Efficacy of a Fluorescent-Antibody Procedure for Identifying Bacillus cereus in Foods

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One hundred and seventeen strains of Bacillus were examined by the fluorescent-antibody technique by using the globulin fraction of serum prepared against spores of B. cereus T. All but one strain of the 59 B. cereus tested fluoresced at the exosporium surface. Fluorescent staining of B. anthracis, B. thuringiensis, and B. mycoides was also observed. Absorption of the globulin fraction with B. anthracis and B. mycoides resulted in the elimination of staining of these organisms. Absorption with B. thuringiensis ATCC 10792 removed antibodies reacting with 6 of the 12 strains of B. thuringiensis tested. Absorption with B. thuringiensis var. galleriae removed antibodies against B. cereus to such a degree that the globulin fraction was unusable.

Since Bacillus cereus was described as an etiological agent of foodborne disease by Hauge (4, 5), there has accumulated increasing evidence that this organism is indeed pathogenic, causing illness when ingested in large numbers. Thus, it is desirable to have at hand rapid and specific means of identifying and enumerating B. cereus in food and other materials derived from epidemiological investigations. Recently, two media have been formulated that permit a presumptive enumeration of B. cereus. Both the MYP medium of Mosserl, Koopman, and Jongerius (9) and the KG medium of Kim and Goepfert (7) utilize the lecithinase reaction as the means of differentiating B. cereus from other Bacillus spp. Both media have limitations in that (i) certain lecithinase-negative strains of B. cereus have been isolated, (ii) at least one temperature-sensitive lecithinase mutant of B. cereus has been isolated (H.U. Kim and J.M. Goepfert, unpublished data), and (iii) certain other species of Bacillus (e.g., B. mycoides, B. thuringiensis, B. anthracis, B. laterosporus, B. alvei, B. circulans, B. brevis, and B. pasteurii) and some B. polymyxa strains also produce lecithinase (1). For these reasons, it is desirable to have a rapid confirmatory test for B. cereus.

Traditionally, most approaches to identification have involved biochemical testing. Most commonly, nitrate reduction, starch hydrolysis, sugar fermentation tests, production of acetylmethylcarbinol, and growth on chloral hydrate agar and phenethyl alcohol agar have been used to characterize B. cereus. However, not only is this time consuming, but there apparently exists a significant degree of physiological heterogeneity among strains of B. cereus.

Another approach has involved agglutination and precipitin tests by using various vegetative cell and spore antigen preparations (10). Although all attempts to devise an agglutination test for B. cereus have failed, these studies have shown that there are antigens that are specific to B. cereus spores and which are present in vegetative cells. Norris and Wolf reported that a heat-resistant, hot formamide-extractable precipitinogen that is species specific was present in spores of B. cereus (11). In an earlier study we attempted to use this test as a diagnostic aid for B. cereus identification (6). However, we were unable to confirm the species specificity of this antigen and, indeed, found it to reside in a considerable number of non-B. cereus isolates from various sources.

Preliminary studies with the fluorescent-antibody (FA) technique indicated that the sensitivity of the procedure might permit detection of specific antigens on the spore surface that would make this a feasible approach to confirmatory testing. This report describes the progress that has been made and the limitations of the FA procedure for identifying B. cereus.
MATERIALS AND METHODS

Cultures. The strains of B. cereus and other Bacillus species used in this study and their sources are listed in Table 1. Cultures were maintained on nutrient agar slants in screw-capped tubes at room temperature.

Preparation of spores. Spores were prepared by growing the culture in G medium (3) with shaking at 32 C for 4 days. Spores were harvested by centrifugation at 10,000 x g and washed with sterile, distilled water. Spores used in preparation of antiserum were washed repeatedly as described previously (8) and resuspended in distilled water in a concentration of 30 mg/ml. Spores used for absorption of the antiserum preparations were washed with sterile, distilled water five times. Washed spores were diluted in distilled water to a concentration of 30 mg of spores per ml for absorption.

