Monoclonal Antibodies for Use in Detection of Bacillus and Clostridium Spores†

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Five monoclonal antibodies against bacterial spores of Bacillus cereus T and Clostridium sporogenes PA3679 were developed. Two antibodies (B48 and B183) were selected for their reactivity with B. cereus T spores, two (C33 and C225) were selected for their reactivity with C. sporogenes spores, and one (D89) was selected for its reactivity with both B. cereus and C. sporogenes spores. The isotypes of the antibodies were determined to be immunoglobulin G2a (IgG2a) (B48), IgGl (B183), and IgM (C33, C225, and D89). The antibodies reacted with spores of B. cereus T, Bacillus subtilis subsp. globigii, Bacillus megaterium, Bacillus steatothermophilus, C. sporogenes, Clostridium perfringens, and Desulfitomaculum nigrificans. Antibody D89 also reacted with vegetative cells of B. cereus and C. sporogenes. Analysis of B. cereus spore extracts showed that two of the antibodies with which the anti-Bacillus antibodies reacted had molecular masses of 76 kDa and approximately 250 kDa. Immunocytochemical localization indicated that antigens with which B48, B183, and D89 react are on the exosporium of the B. cereus T spore. Antibody D89 reacted with the exosporium and outer cortex of C. sporogenes spores in immunocytochemical localization studies but did not react with extracts of C. sporogenes or B. cereus spores in Western blotting. Some C. sporogenes antigens were not stable during long-term storage at −20°C. Antibodies B48, B183, and D89 should prove to be useful tools for developing immunological methods for the detection of bacterial spores.

Spore-forming bacteria are responsible for a variety of food spoilage and food-borne illness problems. As the production of minimally processed refrigerated products becomes more efficient and aseptic, background microflora are eliminated, and it is the spore-forming organism which eventually may limit the shelf-lives of these products. Sporeformers have been responsible for the spoilage of canned foods, bread (2), vacuum-packed meats (16, 17, 21), pasteurized dairy products (13, 24), and fruit juices (23). The presence of spores at high levels in ingredients going into any of these types of products may increase the potential for spoilage of the finished product. The availability of a rapid method to detect the total spore load in raw ingredients would allow selection of the highest-quality ingredients for use in foods which have potential for spoilage by sporeformers.

Enzyme-linked immunosorbent assays (ELISAs) and other immunologically based detection systems are widely used in the food industry for quality control purposes, including detection of pathogens and toxins. The current technology for spore detection, however, still relies on a variety of cultural procedures for detection of the different classes of sporeformers (20, 26, 32).

Previous research has demonstrated the feasibility of detecting a broad range of bacterial spores by using a polyclonal antibody-based ELISA (11). Broad-range detection was possible because of cross-reactivity between species (25) and because of the use of a polyclonal antibody. In order to be practical, such an ELISA would require monoclonal antibodies, since they are less variable and are available in an unlimited supply through cell culture techniques.

In the study reported here, monoclonal antibodies against both Bacillus and Clostridium spores were developed. These antibodies were selected on the basis of having a broad range of reactivity with spores of a variety of species within and across genera. The reactivities of antibodies against both vegetative cells and spores of Bacillus cereus and Clostridium sporogenes were determined in order to identify antibodies which were spore specific.

MATERIALS AND METHODS

Bacterial strains and sporulation. Spore stocks were prepared as indicated in Table 1. Vegetative cell cultures of B. cereus T, Bacillus subtilis A, B. subtilis subsp. globigii, and Bacillus megaterium were grown in nutrient broth at 30°C. Bacillus steatothermophilus was grown in nutrient broth at 55°C, and Bacillus coagulans was grown in Trypticase soy broth at 43°C with shaking. C. sporogenes cells were grown in cooked meat medium at 35°C. Clostridium perfringens cells were grown in fluid thioglycolate medium at 37°C under anaerobic conditions, and Desulfitomaculum nigrificans cells were grown in BEFI (22) at 55°C under anaerobic conditions.

