the fate of the PST when applied at concentrations of (1 ± 0.27) ×

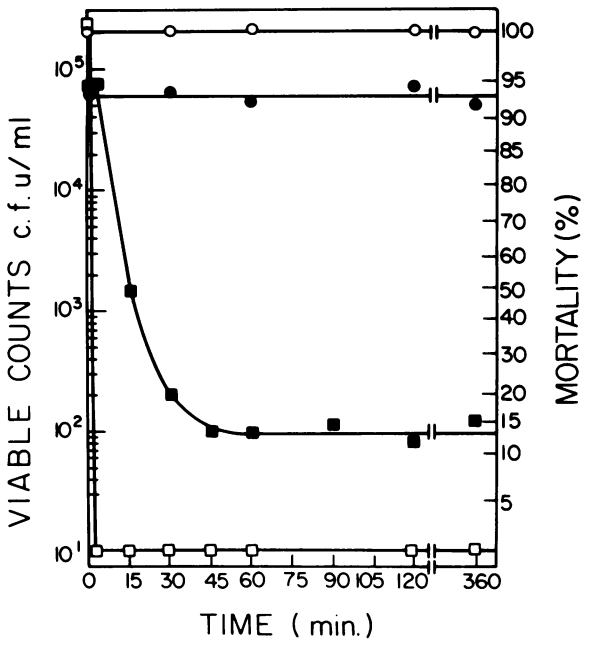

FIG. 2. Viability counts (closed symbols) and larvicidal activity (open symbols) of the PST mutant of *B. thuringiensis* subsp. *israelensis* in stagnant water (○) and in stagnant water plus mud (□). Duplicate samples were taken from the upper water layers without any stirring. Larvicidal tests were performed in 100-ml portions to which 20 third instar *A. aegypti* larvae were added. Viability counts were carried out by placing 0.1 ml of the proper dilution on LB plates containing streptomycin (100 μg/ml) and tetracycline (20 μg/ml).

10^3 and (1.6 ± 0.65) × 10^3 CFU/ml, both only in the range of the 95% lethal concentration of the bacteria. Although the CFU concentration remained practically constant for 30 days, larvicidal activity started to fall between days 3 and 4 at the lower PST concentration and on day 4 at the higher concentration (Fig. 4). The activity fell at a slope of −25 and −30% mortality per day, respectively. Continuous mixing of the stagnant water by the introduction of air bubbles caused 20% mortality on day 10 and 0% on day 11.

DISCUSSION

We studied the fate of *B. thuringiensis* subsp. *israelensis* by using the PST mutant which is resistant to three different antibiotics. Contact of PST with mud resulted in an immediate cessation of larvicidal activity but had no discernible effects on viability. These results do not agree with those of Van Essen and Hembree (7), who reported less than 20% reduction in toxicity of *B. thuringiensis* subsp. *israelensis* 1 day after application of the bacteria to water containing mud. A possible explanation for this discrepancy is that they used formulated powders, whereas we used pure cultures of spores.

The disappearance of the larvicidal activity of *B. thuringiensis* subsp. *israelensis* is caused mainly by its adsorption on mud particles. This is deduced from our findings that within 45 min, 99.8% of the viable counts were found in the mud fraction, with concurrent disappearance from the supernatant. All colony forming ability present in the beaker could be detected when the mud which had settled out was restirred immediately. The possibility that the *B. thuringiensis* subsp. *israelensis* had settled at the bottom of the jar independently of the mud particles in the suspension can be excluded, since we could detect both larvicidal activity and full viability of *B. thuringiensis* subsp. *israelensis* in the upper layers of stagnant water jars, i.e., jars containing no mud, for at least 6 h after application of the bacteria (Fig. 2).

In this study, we did not investigate the nature of the adsorption of the *B. thuringiensis* subsp. *israelensis* to the mud particles. Nevertheless, it became evident that at least some of the cells were only weakly attached to the mud, since 8% of the CFU could be released from the mud by strong continuous stirring followed by immediate filtration. Although the concentration of the spores in the filtrate was about 2.5 times higher than that which would have caused 100% mortality of larvae in stagnant water (Fig. 1), the larvicidal effect of the filtrate showed only about 90% activity during the 22 days of the experiment. A possible explanation for this phenomenon is that at least some of the spores may not be in physical association with the toxic crystal, and thus, release of the CFU from the mud does not necessarily result in a release of the toxin. Alternatively, the bacteria released from the mud may not be predominantly those containing the toxic crystals. Another possibility, and probably a more acceptable one, is that the filtrate may not become completely void of all mud particles, and the residual mud particles could still bind some of the toxin present. A further support for the notion that the larvicidal activity of *B. thuringiensis* subsp. *israelensis* can be released from the soil particles by a strong stirring process is provided by the finding that when the mud suspension was continuously stirred during the bioassay (24 h), 100% mortality was always obtained.