Preparation of antiserum. An autoclaved, aqueous spore suspension containing 30 mg of spores per ml was homogenized with an equal volume of Freund complete adjuvant. A 1-ml amount of this mixture was injected intradermally into the shoulder of New Zealand white rabbits. The rabbits were given three consecutive injections at 1-week intervals, followed by a booster injection 2 weeks later. Serum was harvested 2 weeks after the booster injection, and in most cases the rabbits were given another booster injection after four consecutive bleedings which were made at weekly intervals.

Fractionation of antiserum. Gamma globulin was precipitated from the serum by addition of ammonium sulfate to 33% saturation at 0 C. The resultant precipitate was resuspended in phosphate-buffered saline at pH 7.2 and reprecipitated with ammonium sulfate. The globulin fraction was resuspended in phosphate-buffered saline (pH 7.2) and dialyzed overnight at 4 C against the same buffer.

FA staining of the spores. The indirect staining procedure with fluorescein-labeled goat anti-rabbit gamma globulin (GAR) (Cappel Laboratories, Downington, Pa.) was employed. A loopful of the spore suspension was placed on agar-coated slides and allowed to dry at 32 C. The slides were then covered with 0.02 ml of anti-B. cereus globulin diluted 1:8 and incubated for 15 min in a moist chamber at room temperature. The slides were washed in 0.02 M phosphate buffer (pH 8.2), rinsed briefly in distilled water to remove excess phosphate, and dried. The smears were covered with fluorescein-labeled GAR and incubated at room temperature for 30 min before being rinsed as described. Fluorescence of the stained spores was observed by using a Leitz microscope with ultraviolet-blue excitation from an HBO2000 Osram lamp. A BG12 exciter filter and a K510 barrier filter were used. The degree of fluorescence was rated on a subjective scale ranging from 1+ to 4+.

Absorption of antiglobulin solution. The globulin fraction was diluted two- to fourfold and mixed with an equal volume of a spore suspension containing 30 mg of spores per ml. The mixture was kept at 37 C with occasional stirring. After 2 hr, the spores were removed by centrifugation, and the absorbed globulin was passed through a 0.45-μm membrane filter (Millipore) to remove residual spores. The absorption was considered complete when spores of the same culture used in absorption did not fluoresce when stained with the absorbed preparation.

RESULTS AND DISCUSSION

One hundred and seventeen strains of Bacillus were examined by the FA procedure by using the globulin fraction of antiserum prepared against autoclaved spores of B. cereus T. Specific fluorescence at the spore surface was obtained with 58 or 59 strains of B. cereus, all 13 strains of B. thuringiensis, each of 4 strains of B. mycoides, and the 7 strains of B. anthracis examined (Table 2). With a single exception, none of the "non-cereus group" bacilli evidenced any staining. The exception was B. pumilus which showed a 2+ fluorescence. Each of the fluorescent-positive strains fluoresced at a level of 2+ or greater, and 64 of 58 of the B. cereus strains stained 4+ or 4+.

Comparative observations of individual spores illuminated by tungsten light and ultraviolet-blue light indicated that the primary staining site was the exosporium structure. To confirm this, spores of B. cereus T were sonically treated by the method of Berger and Marr (2) to remove the exosporium layer and stained with the anti-T globulin and GAR reagent. Spores treated in this fashion fluoresced at a 2+ level (as opposed to 4+ for the untreated spores), and the staining appeared to be associated with the outer surface of the spore. These observations strongly suggest that the exosporium is the primary site of staining and that the sonically-treated spores stained at the coat surface because (i) the exosporium layer was not completely removed or (ii) because the exosporium layer was broken down in the rabbit, albeit slowly, and the antibody against the coat was also present in the globulin fraction employed. It is also possible that the spore coat and the exosporium share a common antigen. Moreover, autoclaving the spore preparation prior to inoculation into the rabbit may have altered the antigenic structure of the coat material to such an extent that antibodies against the heated material did not react at all, or only poorly, with the antigens in the unheated spore coat. The experiments necessary to resolve these problems were not performed.

Interestingly, the single strain of B. cereus (B2-AC) that did not stain with the T-globulin fraction did not have an apparent exosporium.
| Cultures | Source | Comments |
|----------|--------|----------|
| *Bacillus cereus* T | R. S. Hanson, U. of Wis., Madison | |
| *B. cereus* 201 | W. B. Sarles, | |
| *B. cereus* 5065 | T. Midura, State Dept. of Public Health., Berkeley, Calif. | Isolated from food poisoning outbreak |
| *B. cereus* B2-AC, B4-AC, B9-AC, B5-AC, B6-AC, B7-AC | D. A. A. Mossel, Louvain, Belgium | Isolated from food poisoning outbreaks |
| *B. cereus* F-38, F-39, F-42, F-47, F-54, F-71, F-77 | C. V. Hall, Public Health, Labs, Seattle, Wash. | Isolated from food poisoning outbreak |
| *B. cereus* 17 Benz, 27 Benz, 39 Benz, D1, D4 | U. de Barjac, Inst. Pasteur, Paris, France | Received as lecithinase-negative strains |
| *B. cereus* B47 | A. Krieg, Inst. für Biologische Schadlingsbekämpfung, Darmstadt, West Germany | Received as lecithinase-negative strain |
| *B. cereus* 2280 | G. W. Gould, Unilever Research, Bedford, England | Received as lecithinase-negative strain |
| *B. cereus* 318, 319, 622, 625, 629, 1940, 1977, 1978, 1979, 1980 | G. W. Gould, Unilever Research, Bedford, England | Isolated from dried foods |
| *B. cereus* A-1, A-2, A-8-1, A-4-1, A-10-2, A-16-1, A-17-1, B-12, C-5, D-1, D-11, D-13-1, D-13-2, E-1, F-3, F-19-2, F-24-3, G-1, G-8, G-3, G-13-1, G-11, H-14, F-8 | Food Research Institute, U. of Wis., Madison | |
| *B. anthracis* WBS | W. B. Sarles, Dept. of Bacteriology, U. of Wis., Madison | |
| *B. anthracis* 10, 240, 4728, 11949, 11966, 14186, 14578 | R. E. Gordon, Institute of Microbiology, Rutgers Univ. | |
| *B. thuringiensis* ATCC 10792 | W. B. Sarles, Dept. of Bacteriology, U. of Wis., Madison | |
| *B. thuringiensis* var. galleriae, entomocidus, dendrolimus, berliner, alesti, sotto, subtoxicus, tolworth, galleriae (B), finitimus | A. A. Yousten, Growth Science Center, International Minerals and Chemical Co. | |
| *B. thuringiensis* F-14-2, F-14-3 | Food Research Inst., U. of Wis., Madison | Isolated from dried foods |
| *B. mycoides* WBS | W. B. Sarles, Dept. of Bacteriology University of Wis., Madison | |
| *B. mycoides* 628 | G. W. Gould, Unilever Research, Bedford, England | Isolated from dried foods |
| *B. mycoides* GK-1 | Food Research Inst. Univ. of Wis., Madison | Isolated from dried foods |
| *B. mycoides* 5119 | U. de Barjac, Inst. Pasteur, Paris, France | Received as nonrhizoidal strain |
However, debris in the preparation did fluoresce, and it is possible that this debris represented exosporium material that was synthesized but not completely assembled prior to lysis of the sporangium. Time course studies employing electron microscopy might provide the information necessary to explain this observation.

In the initial phase of this investigation, it was hoped that the FA procedure could provide a species-specific identification of *B. cereus* and possibly a means of classifying the pathogenic and nonpathogenic strains. To this end, a series of absorption trials were performed.

Anti-*B. cereus* T globulins were absorbed with spores of *B. mycoides* WBS and *B. thuringiensis* ATCC 10792. The absorbed globulins were then tested for ability to effect staining of *B. cereus*, *B. mycoides*, *B. thuringiensis*, and *B. anthracis*. As shown in Table 3, the adsorbed reagent was able to react with all 33 strains of *B. cereus* examined, each of the *B. anthracis* strains, six strains of *B. thuringiensis*, and *B. mycoides* 5119. This latter strain was received as *B. mycoides* but did not exhibit the rhizoidal growth typical of these organisms and, indeed, was culturally indistinguishable from *B. cereus*. It is evident that two broad groups of *B. thuringiensis* exist with respect to serorelatedness to *B. cereus*.

An attempt was made to remove the staining of the remaining six strains of *B. thuringiensis* by absorbing the treated globulin preparation with *B. thuringiensis* var. *galleriae*. Although this absorption did remove the cross-staining reactions, it also removed the antibodies that combine with *B. cereus*,

| Cultures                  | Source                                      | Comments                        |
|---------------------------|---------------------------------------------|---------------------------------|
| *B. licheniformis* A-5    | R. S. Hanson, Dept. of Bacteriology, Univ. of Wis., Madison |                                |
| *B. licheniformis* V-37   | E. Kampelmacher, Utrecht, The Netherlands   |                                |
| *B. megaterium* USDA 234  | W. B. Sarles, Dept. of Bacteriology, Univ. of Wis., Madison |                                |
| *B. subtilis* 10, Mol, Marburg 168 | W. B. Sarles, Dept. of Bacteriology, Univ. of Wis., Madison |                                |
| *B. subtilis* var niger   | W. B. Sarles, Dept. of Bacteriology, Univ. of Wis., Madison |                                |
| *B. pumilus* J. L.        | W. B. Sarles, Dept. of Bacteriology, Univ. of Wis., Madison |                                |
| *B. brevis* Dubos         | W. B. Sarles, Dept. of Bacteriology, Univ. of Wis., Madison |                                |
| *B. polymyxa* WBS 1947    | W. B. Sarles, Dept. of Bacteriology, Univ. of Wis., Madison |                                |
| *B. coagulans* WH-9       | W. B. Sarles, Dept of Bacteriology Univ. of Wis., Madison |                                |
| *B. popilliae*            | A. A. Yousten, Growth Science Center, International Minerals and Chemical Co. |                                |
| Miscellaneous unidentified *Bacillus* spp.: A-8S, A-9S, A-15S, B-12S, B-18S, B-20S1, C-12S, C-13S, C-23S, D-5S, D-6S, D-11S1, E-11S, E-12S, F-6S, F-9S, F-10S, F-21S, G-13S1, G-15S, G-19S | Isolated from dried foods |
experiments were performed, a very complex picture of the antigenic nature of the *B. cereus* spore surface was encountered. These trials revealed that the exosporium layer contained at least three to five antigens in addition to the antigen common to all *B. cereus* strains. Because only five strains were involved in this experiment, it may be that the number of exosporium antigens eventually uncovered will approach the number of strains examined.

To test the efficacy of the FA confirmatory procedure, 24 strains of *B. cereus* were chosen for further study. Three-month-old nutrient agar slant cultures of each strain were used as a source of inoculum. Each culture was streaked directly onto the surface of fresh KG agar plates. The plates were incubated at 37°C for 24 hr. After incubation, a small amount of culture was taken from the center of a well-isolated colony and suspended in 1 ml of sterile, distilled water. Smears were made from these suspensions and stained with anti-*B. cereus* T globulins by the indirect FA procedure. Each of the 24 strains showed free spores that fluoresced at 3+ or greater. At this time, it would appear that the use of the FA procedure for the rapid confirmation of an isolate as *B. cereus* is workable. Implementation of the technique on smears made from colonies appearing on KG agar will permit the confirmation to be completed within 24 to 30 hr after initiation of the analysis of the specimen.

The FA confirmatory procedure affords several advantages over other methods previously suggested. First, it is certainly the most rapid. Second, it is seemingly as specific (if not more so) than biochemical testing because the species appears to be relatively heterogeneous in physiological characteristics (6). Third, it enables the rapid investigation of egg yolk-negative, morphologically similar organisms, which

| Species | No. of strains tested | No. positive | No. negative |
|---------|-----------------------|-------------|-------------|
| *Bacillus cereus* | 33 | 33 | 0 |
| *Bacillus mycoides* | 4 | 1 | 3 |
| *Bacillus anthracis* | 7 | 7 | 0 |
| *Bacillus thuringiensis* | 12 | 6* | 6* |

* Includes *Bacillus var. galleriae, finitimus, tolworth, entomocidus*, and strains F-14-2 and F-14-3

* Includes *Bacillus var. dundraloimius, berliner, alesti, sotto, subtoxicus*, and strain ATCC 10792

and the absorbed globulin fraction became useless. At about this time, it was noted that certain strains of *B. thuringiensis* were able to elicit fluid accumulation in the rabbit ileal loop (W. M. Spira and J. M. Goepfert, in press). Because, at present, this is presumptive evidence for the ability of an organism to cause diarrhea in humans, it was decided that detection of *B. thuringiensis* by the FA procedure might indeed be a fortuitous advantage. Thus, adsorption of the anti-*B. cereus* T globulins with *B. thuringiensis* was discontinued.