Antigen preparation. Antigen was prepared by formalin inactivation (28) of B. cereus T and C. sporogenes PA3679 spore stocks which were free of vegetative cell material as observed by phase-contrast microscopy. A total of 2 × 10⁷ inactivated bacterial spores (1 × 10⁷ B. cereus spores and 1 × 10⁷ C. sporogenes spores) were used as the antigen.

Monoclonal antibody production. Monoclonal antibody production was done according to the procedures of Kohler and Milstein (18). Hybridomas were screened for reactivity with spores of B. cereus T and C. sporogenes. Hybridomas found to be reactive with either spore type were screened for reactivity with the vegetative cells of that spore type.

Isotyping of monoclonal antibodies. Isotyping of monoclonal antibodies was performed by using a dot blot immunooassay format. Spores (10⁷ of the spores indicated as antigens in Table 2) were applied to an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) in a dot blot apparatus. The spores were then incubated with 100 µl of the appropriate tissue culture supernatants. Monoclonal antibody bound to spores was isotyped by using rabbit anti-mouse subclass-specific antiserum obtained from Bio-Rad (Hercules, Calif.).

Reactivities of monoclonal antibodies with bacterial spores and vegetative cells. Antibodies were screened for reactivity with spores from a range of Bacillus and Clostridium species. Spores (10⁷ of the spores indicated in Table 3) were applied to an Immobilon-P membrane, followed by incubation with the indicated antibodies. Similarly, 10⁶ of the indicated vegetative cells were applied to the membrane, followed by incubation with the indicated antibodies. Reactivity was determined by using a dot blot immunooassay format.
detected with a goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Sigma A4416) or a goat anti-mouse IgM-horseradish peroxidase conjugate (Sigma A0786). Color development was with the insoluble substrate 3-amino-9-ethyl-carbazole (Biomedica Corp.).

**Adsorption of monoclonal antibodies.** Culture supernatants were diluted 1:32 and 1:128 with Tris-buffered saline (TBS) (0.01 M Tris, 0.15 M NaCl, pH 7.4). Aliquots of diluted culture supernatants were mixed with an equal volume of the spore antigen (4 × 10^7 spores/ml) by gentle inversion overnight at 4°C. Diluted culture supernatants mixed with sterile distilled water served as a positive control. Antibodies adsorbed to spores were removed by centrifugation (ca. 4,000 × g, 10 min). The supernatant fluid was used in immunoblot assays as detailed above. The antigen used in the immunoblot assays included each spore with which the nonadsorbed antibody showed reactivity.

**Purification and biotinylation of monoclonal antibodies.** Antibodies were concentrated by ammonium sulfate precipitation of tissue culture supernatants (14). The concentrated protein was biotinylated by incubation with N-hydrosuccinimidobiotin (Sigma Chemical Co.) (ca. 100 μg/ml of antibody in the sample) at room temperature for 4 h. One molar ammonium chloride (20 μl/μg of biotin) was added to stop the reaction, and the biotinylated protein was dialyzed extensively against phosphate-buffered saline (0.1 M sodium phosphate, 0.8% NaCl) (PBS), pH 7.0.

For immunocytochemical localization studies, IgG antibodies were purified out of tissue culture supernatants by using a protein A column (Pierce Chemical Co., Rockford, Ill.) according to the manufacturer’s directions. IgM antibodies were purified from the culture supernatant by using a goat anti-mouse IgM antibody column (Pierce Chemical Co.). The tissue culture supernatant was diluted 1:1 in PBS. This dilution was applied to the goat anti-mouse IgM column, which had been equilibrated with PBS. Washing and elution of antibody off the column were according to the manufacturer’s directions. Fractions containing protein were pooled and concentrated with a Centricon 10 microconcentrator (Amicon, Danvers, Mass.) and centrifugation at 3,000 × g for 30 min.

**Purification and biotinylation of monoclonal antibodies.** Spores were extracted by the method of Aronson and Pringle (3) to remove spore coat and exosporium components. Spores of *B. cereus* T (2 × 10^10) or *C. sporogenes* (1 × 10^10) were extracted in 5 mM cyclohexylaminomethane sulfonic acid–8 M urea–30 mM β-mercaptoethanol–0.5% sodium dodecyl sulfate (SDS) at pH 9.8 for 90 min at 37°C. Spores were centrifuged at 12,000 × g for 10 min, and the supernatant extract was boiled for 5 min. Extracts were applied directly to SDS-polyacrylamide gels containing 6 M urea. For molecular weight determination, gels were transferred to 0.2-μm-pore-size nitrocellulose. Blots were blocked with 3% bovine serum albumin in TBS, pH 7.4. The blots were then incubated with the appropriate biotinylated antibody (B48, B183, or D89 for *B. cereus* spore extracts and C33, C225, or D89 for *C. sporogenes* extracts) diluted 1:1,000 in TBS containing 0.05% Tween 20. Avidin-labeled peroxidase (Sigma A7419) diluted 1:1,000 in TBS-0.05% Tween 20 was used to detect the antigens with the insoluble substrate 4-naphthol (Bio-Rad). Glycoprotein determination involved periodic acid-Schiff staining (30). The gels were fixed overnight and treated with 0.5% periodic acid for 2 h and then with 0.2% metabsilfite for 2 h. Gels were then exposed to Schiff reagent (basic fuchsin, sodium metabsilfite, and hydrochloric acid), purchased from Fisher Scientific, Pittsburgh, Pa. The mobilities of the proteins which stained positive as glycoproteins from the spore extracts were determined and compared to the mobilities of the antigens determined by Western blotting.

**Immunocytochemical localization.** Ultrathin (ca. 100-μm) cryosections of spores for immunological labeling with colloidal gold were prepared according to the method of Chang and Foegeding (5). *B. cereus* T and *C. sporogenes* PA3679 spores from the same stock as had been used for the antigen were used for these experiments. The cryosections were floated on PBS containing 5% fetal calf serum, followed in sequence by PBS, PBS containing 0.1 M ammonium chloride, and PBS, each for 5 to 10 min at room temperature. Sections were floated on drops of primary reagent (the appropriate monoclonal antibody diluted 1:100) for 1 to 2 h, followed by washing in PBS six times for 5 min each. Sections were then floated on drops of secondary reagent for 20 to 30 min. The secondary reagent was goat anti-mouse IgG plus goat anti-mouse IgM conjugated to 10-nm-diameter colloidal gold used at a dilution of 1:10. Sections were again washed six times in PBS, followed by three washes with distilled water. They were stained and embedded in methylcellulose by floating them on drops of 2.3 M methylcellulose containing about 20% (vol/vol) saturated aqueous uranyl acetate. Grids were picked up in loops, drained, and air dried. They were viewed in a Philips EM 300 electron microscope. Negative controls were spores treated with monoclonal antibodies of the IgG1 and IgM isotypes which did not react with bacterial spores.

**Removal of exosporia.** Exosporia were removed from *B. cereus* T spores essentially by the procedure of Du and Nickerson (9), except that homogenization in a Braun homogenizer was for 30-s intervals up to a total of 150 s, with 120 to 150 s required for optimal removal of exosporia as determined by crystal violet staining and light microscopy (9).

**RESULTS**

Following immunization of mice with a 1:1 mixture of *B. cereus* T spores and *C. sporogenes* PA3679 spores, 397 hybridomas were screened for their reactivities with 10^7 *B. cereus* spores and for their reactivities with 10^7 *C. sporogenes* spores. Nine hybridomas produced antibodies which reacted with both *B. cereus* T spores but not with either *C. sporogenes* spores or *B. cereus* vegetative cells. Fifteen hybridomas produced antibodies which reacted with *C. sporogenes* spores but not with either *B. cereus* T spores or *C. sporogenes* vegetative cells. Seven hybridomas produced hybridomas which reacted with both *B. cereus* T and *C. sporogenes* spores. Of these seven, one hybridoma which reacted with both types of spores was subcloned and was also found to react with both types of vegetative cells. This antibody was designated D89.

Two of the hybridomas producing antibodies which reacted only with *B. cereus* T spores were subcloned successfully, and the resulting antibodies are referred to as B183 and B48. Two of the hybridomas producing antibodies which reacted only with *C. sporogenes* spores also were subcloned successfully, and the resulting antibodies were designated C33 and C225. The antibodies were isotyped, and the two antibodies which were reactive with *B. cereus* spores, B183 and B48, were found to be IgG1 and IgG2a, respectively. Both antibodies (C33 and C225) which were reactive only with *C. sporogenes* spores were IgM isotypes. The antibody which reacted with both types of spores and both types of vegetative cells, D89, was also of the IgM isotype (Table 2). Light chains of all five antibodies were kappa chains.

The antibodies were examined qualitatively (visually) for their reactivities with a range of *Bacillus*, *Clostridium*, and *Desulfotomaculum* spores by using a dot blot format (Table 3). Monoclonal antibody B183 reacted very strongly with *B. cereus* T spores and reacted weakly with *B. megaterium* and *B. subtilis* subsp. *globigii* spores. Antibody B48 reacted strongly with *B. megaterium*.TABLE 1. Bacterial strains used for production of spore crops

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sporulation medium(a) (reference for method)</th>
<th>Temp (°C)</th>
<th>Time (days)</th>
<th>Culture source(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em> T</td>
<td>FNA (15)</td>
<td>35</td>
<td>2</td>
<td>NCSU</td>
</tr>
<tr>
<td><em>B. subtilis A</em></td>
<td>FNA (15)</td>
<td>30</td>
<td>2</td>
<td>NCSU</td>
</tr>
<tr>
<td><em>B. subtilis subsp. globigii</em></td>
<td>FNA (15)</td>
<td>30</td>
<td>2</td>
<td>AMSCO</td>
</tr>
<tr>
<td><em>B. megaterium</em> 12072</td>
<td>FNA (15)</td>
<td>30</td>
<td>2</td>
<td>NCSU</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em> 7953</td>
<td>Supplemented nutrient agar (6)</td>
<td>35</td>
<td>3</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>B. coagulans</em> 56177</td>
<td>BH1A (19)</td>
<td>43</td>
<td>21</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>C. sporogenes</em> PA 3679</td>
<td>TSP (34)</td>
<td>35</td>
<td>4</td>
<td>NCSU</td>
</tr>
<tr>
<td><em>C. perfringens</em> 3624</td>
<td>Duncan-Strong (10)</td>
<td>37</td>
<td>1</td>
<td>NCSU</td>
</tr>
<tr>
<td><em>D. nigricans</em> 7546</td>
<td>Mushroom compost (8)</td>
<td>55</td>
<td>14</td>
<td>F. Busta, University of Minnesota</td>
</tr>
</tbody>
</table>

(a) FNA, fortified nutrient agar; BH1A, brain heart infusion agar; TSP, Trypticase-sodium chloride-peptone medium. (b) NCSU, North Carolina State University; AMSCO, American Sterilizer Company, Apex, N.C.; ATCC, American Type Culture Collection, Rockville, Md.
**TABLE 2. Monoclonal antibodies against bacterial spores**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>B183</td>
<td>IgG1</td>
<td>B. cereus T spores</td>
</tr>
<tr>
<td>B48</td>
<td>IgG2a</td>
<td>B. cereus T spores</td>
</tr>
<tr>
<td>C33</td>
<td>IgM</td>
<td>C. sporogenes (PA 3679) spores</td>
</tr>
<tr>
<td>C225</td>
<td>IgM</td>
<td>C. sporogenes (PA 3679) spores</td>
</tr>
<tr>
<td>D89</td>
<td>IgM</td>
<td>Antigen common to both Bacillus and Clostridium spores and vegetative cells</td>
</tr>
</tbody>
</table>

**TABLE 3. Reactivities of monoclonal antibodies with bacterial spores and vegetative cells**

<table>
<thead>
<tr>
<th>Spores or cells</th>
<th>Reactivity* of monoclonal antibody:</th>
<th>B183</th>
<th>B48</th>
<th>C33</th>
<th>C225</th>
<th>D89</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus T spores</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>B. megaterium spores</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B. stearothermophilus spores</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>+++</td>
<td>−</td>
</tr>
<tr>
<td>B. subtilis A spores</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B. subtilis subsp. globigi spores</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>C. perfringens spores</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D. nigrificans spores</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>?</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>B. cereus T vegetative cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C. sporogenes vegetative cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Reactivities of antibodies were determined by applying 10^7 of the indicated spores or 10^8 of the indicated cells on Immobilon-P membranes and determining detection by using the dot blot procedure detailed in the text.

Western blots of B. cereus T spore extracts (Fig. 1) showed that the antigen with which B183 reacts has a molecular mass of approximately 250 kDa. Antibody B48 reacted with a 76-kDa protein. The molecular mass of the antigen with which D89 reacted could not be determined by the spore extraction procedure of Aronson and Pandey (1). Periodic acid-Schiff staining indicated that the proteins which react with B183 and B48 may be glycoproteins. The presence of glycoproteins in bacilli is controversial, and it has been demonstrated that some covalently attached sugars on Bacillus proteins are a result of nonenzymatic glycosylation (4). It is not known whether the presence of sugars on the antigens arises from enzymatic glycosylation or is a result of environmental factors. It has been observed in our lab, however, that the antibodies react with B. cereus spores sporulated at different times and of various ages. Therefore, it appears that even if the glycosylation of the antigens is a result of environmental factors, it does not affect the ability of the antibody to detect the antigens. Immunocytochemical localization indicated that the antigens with which B48, B183, and D89 react are each located in the exosporium (Fig. 2). This reactivity was very specific, because none of the antibodies demonstrated cross-reactivity with any other components of the spore when they were used to probe cross sections of the spores. The location of the antigens in the exosporium was confirmed by conducting dot blot immunoassays on spores from which the exosporium had been removed with a cell homogenizer. The exosporium was nearly completely removed after 120 to 150 s of homogenization, and these spores did not react with the antibody (data not shown).

Western blotting of C. sporogenes spore extracts was performed with antibodies D89, C33, and C225 exactly as for Bacillus spore extracts. Coomassie blue staining of the extracts showed that proteins were present, but none reacted with the anti-Clostridium antibodies in Western blotting. Monoclonal antibodies C33 and C225 did not react with C. sporogenes spores in immunocytochemical localization studies. Monoclonal antibody D89 did react with C. sporogenes spores in immunocytochemical localization, and the antibody appeared to be...
specific for the exosporium and the outer regions of the spore cortex (Fig. 3).

DISCUSSION

The two monoclonal antibodies which react with *B. cereus* T spores, B48 and B183, appear to react with different antigens on the spore surface. Both antigens are located predominantly on the exosporium of the spore. Immunoblotting experiments indicate that the antigens are specific to the spore, as the antibodies did not react with vegetative cells of *B. cereus* T. The presence of spore-specific proteins has been demonstrated in the model system, *B. subtilis*, where spore coat proteins are known to be synthesized in a defined temporal order (31). We were primarily interested in reactivity with spores and not with vegetative cells, as the latter are likely to be eliminated with mild heat treatment. Their specificity for spore antigens should make these antibodies valuable for capturing spores but not vegetative cells in an immunoassay. B48 and B183 were also found to cross-react with spores of *B. megaterium* and *B. subtilis* subsp. *globigii* with slightly different affinities.

