

Selective Process for Efficient Isolation of Soil *Bacillus* spp.

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We were able to isolate *Bacillus thuringiensis* from environmental samples with a background of 10^9 bacteria per g of soil. Our selection process differed significantly from classical selection methods which permit only the desired organism to grow. In our process, germination of *B. thuringiensis* spores was selectively inhibited by sodium acetate, while most of the undesired sporeformers germinated. Next, all of the nonsporulated microbes were eliminated by heat treatment at 80°C for 3 min. The surviving spores were then plated on a rich agar medium and allowed to grow until they sporulated. Of random colonies picked from agar, 20 to 96% were crystal-forming *Bacillus* species. *B. thuringiensis* and *B. sphaericus* were routinely selected by this method.

The study of microbial interactions in nature is severely hampered by the lack of efficient methods to isolate specific bacteria from environmental samples. Enrichment techniques of isolation, until now the most powerful tool available to the microbial ecologist, are dependent on the ratio of desired bacteria to unwanted bacteria and have a theoretical lower limit of detection of ca. 1,000 bacteria per g of soil. This level is usually not detected in environmental samples (9). Additionally, these methods do not allow direct enumeration. Immunofluorescence methods that allow direct enumeration (13) have a lower detection limit of ca. 10,000 cells per g of soil. The method reported here describes a new process for isolating *Bacillus* spp. from the environment which avoids the problems inherent in enrichment culture. By using this process, we were able to isolate desired bacteria to a level of one spore per gram of soil and to enumerate the unwanted bacteria that were eliminated. We used this method to isolate *Bacillus thuringiensis* from soil samples in an effort to determine the quantitative environmental significance of the bacteria.

B. thuringiensis produces an insecticidal protein toxin during sporulation. This toxin is effective against three orders of insect pests (Lepidoptera, Diptera, and Coleoptera). In the more than 80 years since the discovery of the bacterium (5), only 25 varieties of *B. thuringiensis* have been found. The majority of these varieties were isolated from dead or moribund insects. Commercial uses of this bacterium represent a \$40-million/year industry worldwide, but further enlargement of the market for *B. thuringiensis* is limited now by a lack of new insecticidal strains.

The normal method for the isolation of these bacteria is to look for diseased or dead insects in insectaries or in areas with high levels of insect infestation (7). One major exception to this approach was a study (3) of the distribution of *B. thuringiensis* strains in soil in the United States. *B. thuringiensis* represented between 0.5 and 0.005% of all *Bacillus* spp. isolated from the soil samples tested. In this study, the selective medium described by Saleh et al. (9) was used to reduce the number of non-*Bacillus* organisms from soil samples. This medium employed polymyxin B sulfate (5 ppm [5 µg/ml]) and penicillin G (4 ppm [4 µg/ml]) as the basis for selection. Saleh et al. (9) proposed that their method could detect *B. thuringiensis* to a lower limit of 1,000 bacteria per

g of soil with a background of ca. 2.0×10^5 organisms per g of soil.

During the course of a project to develop a chemically defined sporulation medium, R.S.T. observed that *B. thuringiensis* spores did not germinate in the presence of a high concentration (0.25 M) of sodium acetate (R. S. Travers, Ph.D. dissertation, University of Maryland, College Park, 1982). It was this finding that led to the development of the new and more efficient process to isolate *B. thuringiensis* from environmental samples that we describe here.

MATERIALS AND METHODS

Organisms and growth conditions. Known strains of *B. thuringiensis* were supplied by H. de Barjac (Institut Pasteur, Paris, France) and were incubated on T3 medium (per liter: 3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 0.05 M sodium phosphate [pH 6.8], and 0.005 g of MnCl₂; Travers, Ph.D. dissertation) until sporulation was complete. New strains of *B. thuringiensis* were isolated from a random selection of colonies developing on L agar (per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar) seeded with environmental samples. After isolation, these organisms were grown on T3 agar until sporulation was complete. The remainder of the standard *Bacillus* strains were a gift from T. Cook (University of Maryland, College Park; Table 1). These latter strains were grown on L agar at 30°C until they sporulated.

Because *B. thuringiensis* and *B. anthracis* are closely related (4), four pathogenic strains of *B. anthracis* were tested for coselection by this method. These tests were carried out by B. Ivins (U.S. Army Infectious Diseases Laboratory, Ft. Detrick, Md.). In all cases, the incubation temperature was 30°C.

Spores used in the project were floated from agar plates with 10 ml of sterile water and washed three times with sterile distilled water. After being washed, the spores were suspended in 10 ml of sterile distilled water and heat shocked as 1.5-ml samples in a flowthrough heat treater (Fig. 1) at 80°C for 3 min. Two (40 µl) drops of this spore suspension, which contained approximately 5×10^7 bacteria, were used to seed agar plates in experiments testing the ability of axenic cultures to germinate in the presence of acetate. *B. anthracis* cultures were heat shocked at 75°C for 1 h.

