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Clostridium perfringens Type A: In Vitro System for Sporulation and Enterotoxin Synthesis

WALTER P. SMITH† AND JAMES L. McDONEL*

Department of Microbiology, Cell Biology, Biochemistry and Biophysics, Pennsylvania State University, University Park, Pennsylvania 16802

Polysomes were isolated from an enterotoxigenic strain of *Clostridium perfringens* during vegetative growth and at 1-h intervals after transfer into Duncan-Strong sporulation medium. During vegetative growth, about 67% of the ribosomes were in polysomal complexes. This proportion decreased to about 20% during the first 2 h in sporulation medium and then gradually increased to a maximum of 45% at 6 h. Ribosomes isolated from cells in vegetative or in sporulation phase could equally translate vegetative, sporulation, and natural viral R17 messenger ribonucleic acid with either vegetative or sporulation initiation factors. When polysomes were allowed to complete their nascent chains with labeled amino acids in vitro, most of the polypeptides synthesized by the vegetative phase and by the sporulation phase polysomes appeared to be identical. There were, however, notable differences upon further investigation. Specifically, when antiserum against the enterotoxin was reacted with the completed polypeptides, no counts were precipitated from the vegetative products. On the other hand, up to 12% of the total labeled protein was precipitated from the products obtained with the sporulation phase polysomes. Upon electrophoresis on sodium dodecyl sulfate, the putative enterotoxin synthesized in vitro ran as a major band with a molecular weight of 35,000, and as two minor bands with molecular weights of 17,000 and 52,000, respectively.

The study of cellular differentiation, a highly regulated process, has been an area of intense research in recent years. Many factors, such as specificity of translation (1, 19), modification of the translation apparatus (1, 14, 19-21), and transcription modification (2, 3, 11), have been implicated in the differentiation process. The process of sporulation is a relatively simple developmental system in which a vegetative cell, after exhaustion of necessary nutrients, differentiates through a unique series of events into a spore (23). One common approach used to study sporulation has involved the extraction of protein synthetic systems from vegetative and sporulating cells, and assessment of differences between the two. In studies with *Bacillus cereus* spores, ribosomes were characterized as defective by several different assays (1). However, modification during isolation may contribute to those measurable differences (16).

A disparity of information exists concerning ribosomal modification during sporulation. No definitive answer exists as to whether sporulation ribosomes are modified during sporulation or during isolation. Accordingly, it is desirable to have a specific probe to analyze transcriptional regulation during sporulation. Spore coat protein is such a probe, and one of the few sporulation-specific gene products that have been well characterized. The characterization of its properties and time of synthesis during sporulation would aid in its use to monitor the expression of a sporulation-specific gene.

We have examined *Clostridium perfringens* as a system for studying sporulation for the reasons mentioned above, and also because of its widespread medical and industrial importance. *C. perfringens* produces a sporulation-specific protein enterotoxin that is responsible for one of the most common forms of food poisoning in the United States (12). This enterotoxin is thought to be serologically homologous to spore coat protein (9, 10) in toxigenic and nontoxigenic strains. In this paper we have examined the polypeptide synthesizing system from vegetative and sporulating *C. perfringens* cells to outline the elaboration of enterotoxin by toxigenic strains, as well as to delineate the fundamentals of sporulation in this species.

MATERIALS AND METHODS

Organism and culture. *C. perfringens* type A, strain NCTC 8239 (Hobbs serotype 3), was inoculated from cooked meat medium (Difco Laboratories) into 10 ml of fluid thioglycolate medium (Baltimore Biological Laboratories) and incubated overnight (16 to 18 h) at 37°C. A 2% inoculum was then made into either Duncan-Strong sporulation medium (6) for

† Present address: Dermatological Research Department, Vick Divisions Research and Development, Richardson-Merrell Inc., Mt. Vernon, NY 10553.

sporulation or fluid thioglycolate medium for vegetative cells. The percentage of cells in Duncan-Strong sporulation medium that entered into the sporulation process ranged from 85 to 90%.

Preparation of cell fractions. Chloramphenicol (200 μg/ml) was added to exponentially growing or sporulating cultures (at the ages given); the cultures were poured onto excess ice, and the cells were pelleted by brief centrifugation. Cells were suspended in 25 ml of buffer A [10 mM $Mg(OAc)_2$, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.0] and lysed by sonication (five 1-min bursts at a 60 setting with a Versonic cell disruptor (Vertis Co.). DNase (5 mg/ml) was added to the lysates, and cell debris was pelleted by centrifugation at 30,000 × *g* for 20 min. The supernatant was centrifuged at 160,000 × *g* for 3 h to sediment the ribosomes/polysomes. Polysomes were separated from free ribosomes by Sepharose 2B chromatography (28). Preincubated S-30 fractions from *Escherichia coli* were prepared by standard methods (22). Crude initiation factors (IF), IF-free ribosomes (18), and mRNA (26) were prepared by methods described previously.

In vitro protein synthesis (polysomal) and identification of products. Two units with absorbance at 260 nm (A_{260}) of purified *C. perfringens* polysomes were incubated with 3 A_{260} U of an *E. coli* S-100 cell extract for 45 min at 37°C in a 0.1-ml protein-synthesizing system as described previously (26), except that 5 μCi of [^{35}S]methionine (600 Ci/mmol) and the 19 other standard unlabeled amino acids were substituted for a mixture of ^{3}H-amino acids. Protein synthesis was terminated by diluting the entire reaction mixture in 0.9 ml of cold buffer B (0.14 M NaCl, 0.1 M $NaPO_4$, pH 7.4) with 3 mM phenylmethylsulfonyl fluoride and 5 mM ortho-phenanthroline (pH 8.0). The mixture was clarified by centrifugation, and total protein was either fractionated by electrophoresis (27) or quantitated by trichloroacetic acid precipitation with a subsequent counting of radioactivity.

In some experiments enterotoxin was precipitated from the reaction mixture by the following method: an excess of rabbit anti-enterotoxin serum (5 μl) prepared against highly purified (>99%) enterotoxin was added to the 1-ml reaction mixture and incubated for 90 min at 37°C. Goat anti-rabbit serum was added at a concentration predetermined to precipitate maximum amounts of the anti-enterotoxin. The mixture was incubated an additional 12 h at 4°C. The precipitate was collected by centrifugation at 15,000 × *g* for 10 min, washed three times with buffer B, and after final precipitation, assayed for total radioactivity or analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22).

In vitro protein synthesis (ribosomal) and identification of products. One A_{260} unit of IF-free ribosomes was incubated with 0.1 A_{280} U of IF and 0.1 A_{260} U of endogenous or R17 mRNA at 37°C in a 0.1-ml protein-synthesizing system as described above.

Electrophoresis. One-dimensional sodium dodecyl sulfate electrophoresis was done with 25-cm slabs containing 10% acrylamide (24).

RESULTS

Ribosome/polysome distribution and activity in vegetative and sporulating cells. The activity of crude polysome preparations isolated from vegetative and sporulating cultures was examined to determine whether active polysome preparations could be isolated during different stages of sporulation. *E. coli* S-100 fractions were found to be more active than *C. perfringens* S-100 fractions in in vitro protein-synthesizing enzymes. Accordingly, *E. coli* S-100 was used in all subsequent experiments. When crude *C. perfringens* polysome preparations were incubated with an *E. coli* S-100 fraction, polysomes isolated from sporulating cultures during the first few hours after transfer into sporulation medium supported very little protein synthesis. Vegetative preparations, however, were quite active (Table 1). Maximal activity of polysomes (i.e., sporulation) was obtained at 6 h after transfer into sporulation medium.

Since it is plausible that the apparent change in activity of sporulating and vegetative polysomes may reflect a change in the proportion of 70S monomers and ribosomal subunits to polysomes, their distribution was examined. During vegetative growth, most of the ribosomes (67%) were complexed with mRNA in the polysome form (Table 1). Upon transfer to Duncan-Strong sporulation medium, for the first several hours there was a very low content of polysomes (17% at 1 h). This value reached a maximum of about 45% polysomes at 6 h. At 8 and 10 h sporulating cells were beginning to lyse and, consequently, very poor polysome preparations were obtained and no interpretations could be made.

These data suggest that upon transfer into sporulation medium the vegetative cells' metabolic machinery undergoes a dramatic shift so that polysomes break down immediately and then reform. To verify that the 70S particles were free ribosomal particles and not fragmented polysomes attached to short segments of mRNA, cultures were pulse-labeled with [^{3}H]uracil. Under such conditions no appreciable activity was observed in the 70S peak (data not shown).

When polysomes were separated from the free 70S monomers by Sepharose 2B chromatography and examined for protein synthetic capability (Table 1), essentially identical activity was obtained whether purified polysomes from vegetative or sporulating cultures were tested. These results suggest that no modifications of the polysomes (ribosomes) themselves take place during sporulation. It appears that activity differences observed with crude vegetative and sporulation phase extracts reflect the ability to find available mRNA.

Specificity of translation of vegetative and sporulating polysomes. To test the possibility that the breakdown of polysomes upon

TABLE 1. *Incorporation of [^{35}S]methionine into protein by various protein-synthesizing systems*[a]

S-100	*C. perfringens* polysomes	% Polysomes[b]	Activity[c] (10^3 cpm)	
			Crude	Purified
C. perfringens (vegetative)	Vegetative		671	1,007
E. coli	Vegetative	67	1,063	1,279
E. coli	Sporulating + 1 h	17	214	1,171
E. coli	Sporulating + 2 h	23	241	1,231
E. coli	Sporulating + 3 h	35	472	1,417
E. coli	Sporulating + 4 h	37	427	1,201
E. coli	Sporulating + 6 h	45	786	1,137
E. coli	Sporulating + 8 h	10[d]	121	—[e]
E. coli	Sporulating + 10 h	6[d]	103	—[e]

[a] Protein was synthesized in vitro as described in the text.

[b] Calculated as: polysomes/polysomes + ribosomes (70S) × 100.

[c] Crude polysomes were prepared as described in the text. Purified polysomes were prepared by Sepharose 2B chromatography as described. Values given are total trichloroacetic acid-precipitable counts corrected for background.

[d] Approximations due to depleted state of system.

[e] No polysomes could be isolated at these times.

transfer into sporulation medium results from an altered translational specificity of the vegetative or sporulation phase protein-synthesizing components, IF, mRNA, and washed ribosomes were isolated from the sporulating and vegetative cultures. The ribosomes from vegetative and sporulating cultures appeared identical in their ability to translate either vegetative or sporulation phase cultures. Moreover, vegetative and sporulating systems could equally well translate (R17) viral mRNA (data not shown).

Synthesis of different proteins by vegetative and sporulation phase polysomes. Since *C. perfringens* enterotoxin accumulates during spore formation, it should be possible to detect specific differences in the proteins synthesized by the polysomes isolated from different growth phases. Proteins synthesized by each polysome fraction were examined by single-dimension sodium dodecyl sulfate electrophoresis (Fig. 1). Although several distinct differences in gel banding patterns were observed, the majority of the protein bands appeared in both the vegetative and sporulating cultures. One such difference was a major band at a molecular weight of about 35,000 in the sporulation phase extracts. Since enterotoxin has been reported to have a molecular weight of about 35,000 and is synthesized only in sporulating cells (7), studies were undertaken to characterize this unknown band.

Synthesis of enterotoxin by polysomes from sporulating *C. perfringens* cells. Polysomes were incubated with *E. coli* S-100 fractions in in vitro protein-synthesizing systems, and the proteins synthesized were incubated with antisera to *C. perfringens* enterotoxin (Table 2). Polysomes from vegetative *C. perfringens* synthesized very little enterotoxin (less than 1% total protein) whereas polysomes from sporulating cultures synthesized appreciable amounts of enterotoxin (up to 12% of total protein). The maximal synthesis of enterotoxin by polysomes isolated from cultures, i.e., 6 h after transfer into Duncan-Strong sporulation medium, corresponds well with data obtained for the in vivo-synthesized enterotoxin (4, 17). Figure 2 shows the sodium dodecyl sulfate-polyacrylamide gel analysis of immunoprecipitated enterotoxin. Three bands were observed: the major band appeared at a molecular weight of 35,000; two minor bands were detected at molecular weights of 17,000 and 52,000, respectively.

DISCUSSION

We have studied the protein-synthesizing machinery from vegetative and sporulating cells to develop an in vitro system to examine sporulation and the synthesis of the enterotoxin produced by *C. perfringens* type A. When crude ribosomal fractions were isolated from vegetative and sporulating cells, vegetative extracts were quite active in protein synthesis, whereas sporulation phase extracts (when isolated 1 or 2 h after transfer into sporulation medium) displayed only 20% of the level of vegetative activity. When isolated at 6 h after transfer into sporulation medium, the extracts reached their maximal activity, which was about 75% that of the vegetative extracts.

The polysome/ribosome distribution profile of the cell extracts was analyzed, and most (75%) of the ribosomes in vegetative cells were found in the polysomal form. However, soon after transfer into sporulation medium, most of the ribosomes appeared free of mRNA as monomers

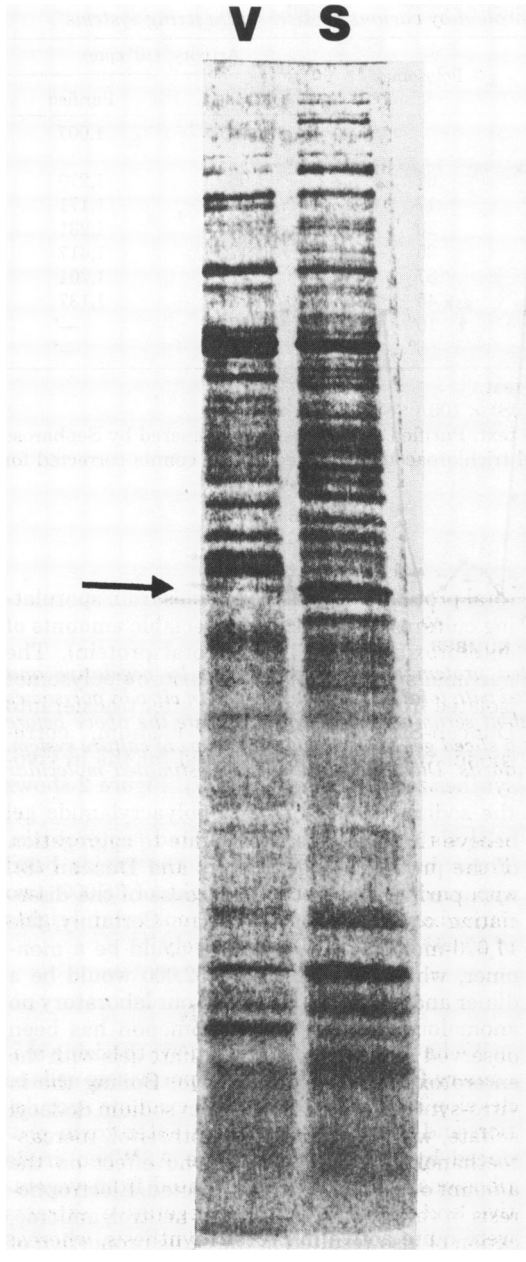

FIG. 1. *Gel electrophoresis (sodium dodecyl sulfate) of total proteins synthesized in vitro by polysomes from vegetative and sporulating cultures. Electrophoresis was performed as described in the text. Arrow indicates location of 35,000-molecular-weight band. Abbreviations: V, vegetative system; S, sporulation system.*

or ribosomal subunits. Only 15% appeared as polysomes. Polysomes gradually reformed, and at 6 h after transfer into sporulation medium the maximal sporulation phase polysomal content (45%) was seen. This time correlates well with the maximal rate of protein synthesis in vivo (9).

TABLE 2. *Synthesis of enterotoxin by vegetative and sporulation polysomes*

Polysomes	Immunoprecipitated enterotoxin (10^3 cpm)[a]	% Total protein synthesized
Vegetative	5.5	0.5
Sporulation		
1 h	45.5	4.3
2 h	66.6	6.3
6 h	131.2	12.3

[a] Enterotoxin was immunoprecipitated from 1,063 × 10^3 cpm of in vitro-synthesized protein from each system. Values given are corrected for background counts (5,200 cpm) precipitated when all in vitro conditions were similar but in the absence of added polysomes.

When polysomes were separated from free ribosomes by Sepharose 2B chromatography and used with S-100 fractions in an in vitro protein-synthesizing system, similar activity was observed with either vegetative or sporulation phase polysomes. Cell extracts prepared from sporulation or vegetative phase cultures appear to differ in activity due solely to the availability of mRNA (i.e., the degree of ribosome association with mRNA).

These results can be interpreted in at least two ways. Upon transfer into sporulation medium, the cells may undergo a nutritional or energy shutdown. As a result, polysomes may complete protein chains already initiated, but fail to reinitiate synthesis due to energy requirements. Alternatively, it is possible that transfer into sporulation medium results in some modification of the translation apparatus. However, the rapidity of the polysome breakdown makes this possibility less likely.

This second possibility, the specificity of translation, was examined by preparing crude mRNA, IF-free ribosomes, and IF from vegetative and sporulation phase cultures and assessing the protein-synthesizing capability of the various possible combinations. The source of ribosomes, IF, and mRNA seemed to have no effect on protein-synthesizing capabilities. Initiation factors from vegetative cultures worked with both vegetative and sporulation phase mRNA and ribosomes. The same was true for sporulation phase IF. Moreover, both vegetative and sporulation systems could efficiently translate natural R17 viral RNA. Apparently, no major translational specificity differences are detectable between components of vegetative and sporulation phase protein-synthesizing systems. In a crude system such as this one, minor differences, such as the induction of new mRNA species, may be neglected. The products synthesized by

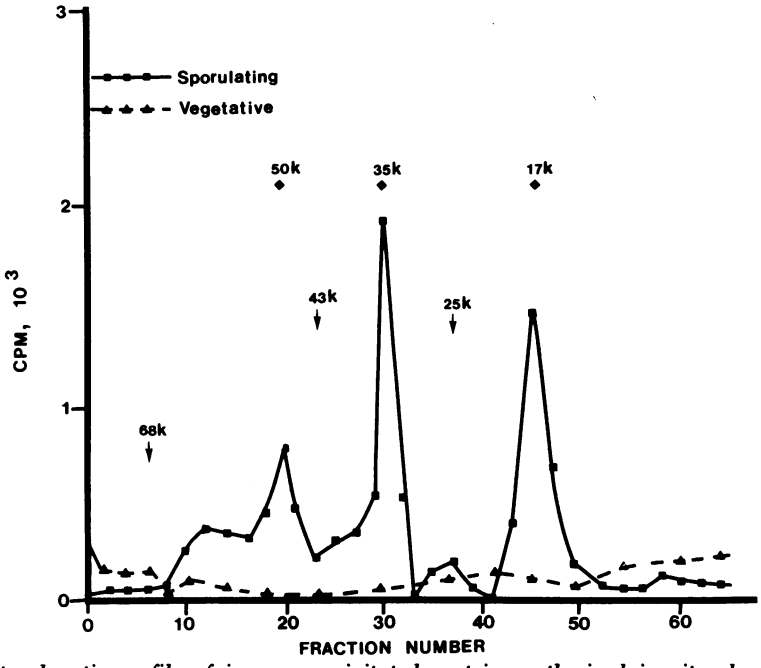

FIG. 2. *Electrophoretic profile of immunoprecipitated protein synthesized in vitro by vegetative and sporulation cultures. Anti-enterotoxin (rabbit) was reacted with total protein synthesized in vitro by polysomes from vegetative and sporulation cultures. Goat anti-rabbit serum was used to precipitate the above before sodium dodecyl sulfate gel electrophoresis. Counts from a sliced gel are given for each type of culture system. Arrows indicate the location of molecular weight standards. Diamonds indicate the estimated molecular weight standards.*