The exosporium of the spore is composed primarily of protein, with carbohydrate and lipid present (33). It is divided into a basal layer, an intermediate layer, and an outer layer of hair-like projections (12). Previous research with *B. cereus* spores has shown the exosporium to contain spore-specific antigens which are not present on the cells (7). Polyclonal antibodies made against *B. cereus* spores which reacted with a range of *Bacillus* spores, but not vegetative cells, were found to be located primarily on the exosporium (5). *B. cereus* and *B. megaterium* spores have similar hexagonally ordered lattice structures in the basal layers of their exosporia (3). Similar tertiary structures of the proteins which make up this basal layer may contribute to the cross-reactivity of the exosporium-reacting antibodies with *B. megaterium* spores.

The hybridoma producing monoclonal antibody D89 was subcloned because it produced the only antibody which reacted with both spores and vegetative cells of both the *Bacillus* and *Clostridium* genera. Such an antibody could be useful in an immunoassay because it could help to eliminate the need for an expansive cocktail of monoclonal antibodies to detect a broad range of spores. It is also of interest because it may prove to be a useful tool in studying an antigen which is common to two different genera of sporeformers and present on

![FIG. 2. Immunocytochemical localization of *B. cereus* T spore antigens. Ultrathin cryosections of *B. cereus* T spores were incubated with a negative primary antibody control (a), B48 (b), B183 (c), or D89 (d) and then with a colloidal gold-labeled secondary antibody. Bars, 0.1 μm.](http://aem.asm.org/)

...
Antigenic determinants may be lost or altered over long-term storage at \(-20^\circ\text{C}\). C. sporogenes spores were reactive with antibodies C33, C225, and D89 in dot blot assays up to 18 months after the original preparation of the spore stock. Between 18 and 24 months of storage at \(-20^\circ\text{C}\), it was observed that the antibodies were not reactive in Western blots of C. sporogenes spore extracts. It was unclear whether the problem was with insufficient extraction of the antigens, alteration of the antigens upon extraction, or loss of the antigens in the stored spores. After approximately 24 months of storage of the spores, immunocytochemical localization of the antigens was performed to identify where on the spores the antigens were located. The antigens with which C33 and C225 reacted were no longer reactive when cross-sections of Clostridium spores were probed with the antibodies C33 and C225. After the same period of storage, the antigen which reacted with D89 was still reactive in immunocytochemical localization studies. Two new spore stocks were prepared and held at 4°C for \(<6\) months. Spores of each of these stocks reacted with C33, C225, and D89 in dot blot immunoassays. These spores could also be detected by C33 in an ELISA at levels of \(\geq10^7\) spores/ml when spores of the original spore stock could not be detected even at levels of \(10^6\) spores/ml (data not shown). These results indicated that the antibody was still able to react with antigen, but they led us to conclude that some antigens on spores from the original spore stock were altered such that they were no longer recognized by the antibodies C33 and D225. The loss of bacterial spore antigen over long-term refrigerated storage of spores has previously been reported for Bacillus anthracis (27).

Together, three of these antibodies (B48, B183, and C33) are able to detect spores of B. cereus, B. megaterium, B. subtilis subsp. globigii, B. stearothermophilus, C. perfringens, and D. nigriicans. Future research will focus on the use of these antibodies, along with antibody D89, to develop immunoassays which will detect a range of bacterial spores of significance in food products and ingredients. Our results indicate that broad-range detection of spores with a monoclonal antibody-based detection system would most likely require a cocktail of monoclonal antibodies, as no one antibody was found to react with the entire range of spores examined. One antibody found to react with both Bacillus and Clostridium spores also reacted with vegetative cells of both genera. Such an antibody may be useful as a detector antibody once spores are isolated from a food or ingredient by using a more specific capture antibody.

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