Absorption of anti-*B. cereus* T globulins with *B. anthracis* WBS removed the staining of three strains of *B. anthracis* and reduced the fluorescence level of the other five *B. anthracis* strains to 1+, but did not alter the fluorescent staining of *B. cereus*.

When antisera were prepared against five strains of *B. cereus* and reciprocal absorption

### Table 2. Results of FA staining of the Bacillus spores by using anti-*B. cereus* T globulin

| Fluorescence-positive strains | Fluorescence-negative strains |
|------------------------------|------------------------------|
| *Bacillus cereus*: T 201, B4-AC, B5-AC, B6-AC, B7-AC, B9-AC, 5065, 17 Benz, 27 Benz, 39 Benz, D1, D2, B47, 318, 319, 622, 625, 629, 1977, 1978, 1979, 1980, 1940, 2280, A-3-1, A-10-2, G-11, A-4-1, D-1, E-1, H-14, G-8, A-1, A-2, F-19-2, F-24-3, G-13-1, D-13-2, G-1, G-3, D-13-1, F-39, F-38, F-42, F-47, F-77, F-66, F-71, F-54, A-16-1, B-12, A-17-1, C-5, D-11, F-3, F-8 | *Bacillus coagulans* WH-9 |
| *Bacillus thuringiensis*: 10792, F-14-2, F-14-3, galleriae, entomocidus, dundraloimius, berliner, alesti, sotto, finitimus, subtoxicus, tolworth, galleriae Y. | *B. megaterium* USDA 234 |
| *Bacillus mycoides*: WBS, 628, 5119, G-1 | *B. licheniformis* A-5, V-37 |
| *Bacillus anthracis*: WBS, 10, 240, 4728, 11966, 14186, 14578 | *B. subtilis* Marburg 168, 10, Mol. |
| *Bacillus pumilus* J. L. | *B. polymyxa* WBS 1947 |

TABLE 3. Reaction of *Bacillus* species in the FA test by using the globulin fraction of anti-*B. cereus* T serum absorbed with *B. mycoides* WBS and *B. thuringiensis* ATCC 10792

| Species | No. of strains tested | No. positive | No. negative |
|---------|-----------------------|-------------|-------------|
| *Bacillus cereus* | 33 | 33 | 0 |
| *Bacillus mycoides* | 4 | 1 | 3 |
| *Bacillus anthracis* | 7 | 7 | 0 |
| *Bacillus thuringiensis* | 12 | 6* | 6* |

* Includes *Bacillus var. galleriae, finitimus, tolworth, entomocidus*, and strains F-14-2 and F-14-3

* Includes *Bacillus var. dundraloimius, berliner, alesti, sotto, subtoxicus*, and strain ATCC 10792

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is an important feature because lecinthinase-negative mutants do occur and certain strains have the ability to induce fluid accumulation in the rabbit ileal loop (W. M. Spira and J. M. Goepfert, in press). In this connection, we have also isolated lecinthinase-positive group 2 bacilli from foods as well as B. cereus strains that had temperature-sensitive lecinthinase systems that may go undetected on either isolation medium.

It must also be noted that the FA procedure is not 100% efficient at this point and, indeed, may never be. Obviously, asporogenic mutants cannot be confirmed by this procedure. B. thuringiensis will also be detected, but these may be differentiated by observation of crystals in the preparation. In addition, it must be remembered that this test does not provide information on the pathogenicity of a given isolate but could be used to indicate whether such a test should be undertaken.

Unfortunately, it is not possible with the data now available to group the various strains of B. cereus on the basis of exosporium antigens. Additional work in this direction is in progress.

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