sporulation phase and vegetative polysomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Although most of the proteins synthesized by vegetative and sporulation phase polysomes were found to be similar, several marked differences were noted. Most noticeable was the appearance of a band in the sporulation phase protein gel at a molecular weight of about 35,000. This corresponds to the molecular weight of the enterotoxin produced in vivo by *C. perfringens* (8). Accordingly, the amount of enterotoxin synthesized by vegetative and sporulation phase polysomes was examined and, as expected, only the polysomes from sporulating cultures produced appreciable amounts of enterotoxin. In fact, enterotoxin comprised about 12% of total protein synthesized by polysomes isolated from cultures at 6 h after transfer into sporulation medium.

It has been reported previously (15) that *C. perfringens* enterotoxin exhibits anomalous behavior in sodium dodecyl sulfate and appears as large molecular weight aggregates in gels. Immunoprecipitated enterotoxin from sporulation phase proteins synthesized in vitro appeared as three bands in sodium dodecyl sulfate gels: a major band at a molecular weight of 35,000 and two minor bands, one with a molecular weight at 17,000 and the other at 52,000. We do not believe that these bands are due to aggregation of the protein, as did Enders and Duncan (8), with purified enterotoxin, because of the dissociating conditions of the assay. Certainly, the 17,000-molecular-weight band could be a monomer, while the 35,000 and 52,000 would be a dimer and trimer. However, in our laboratory no anomalous aggregation phenomenon has been observed in sodium dodecyl sulfate gels with the enterotoxin synthesized in vivo. Boiling the in vitro-synthesized enterotoxin in sodium dodecyl sulfate, with or without dithiothreitol, mercaptoethanol, or 8 M urea had no effect on the amount of the three bands detected. Electrophoresis in various detergents (i.e., neutral, amionic, cation c) also resulted in the same proportion of the three bands, and we did not observe multiples of the 35,000 unit which has previously been reported (15, 25). Moreover, enterotoxin isolated from sporulating cells appeared predominately as a 35,000-molecular-weight band when run on the same sodium dodecyl sulfate gels. These results suggest that either the immunoprecipitated enterotoxin is a complex mixture of labile polypeptides or cross-reactive peptide fragments in *C. perfringens* cells exist. It is possible that the 17,000- and 35,000-molecular weight species are spore coat proteins immunoprecipitated by enterotoxin (9). This issue may be partially clar-

ified by reacting antisera to purified coat protein with the in vitro-synthesized products. It is plausible that the 52,000-molecular-weight product represents the primary translation product and the 35,000- and 17,000-molecular-weight proteins represent cleavage products. The enterotoxin may be synthesized in vivo as a precursor molecule that is normally cleaved into units of 17,000 and 35,000. The 17,000 fragment may be inserted into the spore coat membrane as an integral component. In fact, Frieben and Duncan (9) have shown that spores from toxigenic and nontoxigenic strains of *C. perfringens* contain proteins with molecular weights of 14,500, 23,000, and 36,000 which have serological and biological activities similar to those of purified enterotoxin.

Since spore coat protein is highly insoluble (13), it is possible that a 35,000-molecular-weight tail is necessary to make the entire protein soluble before its insertion into the spore coat. It appears that strains of *C. perfringens* which are not enterotoxigenic may produce low-level amounts of enterotoxin, which are incorporated into the spore coat. It may be that an overproduction of this protein in enterotoxigenic strains overloads the insertion system and results in the intracellular accumulation (5) of this molecule. It is very important that further studies be performed to determine the serological and biological activities of these different molecular weight forms.

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