Two media were used in these experiments. The basal medium used for *B. thuringiensis* was T3. All other strains of bacteria were grown on L broth medium because T3 medium

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TABLE 1. Growth of environmental isolates of *Bacillus thuringiensis* in acetate-buffered medium

Isolate code	Growth ^a at final acetate concn of:		
	0.25 M	0.12 M	0.06 M
<i>B. thuringiensis</i> subsp. <i>canadensis</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>finitimus</i>	+	+	+
<i>B. thuringiensis</i> subsp. <i>dakota</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>indiana</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>kurstaki</i> (3) ^b	-	+	+
<i>B. thuringiensis</i> subsp. <i>kyushuensis</i>	-	-	+
<i>B. thuringiensis</i> subsp. <i>morrisoni</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>pakastani</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>sotto</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>subtoxicus</i>	-	-	+
<i>B. thuringiensis</i> subsp. <i>thompsoni</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>tochingiensis</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>tokakuensis</i>	-	+	+
<i>B. thuringiensis</i> subsp. <i>toumanoffi</i>	-	+	+
CNW 17	-	-	+
CNW 37	-	+	+
DNT 1	+	+	+
GNC 1	-	+	+
JNJ 8	-	+	+
JOL 3	-	+	+
LNL 1	+	+	+
MOL 1	-	-	+
MOL 44	-	+	+
TNC 1	-	+	+
ZOE 5	-	+	+

^a Growth (+) or no growth (-) after incubation for 24 h at 30°C. No isolate grew in 0.50 M acetate.

^b Number in parenthesis indicates the number of strains tested.

partially inhibited growth and sporulation of *Bacillus* spp. other than *B. thuringiensis*. A total of 5 ml of 2× basal medium was placed in a test tube, and sufficient 2.0 M sodium acetate buffer (pH 6.8) to produce the desired concentration of acetate was added. The volume was then adjusted to 10 ml with sterile distilled water. The acetate concentrations tested were 0.5, 0.25, 0.12, and 0.06 M. Completed media were incubated in tubes at 30°C for 24 h before being used to monitor sterility.

After the sterility check was completed, the media were inoculated and incubated at 30°C. After incubation, the tubes were checked visually by a comparison of uninoculated and positive control tubes. A large inoculum (5×10^7 CFU) was used, which prevented optical density determinations to estimate growth. Therefore, germination was determined by pellicle formation at the surface and by turbidity of the medium after overnight incubation. Spores which did not germinate settled out overnight.

To isolate *B. thuringiensis* from dirt, 0.5 g of dirt was added to 10 ml of L broth in a 125-ml triple-baffled flask. The L broth was buffered with 0.25 M sodium acetate. This mixture was shaken for 4 h at 250 rpm at 30°C. At the end of this time, a sample was taken, heat treated as described above, plated on L agar, and grown overnight at 30°C. A random sample of colonies was picked onto T3 medium (agar dots; 6) and allowed to sporulate overnight at 30°C. Cultures were then checked for the presence of crystals, which was the criterion used to confirm isolates as *B. thuringiensis*.

RESULTS

Thirty-seven strains of sporeforming bacteria were tested for their ability to germinate in acetate-buffered medium. Of

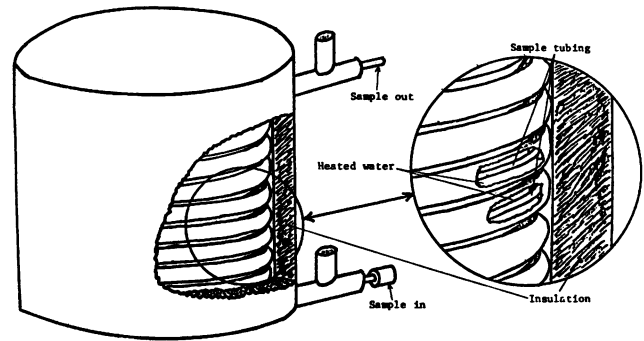


FIG. 1. Tube-in-tube flowthrough pasteurization device.

