Spore Coat Protein and Enterotoxin Synthesis in Clostridium perfringens

RONALD G. LABBE1 AND CHARLES L. DUNCAN2*

Food Research Institute, Department of Food Microbiology and Toxicology, and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 25 April 1977

Polyacrylamide gel profiles of Clostridium perfringens spore coat protein revealed four and occasionally five components. Pulse-chase experiments indicated that synthesis of coat protein polypeptide and enterotoxin was an early sporulation event. However, maximum synthesis occurred coincident with the onset of heat resistance.

Depending on the species involved, previous investigators have reported that spore coat proteins are composed of one to several polypeptides (3, 4, 9, 10, 11, 14). We have obtained evidence for the involvement in Clostridium perfringens of stable messenger ribonucleic acid in the synthesis of enterotoxin and spore coat protein (8). Since C. perfringens enterotoxin is itself a structural component of the spore coat (3), we looked at the polyacrylamide gel profile of coat proteins and the kinetics of synthesis of coat protein and enterotoxin. The production of clean spores of C. perfringens NCTC 8798 and the determination of percent sporulation, levels of protein, and presence of heat-resistant spores were accomplished by previously described techniques (5–7).

For gel electrophoretic profiles of solubilized coat protein, 25 mg of clean, lyophilized spores was suspended in 15 ml of 50 mM dithiothreitol (DTT), pH 10.0. The spores were subjected to sonic treatment for 15 to 20 s to disrupt clumps and then were extracted by gentle stirring at 37°C for 2 h. About 33% of the total spore protein was solubilized by this procedure (3). As seen by electron microscopy, most and occasionally all of the structural material exterior to the cortex was removed by this extraction without apparent alteration of the remaining spore structure (R. Reich and C. L. Duncan, unpublished data). The solubilized spore coat material was centrifuged at 15,000 × g for 20 min at room temperature. The supernatant fluid was concentrated eight- or ninefold by ultrafiltration on a Diaflo PM 10 membrane (Amicon Corp., Lexington, Mass.). A sample of the concentrated coat proteins was immediately analyzed on 5.5% polyacrylamide gels (2) with bromophenol blue as the tracking dye. The balance of the coat material was dialyzed for 16 h at 4°C against 50 mM barbitral buffer, pH 8.6, to remove DTT and then assayed for protein content or used for production of antiserum. Antiserum against both coat proteins and purified enterotoxin was produced as described previously (12).

A representative densitometric tracing of the profiles obtained is shown in Fig. 1. Four peaks were obtained with Rf values of 0.13, 0.24, 0.48, and 0.72. Occasionally, an additional slowly moving, barely stainable band appeared near the top of the gel. The possibility that this represents aggregates cannot be eliminated. Extraction of spores with 0.1 N NaOH or 8 M urea-1% mercaptoethanol (pH 8.5) resulted in essentially the same pattern of four protein bands but with occasional slight smearing of the stained gel. Gel profiles of dialyzed coat material were similar to those of nondialyzed samples. The appearance of multiple protein species in extracted spore coats is in agreement with results obtained by using C. perfringens NCTC 8238 (3) and Bacillus megaterium (14). On the other hand, other investigators using Bacillus thuringiensis (10) and B. cereus (1) reported that spore coat protein was comprised of only one or two protein species.

At least three precipitin arcs (Rf, 0.24, 0.47, and 0.70) consistently formed when anticoat serum was diffused against polyacrylamide gels containing coat proteins separated by electrophoresis (13). The failure to detect a number of precipitin arcs equal to the number of stained protein bands may have resulted from antibody excess via a via the minor protein species.