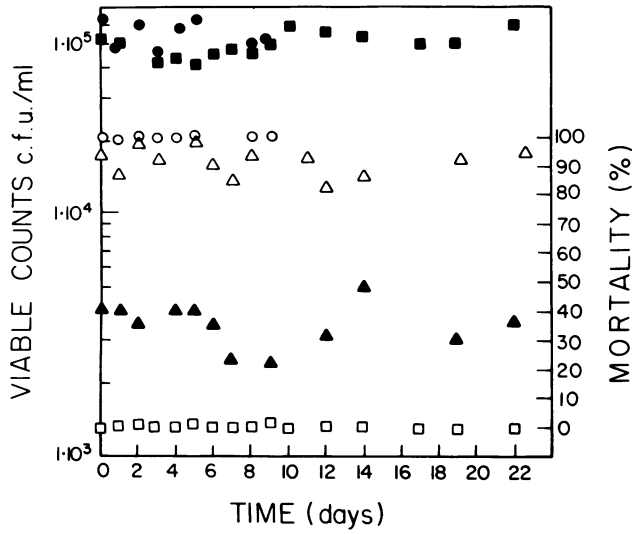

FIG. 3. Viability counts (closed symbols) and larvicidal activity (open symbols) of the PST mutant of *B. thuringiensis* subsp. *israelensis* in stagnant water (○), mud suspension (□), and filtrate (△). Viability counts and larvicidal tests were performed as described in the legend to Fig. 2.

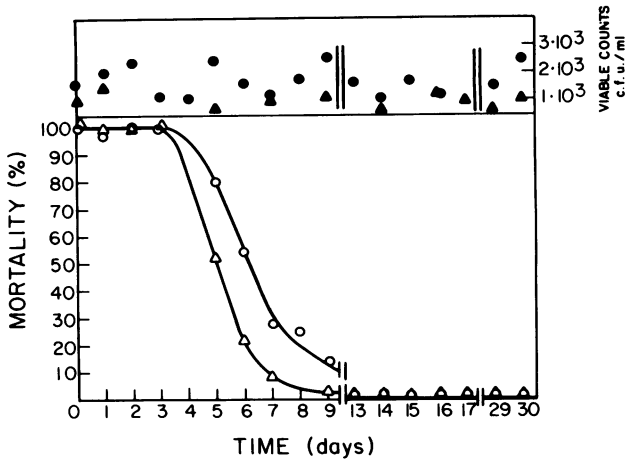

FIG. 4. Viability counts (closed symbols) and larvicidal activity (open symbols) of the PST mutant in stagnant water at (1 ± 0.27) × 10^3 (△) and at (1.6 ± 0.65) × 10^3 (○) CFU/ml. Viability counts and larvicidal tests were performed as described in the legend to Fig. 2.

The above findings suggest that the masking of the toxin caused by the adsorption of the spores or the toxin or both on the mud is a reversible process. Loss of larvicidal activity is mainly due to the rendering of the toxin into a form not accessible to mosquito larvae. This loss could be the result of the larvae being unable to ingest bacteria adsorbed on mud particles.

Although we observed the progressive inhibition of larvicidal activity in stagnant water and in mud suspension, we found no noticeable effects on the viability of the PST mutant. The interference with toxicity was very slow compared with that observed for the mud. These findings could be explained either by a gradual adsorption of the bacteria on soil and organic particles present in the water or by the destruction of the toxin by proteolytic enzymes. However, unlike the situation with the mud suspension, larvicidal activity could not be restored after day 11, even by continuous mixing of air bubbles into the bacterial suspension

during the entire bioassay procedure. These results indicate an irreversible inhibition of toxicity in the stagnant water. On the other hand, the concentration of PST which is released from the particles by the stirring process may have been too low to exert toxic action in the bioassay, as only a small portion (approximately 8% in the mud suspension) of the bacteria was released by this procedure.

The viability of PST measured in CFU per milliliter remained constant in the mud suspension for at least 22 days and in the stagnant water for 30 days. This indicates that the PST mutant did not multiply under our experimental conditions. An essential factor for multiplication, such as certain nutrients, oxygen, etc., was probably missing, or *B. thuringiensis* subsp. *israelensis* or its PST mutant was not able to compete with other bacteria for the nutrients available in the water, or both. Similar results have been reported previously by West and Burges (8) with other strains of *B. thuringiensis*. On the other hand, it was recently found that spores of *B. thuringiensis* subsp. *israelensis* can germinate and multiply in dead larvae (1; A. Zaritsky, K. Khawaled, Z. Barak, D. M. Chipman, and T. Rabi, Acta Microbiol. Pol., in press).

In contrast to our results, the results of Larget-Thiery (2) indicated high residual larvicidal activity in jars containing field water and mud even 60 days after application of the *B. thuringiensis* subsp. *israelensis* powder. Her results may have been due to the presence in the jars of *B. thuringiensis* subsp. *israelensis*-killed larvae in which spores of the bacteria can germinate, multiply, and even complete their life cycle, producing endotoxin and spores (1; B. Ohana, M.S. thesis, Ben-Gurion University, Beer Sheva, Israel, 1985; A. Zaritsky et al., in press). Furthermore, part of the observed high persistance could be the result of living larvae feeding on *B. thuringiensis* subsp. *israelensis*-killed larvae in which the toxin is particularly concentrated (9).

ACKNOWLEDGMENT

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