these strains, 15 were recognized varieties of *B. thuringiensis* (1; Table 1), 11 were environmental isolates of *B. thuringiensis* selected at random from agar plates seeded with environmental samples, and 11 were other sporeformers of the genus *Bacillus* (Table 2; not all data shown). All bacteria examined in this study germinated and grew in the absence of acetate buffer and in medium that was adjusted to 0.06 M sodium acetate. None of the strains tested germinated in medium that contained 0.50 M sodium acetate buffer. In medium buffered with 0.25 M acetate, *B. cereus*, *B. anthracis*, *B. chitinosporus*, *B. subtilis*, *B. polymyxa*, *B. globisporus*, *B. thuringiensis* subsp. *finitimus*, and two *B. thuringiensis* environmental isolates germinated and grew. In medium with 0.12 M sodium acetate, all strains grew except *B. thuringiensis* subsp. *subtoxicus*, *B. thuringiensis* subsp. *kyushuensis*, *B. thuringiensis* subsp. *dakota*, and two environmental isolates of *B. thuringiensis*. Of 40 *B. thuringiensis* strains tested (not all data shown), only 2 strains germinated and grew in media containing 0.25 M sodium acetate. This number represented 5% of the strains tested. Because of these data, 0.25 M acetate was chosen for all of the subsequent environmental isolations.

Growth of soil bacteria in acetate. Isolating *B. thuringiensis* from a mixed population of soil bacteria presented problems different from those in testing isolated pure cultures. It was necessary to determine how quickly non-*B. thuringiensis* sporeformers germinated in a background of nonsporeforming bacteria. One gram of a typical soil sample (from Jackson, Wyo.) examined in this study contained 6.2×10^6 detectable bacteria. Of these bacteria, 27.4% were initially heat-resistant sporeformers (Table 3). When soil was incubated in a medium containing acetate (0.25 M), the spore count remained approximately constant for the first 3 h. At 4 h, the count of heat-resistant sporeformers fell to less than

TABLE 2. Germination of *Bacillus* strains in acetate buffer

Bacillus strain	Growth ^a at acetate concn of:		
	0.25 M	0.12 M	0.06 M
<i>B. anthracis</i> (4) ^b	+	+	+
<i>B. cereus</i>	+	+	+
<i>B. chitinosporus</i>	+	+	+
<i>B. globisporus</i>	+	+	+
<i>B. polymyxa</i>	+	+	+
<i>B. sphaericus</i>	-	+	+
<i>B. subtilis</i>	+	+	+

^a Growth (+) or no growth (-) after incubation for 24 h at 30°C. No isolate grew in 0.50 M acetate.

^b Number in parenthesis indicates the number of strains tested.

0.45% of the initial spore counts. In the next 2 h, these counts increased. Thus, 4 h of incubation in 0.25 M acetate was routinely used to isolate *B. thuringiensis* from the soil.

Comparison of antibiotic versus acetate selection. We used the acetate selection method and the antibiotic selection method described by Saleh et al. (9) on the same soil sample to ascertain any differences between the two methods in efficiency of bacterial isolation. First, we used the antibiotic selection method described by Saleh et al. (ampicillin and polymyxin B; 9). Then we subjected a part of the same soil sample to the method described here. After the prescribed treatment, dilution, and plating of the samples on agar, the following results were obtained. Of 125 colonies which grew on ampicillin-polymyxin B plates, only 1 colony formed a parasporal crystal. Of 140 colonies randomly picked after acetate selection and heat treatment, 47 colonies formed crystals. The single crystal former isolated on antibiotic plates was identified by biochemical tests (6) as *B. thuringiensis* subsp. *tohokuensis*. The 47 crystal formers isolated by acetate selection were identified by the same battery of biochemical tests as 20 strains of *B. thuringiensis*. Nine of the acetate-selected crystal formers failed to grow on plates containing ampicillin and polymyxin B. It is particularly important that colonies picked from the initial agar plate be representative of all colony morphologies on the plate and not just of those that have the typical *B. thuringiensis* fried-egg morphology (8). The acetate-heat treatment often temporarily alters the typical colony morphology, which then quickly (in two or three subcultures) reverts to normal.

Isolation of *B. thuringiensis* from local soil samples. To test the field performance of the new procedure, soil samples were collected in Montgomery County, Md. and screened for *B. thuringiensis* by acetate selection. The criterion for the presence of *B. thuringiensis* in any given sample was the occurrence of one crystal former in the first 60 bacterial colonies examined. Of the first 68 soil samples examined, 67 samples contained *B. thuringiensis*. Crystal formers were present in 10% of the 6,000 colonies examined. As many as four crystal morphologies could be seen in colonies of bacteria from a single soil sample. Subsequent soil surveys indicated that many soil types found in the Washington, D.C., area contained abnormally low levels of *B. thuringiensis*.

DISCUSSION

The method reported here, a novel single-plate method, can select *B. thuringiensis* spores at a level below 100 spores per g of soil. This selection method eliminates most sporeforming bacteria and all nonsporeforming organisms in a soil sample. The method allows the spores of unwanted bacterial species to germinate while preventing the desired bacteria from germinating. The unwanted bacteria, which enter the vegetative state, are eliminated by a controlled heat treatment. This procedure also allows the detection of desired organisms that would otherwise be eliminated if antibiotics were used as a selective factor.

Of the six non-*B. thuringiensis* sporeforming *Bacillus* spp. tested, only *B. sphaericus* responded similarly to the *B. thuringiensis* strains. This bacterium, although devoid of a parasporal crystal, also has insecticidal properties (12). *B. cereus*, the bacterium most closely related to *B. thuringiensis* (11), germinated in 0.25 M acetate-buffered medium, and therefore the resultant vegetative cells were eliminated by heat treatment in our procedure.

Forty *B. thuringiensis* strains were tested for their ability to germinate in media with various concentrations of sodium

TABLE 3. Time course of germination of spores in acetate with and without heat treatment

Time (h)	No. of CFU/g of soil	
	With heat treatment	Without heat treatment
0	1.78×10^6	6.22×10^6
1	1.00×10^6	6.40×10^6
2	1.18×10^6	5.81×10^6
3	1.59×10^6	5.97×10^6
4	8.00×10^3	6.34×10^6
5	3.50×10^4	6.23×10^6
6	2.00×10^4	6.66×10^6

acetate. Of these 40 strains, only 2 (non-acetate-selected) environmental isolates germinated at high levels of acetate. The difference in ability of strains to germinate in the presence of acetate was the only unusual characteristic that separated these isolates from the others that were tested. The strains were similar in crystal morphology, biochemical profiles, and ability to grow and sporulate on minimal media (data not shown). Several isolates of *B. thuringiensis* lacked the ability to germinate in the presence of 0.12 M acetate buffer. Of these, two isolates were recognized subspecies of *B. thuringiensis* and two isolates were non-acetate-selected environmental strains. Neither biochemical characteristics, parasporal crystal morphology, nor toxicity suggests why these strains show these differences in ability to germinate in acetate.

We do not yet know why spores from nearly all *B. thuringiensis* strains were unable to germinate in the presence of sodium acetate even though these bacteria were able to grow vegetatively in an acetate-buffered medium. The inability of most *B. thuringiensis* strains to germinate in the presence of acetate buffer allows the use of this trait to screen for this organism in environmental samples. Placing soil samples in growth media and incubating them allows the maximum germination of unwanted organisms. Subsequent heat treatment eliminates the excess contaminating organisms. The procedure outlined here should be used without modification to isolate *B. thuringiensis*. Subsequent research has indicated that alterations in methodology may not eliminate potentially hazardous soil microbes. Testing showed that *B. anthracis* was removed from soil samples by the method described. Any changes may affect *B. anthracis* recovery.

Using the ampicillin-polymyxin B selection method of Saleh et al. (9), DeLucca et al. (3) examined 46,373 bacillus colonies which yielded 250 isolates of *B. thuringiensis*. These numbers represented 95 soil samples, of which 15 contained *B. thuringiensis*. This method selected primarily against the nonsporeforming bacteria in the soil sample, thus leaving most of the *Bacillus* species viable. Data presented by Saleh et al. (9) suggested that a simple heat treatment would reduce the unwanted microorganisms as efficiently as antibiotics. The method described in this paper could further reduce the number of *Bacillus* isolates to be examined from any soil sample by a factor of 100 over simple heat treatment. Data obtained from this study confirm this assumption. In our work, typically 20 to 96% of the bacterial colonies randomly selected after acetate selection and subsequent heat treatment of a soil sample were *B. thuringiensis*. By using the method we describe and screening the same number of colonies as DeLucca et al. (3), approximately 25,000 crystal formers would be isolated from the same number of *Bacillus* spp. isolates. Using this method,

we have examined ca. 250 dirt samples over the last 3 years. From these samples, we have isolated ca. 8,000 crystal-positive *B. thuringiensis*. These isolates were screened by the method of Martin et al. (6) and found to contain 73 new biochemically distinct varieties of *B. thuringiensis*. When compared with the existing 24 varieties, these new varieties displayed extraordinary diversity and efficacy as insect control agents (G. J. Tompkins, J. J. Linduska, P. A. W. Martin, R. S. Travers, and D. Diggs, Insecticide and Acaricide Tests, in press; G. J. Tompkins, J. J. Linduska, P. A. W. Martin, R. S. Travers, D. Diggs, and M. L. Lanham, Insecticide and Acaricide Tests, in press).

The present study illustrates the advantage of a selection method based on inhibition of growth for desired bacteria under conditions that permit other microorganisms to develop stages sensitive to a subsequent lethal treatment. The desired bacteria are preserved because they remain in a quiescent state. In a logical extension of this principle, the novel selection method described here could be used for isolating specific types of bacteria. New selective media for use with this process are under development in this laboratory.

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