To relate the kinetics of synthesis of enterotoxin and other spore coat proteins to the sporulation process in general, pulse-chase tech-

1 Present address: Department of Food Science and Nutrition, University of Massachusetts, Amherst, MA 01003.
2 Present address: Campbell Institute for Food Research, Camden, NJ 08101.
niques were employed. Cells at different intervals during sporulation were exposed to $[^{14}C]$serine (162 mCi/mmol; final concentration, 0.1 $\mu$Ci/ml) for 10 min followed by a cold-serine (0.35 mg/ml) chase. The cells were immediately centrifuged at 10,000 $\times$ g for 10 min at 37°C. The pellets were gently suspended in 35 ml of culture supernatant fluid from an unlabeled culture of the same age which contained a 5,000-fold excess of cold serine. Incubation was continued for a total of 7 h, at which time all cultures were centrifuged for 20 min at 15,000 $\times$ g at 4°C. This procedure did not decrease the number of heat-resistant spores obtained at 7 h relative to an untreated control culture. The pellets were suspended in 3 ml of cold, distilled water and sonically treated until 95% of the spores were free from the sporangium. The cell extract was cleared by centrifugation and used for immunoprecipitation (see below). Spores were washed 20 to 25 times with cold, distilled water and extracted with DTT. After dialysis against the barbital buffer, the protein content of extracted spore coat material was determined. In addition, 0.5 ml of this material was precipitated with 1 ml of 20% ice-cold trichloroacetic acid. The precipitates were collected, and the radioactivity therein was counted as previously described (7).

Specific activity (counts per minute per microgram of protein) of spore coat material and enterotoxin in the cell extract was determined by mixing 500 $\mu$g of extract with 0.3 ml of anticoat serum or anti-enterotoxin serum. Immunoprecipitation was at 37°C for 1 h followed by immunoprecipitation for 18 h at 4°C. Precipitates were washed three times with cold saline, suspended in 0.4 ml of Soluene 350 (Packard Instrument Co., Inc., Downers Grove, Ill.), transferred to glass scintillation vials, and allowed to solubilize for 12 h. Ten milliliters of Phase Combining System (Packard) was added to each vial. To allow chemiluminescence to subside, the radioactivity in each vial was counted after 48 to 72 h, by using a Packard model 2425 scintillation counter. The kinetics of increase in specific activity of the spore coat material extracted from cleaned spores, immunoprecipitable spore coat material, and enterotoxin in the cell extract from each labeling time are presented in Fig. 2.

![Fig. 1. Densitometric tracing of a representative electropherogram of spore protein extracted from spores of strain NCTC 8798 by DTT.](image1)

![Fig. 2. Kinetics of sporulation, enterotoxin synthesis, cell extract spore coat protein, and DTT-extractable spore coat protein of strain NCTC 8798 growing in Duncan-Strong medium (8). Abbreviations: ent, enterotoxin; CE, cell extract.](image2)
The first significant increase in the specific activity of coat material from both whole spores and cell extracts occurred between 2.5 and 3.5 h after sporulation had begun but before the appearance of heat-resistant spores. After 2.5 h, 85% of the cell population was at stage II or III of sporulation. The specific activity of spore coat protein in the cell extract decreased after 3.5 h, whereas the specific activity of coat material extracted from whole spores continued to increase until 4.5 h. These results indicate that coat polypeptide synthesis is a relatively early event in the sporulation of this organism. Most of the active coat deposition occurs slightly later, coincident with the onset of heat resistance. At this time, coat material is rapidly synthesized and apparently immediately deposited. The kinetics of labeling enterotoxin parallels that of coat protein extracted from whole spores. This is consistent with the intimate relationship known to exist between coat protein and enterotoxin (3). Nevertheless, attempts to equate the various proteins, revealed by gel electrophoresis of coat material, with enterotoxin by immunodiffusion of gels against antitoxin were unsuccessful. It is difficult to make exact comparisons with the kinetics of spore coat protein synthesis in Bacillus species because, in the case of C. perfringens, the sporulation process is much more rapid. However, our results do indicate that, as in the case of the aerobic sporeformers (1, 15), spore coat protein synthesis, including enterotoxin, is a relatively early event during sporulation of C. perfringens and that multiple coat polypeptides are synthesized.

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by research grant P0-00203-06 from the Food and Drug Administration, by Public Health Service research grant AI-11865-06 from the National Institute of Allergy and Infectious Diseases, and by contributions to the Food Research Institute by member industries. C.L.D. is the recipient of Public Health Service Research Career Development Award AI-70721-03 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED