Synthesis of Deoxyribonucleic Acid, Ribonucleic Acid, and Protein During Sporulation of Clostridium perfringens

RONALD G. LABBE* AND CHARLES L. DUNCAN

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

Received for publication 6 October 1975

The kinetics of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis as well as protein breakdown during sporulation by Clostridium perfringens were determined. Maximum levels of DNA and net RNA synthesis occurred 3 and 2 h, respectively, after inoculation of sporulation medium. The rate of RNA synthesis decreased as sporulation progressed. Deoxyadenosine increased uptake of $[^4C]$uracil and $[^4C]$thymine but depressed the level of sporulation and the formation of heat-resistant spores when added at concentrations above 100 µg/ml. Unlike Bacillus species, net protein synthesis, which was sensitive to chloramphenicol inhibition, continued during sporulation. The rate of protein breakdown during vegetative growth was 1%/h. During sporulation this rate increased to 4.7%/h. When added to sporulation medium at 0 time chloramphenicol reduced protein breakdown to 1%/h. If added at 3 h the rate decreased to 2.1%/h. The role of proteases in this process is discussed.

Studies on macromolecular synthesis and turnover (breakdown) by sporulating cells invariably have dealt with Bacillus species. Essentially no information is available on Clostridium species, doubtlessly due to their more exacting cultural conditions.

In the case of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis, the rapid production of these nucleic acids during the exponential phase of growth by Bacillus cereus and B. subtilis ceases near the end of the logarithmic phase (18, 28), although in some cases a slow synthesis of DNA occurs during sporulation (27). Although total DNA does not increase during sporulation, RNA synthesis does continue (18). Net protein levels also plateau during sporulation (7, 24). However, there is active turnover and synthesis of new protein. In the case of B. thuringiensis and B. subtilis at least 70% of the spore protein has been shown to be newly synthesized during sporulation (13, 15, 23). Estimates for the rate of protein turnover range from 6 to 22% (7, 13, 24, 25). A recent report involving B. megaterium (7) indicates that, whereas protein synthesis occurs in both the forespore and mother-cell compartment, only in the latter does protein breakdown occur.

A direct relationship between sporulation and enterotoxin formation has been shown with Clostridium perfringens (6). In addition, an enterotoxin-like protein can be extracted from the spore coats of several strains of this organism (8). As part of an attempt to gain a better understanding of the molecular basis of these observations, and for comparison with Bacillus species, the kinetics of macromolecular synthesis by C. perfringens during sporulation were investigated. The results of our investigation are presented here.

MATERIALS AND METHODS

General. C. perfringens NCTC 8796 (Hobbs serotype 9) was used throughout. Measurement of the levels of sporulation and heat-resistant spores was as previously described (10). Turbidity was measured using a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 600 nm.

Protein determination. For the determination of protein levels during sporulation, samples were removed at hourly intervals and centrifuged at 15,000 x g for 15 min. The pellet was washed twice with saline, and 3.5 ml of 0.5 N NaOH was added to each screw-cap tube containing the pellet. The tubes were then autoclaved for 15 min to solubilize the protein. Insoluble material was spun out, and the protein content of the supernatant fluid was determined by the method of Lowry et al. (12), using bovine serum albumin as standard.

Macromolecular synthesis. DNA, RNA, and protein synthesis during sporulation in Duncan and Strong (DS) sporulation medium were determined as follows. A 0.5-ml amount of an overnight culture of fluid thioglycolate (PTG) was used to inoculate 50 ml of DS medium contained in a screw-cap test tube (2.5 by 20.0 cm). For DNA or RNA synthesis the medium
contained either [2-14C]thymine (specific activity, 47.5 mCi/mmol) or [2-14C]uracil (specific activity, 56.8 mCi/mmol), respectively, both at a final concentration of 0.2 μCi/ml unless otherwise noted. In these experiments 2'-deoxyadenosine was routinely included at a final concentration of 100 μg/ml. Pre-soaking of filters with cold thymine or uracil or including them during trichloroacetic acid precipitation did not affect the counts obtained, and these steps were omitted.

In the case of kinetics of protein synthesis during sporulation, a 14C-labeled amino acid mixture was added at a final concentration of 0.1 μCi/ml unless otherwise noted. Use of single radioactively labeled amino acids resulted in poor incorporation.

Measurement of incorporation of radioactive precursors into DNA, RNA, and protein was determined by pipetting at specified intervals duplicate 1-ml samples into 1 ml of ice-cold 20% trichloroacetic acid. After 0.5 h the precipitates were collected on 2.4-cm glass-fiber filters (Reeve Angel, Clifton, N.J.). The filters were washed with 10 ml of 10% trichloroacetic acid followed by 10 ml of 95% ethanol. The filters were dried under a heat lamp, placed in scintillation vials, and counted in the presence of 5 ml of 0.6% 2,5-diphenyloxazole in toluene, using a Packard model 2425 scintillation counter.

**Assay of protein breakdown.** Protein breakdown during sporulation in DS medium was determined by measuring the decrease in radioactivity by cells prelabeled during vegetative growth in a modified FTG (mFTG) medium. The latter consisted of 0.9% Trypticase (BBL, Cockeysville, Md.), 0.3% yeast extract, 0.5% dextrose, 0.25% sodium chloride, 0.5% sodium thioglycolate, and 0.02% cysteine. The pH was adjusted to 7.3 before autoclaving. One drop of cooked meat stock was added to 10 ml of mFTG, which was heat shocked at 75°C for 20 min. After overnight (11 to 13 h) incubation at 37°C, 1 ml of the culture was inoculated into another 10-ml mFTG tube containing a final concentration of 1 μCi of 14C-labeled amino acid mixture per ml. When the absorbance (600 nm) reached 0.55 (late log phase, 2 x 10⁸ vegetative cells/ml), the culture was centrifuged at 1,600 x g for 15 min. The pellet was suspended in 10 ml of 0.1% peptone (Difco Laboratories, Detroit, Mich.), and the tube was centrifuged at 1,500 x g for 15 min. The supernatant fluid was removed, and the cells were washed once more. After this the pellet was suspended in 10 ml of DS medium. From this 1.5 ml was used to inoculate 50 ml of DS medium. At hourly intervals duplicate 1-ml samples were removed, and radioactivity was determined as above. The percentage of decrease in the amount of radioactivity during sporulation was taken as a measure of protein breakdown.

Protein breakdown during vegetative cell growth was similarly determined, except that 2 ml of labeled log-phase cells was used to inoculate 50 ml of mFTG and 0.5-ml samples were taken for radioactivity measurements.

Radioactivity in the DS culture supernatant fluid during sporulation was determined by centrifuging 5 ml of culture at 10,000 x g for 20 min and counting 1 ml of the supernatant fluid in 10 ml of PCS solubilizer (Amersham/Searle Corp., Des Plaines, Ill.).

**Chemicals.** [2-14C]uracil, [2-14C]thymine, and [U-14C]-labeled L-amino acid mixture were obtained from New England Nuclear Corp., Boston, Mass. Chloramphenicol was purchased from Calbiochem, Los Angeles, Calif. Mitomycin C and 2'-deoxyadenosine were obtained from Sigma Chemical Co., St. Louis, Mo., and vitamin-free Casamino Acids were from Difco Laboratories, Detroit, Mich.

**RESULTS**

**Nucleic acid synthesis.** The time course of DNA and RNA synthesis by C. perfringens was determined during sporulation in DS medium (Fig. 1). Maximal net uracil incorporation into RNA occurred by 2 h, the time sporulating cells were first detected microscopically. Breakdown of RNA presumably accounted for the decline in counts after 3 h. We investigated the possibility that RNA synthesis occurred after 2 h and was masked by breakdown of previously synthesized RNA by following the incorporation of [14C]uracil by 1-, 3-, and 4.5-h cells (Fig. 2). At 1 h all cells were in the vegetative phase; at 3 h the majority were at stage III or IV of sporulation, and at 4.5 h, the majority were at stage V or VI. There was a drastic decrease in the rate of RNA synthesis by 3-h cells as compared to 1-h cells. Even less incorporation was observed using 4.5-h cells. A similar dependence of uracil incorporation on the age of the sporulating culture has been shown by other workers (2, 9). It is reasonable to assume that there would be a diminished requirement for both stable and labile RNA as the sporulation cycle nears completion. In any event it is evident that, whereas no net RNA synthesis occurs after about 2.5 h, the production of this nucleic acid does continue, although at a reduced rate.

The ability of [14C]uracil to be specifically incorporated into RNA was checked by alkaline hydrolysis (0.5 N NaOH for 6 to 8 h followed by trichloroacetic acid precipitation). Greater than 95% of the label incorporated at 25 min was alkali labile.

Incorporation of [14C]thymine showed a slightly different pattern in that net DNA synthesis continued after sporulation was first detected at 2 h (stage II sporulating cells) and reached a maximum at about 3 h. Slow synthesis of DNA during sporulation has been previously reported in B. cereus (27). It is possible, however, that in our system residual [14C]thymine incorporation was due to asynchronous sporulation.

We observed 85 to 90% sporulation (any cell at or beyond stage II of sporulation) by 4 h, which is also the time of first detection of heat-resistant spores. The number of heat-
Fig. 1. Kinetics of sporulation and incorporation of $[^{14}C]$thymine and $[^{14}C]$uracil (trichloroacetic acid-precipitable counts per minute per milliliter) by C. perfringens NCTC 8798 during growth in DS medium.
resistant spores obtained at 6 h, about 2 × 10⁷/ml, was the maximum level observed in this system.

The ability of [14C]thymine to be specifically incorporated into DNA was also determined by alkaline hydrolysis. The same incorporation kinetics were obtained during the 3-h observation period with or without alkali treatment.

Synthesis of DNA during sporulation could be inhibited by the addition of mitomycin C. Figure 3 shows that when added at 1.5 h, a time of rapid DNA synthesis, 1 μg of the antibiotic per ml severely inhibited incorporation of [14C]thymine. Increasing the concentration to 10 μg/ml resulted in a loss of cell-associated radioactivity and prevented a further increase in turbidity 0.5 h after addition.

Incorporation of [14C]uracil and [14C]thymine during growth in DS medium (Fig. 1) was measured in the presence of 100 μg of 2'-deoxyadenosine per ml, a compound often used to increase incorporation of these bases (4, 16, 19). The effect of higher concentrations of this compound on sporulation and incorporation of radioactive bases was also determined. Its ability to stimulate incorporation of [14C]uracil and [14C]thymine was assessed at 3 h by measuring the incorporation of these nucleic acid precursors (Table 1). We too found that inclusion of deoxyadenosine resulted in increased incorporation of both labeled thymine and uracil. However, at a concentration of 300 μg/ml, deoxyadenosine also depressed the levels of sporulation and heat-resistant spores. This effect was even more severe at a concentration of 500 μg/ml. Since turbidity at high deoxyadenosine concentrations was somewhat lower than at 100 μg/ml, the decrease in sporulation and heat-resistant spore levels was not due to additional vegetative growth.

**Protein synthesis and breakdown.** The kinetics of protein synthesis during sporulation was investigated by following the incorporation of a mixture of [14C]-labeled amino acids by 1-, 2-, 3-, and 5-h cultures of cells growing in DS medium (Fig. 4). There was no microscopic evidence of sporulation at 1 h. By 2 h some cells had formed forespore septa (stage II). At 3 h most cells had reached stage IV or V of sporulation; at 5 h most were at stage V or VI. The majority of label was incorporated within 30 min by 1- and 2-h cultures. Three- and 5-h cells showed little incorporation after 15 min, possibly due to the high level of radioactivity incorporated at 0 time, about 1,000 counts/min per ml. Considering the amount of label added (0.1 μCi/ml) and percentage of incorporation (ca. 1%), it is likely that only a few of the 15 amino acids contained in the labeled amino acid mixture were incorporated. Such a specific amino acid requirement could account for the high 0 time incorporation value by 3- and 5-h sporulating cells.

Incorporation of labeled amino acids by a 3-h culture was sensitive to an inhibitor of protein synthesis (data not shown). When added 15 min following addition of label, 60 μg of chloramphenicol per ml totally inhibited protein synthesis within 10 min.

The rate of protein breakdown during sporulation by *C. perfringens* was determined by the percentage of loss of radioactivity by sporulating cells prelabeled during vegetative growth in mFTG. Acid-precipitable counts per minute per milliliter at 0 time after inoculation of DS medium were taken as 100%. Optical density and kinetics of uptake of [U-14C]-labeled amino acid mixture paralleled each other during growth in mFTG. Vegetative cells from late log phase were used to inoculate DS medium. Figure 5 shows the loss in acid-precipitable radioactivity during sporulation and the appearance of label in the supernatant fluid. Again sporulation was first detected at 2 h and reached a maximum of about 90% at 6 h. The rate of protein breakdown was 4.7%/h. This was determined by the decrease in counts per minute per milliliter be-
**Fig. 3.** Effect of mitomycin C on \([^{14}C]\)thymine incorporation and turbidity (600 nm, lower graph) during growth in DS medium. Arrows indicate time of antibiotic addition.
TABLE 1. Effect of deoxyadenosine on incorporation of [14C]uracil and [14C]thymine, growth, and sporulation of C. perfringens NCTC 8798 in DS medium

| Deoxyadenosine (μg/ml) | Counts/min per ml* | Sporulation (%) | Heat-resistant spores/ml* | Optical density*
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]thymine</td>
<td>[14C]uracil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1,922</td>
<td>9,521</td>
<td>89</td>
<td>3.1 x 10^7</td>
</tr>
<tr>
<td>100</td>
<td>3,063</td>
<td>14,392</td>
<td>85</td>
<td>2.6 x 10^7</td>
</tr>
<tr>
<td>300</td>
<td>5,556</td>
<td>17,527</td>
<td>71</td>
<td>2.3 x 10^7</td>
</tr>
<tr>
<td>500</td>
<td>8,762</td>
<td>18,257</td>
<td>56</td>
<td>1.4 x 10^7</td>
</tr>
</tbody>
</table>

* [14C]thymine and [14C]uracil were present in a concentration of 0.2 μCi/ml; incorporation was determined after 3 h.
* Determined after 6 h.

![Fig. 4. Incorporation of 14C-labeled amino acid mixture by 1-, 2-, 3-, and 5-h-old cells growing in DS medium.](image)

between 2 and 5 h. Since there is brief vegetative cell division during early growth in DS medium (11), the acid-precipitable counts at 0 and 1 h were disregarded. The level of radioactivity at 6 h and after was also ignored, since it is about this time that spores become free of their sporangium. On one occasion we attempted to block possible reincorporation of degraded protein by adding cold amino acids at 2 h. This resulted in a lower percentage of sporulation, presumably due to renewed vegetative growth.

Reincorporation of extruded perfringens is probably minimal considering the complex nature of DS medium.

Figure 5 also shows the continual increase in protein level during sporulation. This is unlike the case of some bacilli where net protein levels remain constant during sporulation and attendant protein turnover.

Protein breakdown during vegetative growth in mFTG occurred at 1%/h (data not shown). A rate of 1.4 to 3% during vegetative growth has been reported in other bacterial systems (17, 21, 24, 26).

The close association of proteases with sporulation has been known for some time. It was of interest to determine what effect chloramphenicol would have on protein breakdown (Fig. 6). When added to DS medium at 0 time, the antibiotic reduced protein breakdown to 1%/h and prevented growth. This low rate of breakdown was probably due to residual protease activity carried over in vegetative cells. Addition of chloramphenicol at 3 h also prevented further increase in turbidity and depressed the rate of protein breakdown to 2.1%/h. This demonstrates that protein synthesis after 3 h is necessary if the normal rate of protein breakdown is to occur. The requirement possibly is for the production of a protease.

**DISCUSSION**

Results reported here show that the kinetics of nucleic acid synthesis and inhibition during sporulation of C. perfringens are similar to those observed with Bacillus. Unlike the latter, however, net protein synthesis continued during the sporulation process. In addition, the rate of breakdown was slightly less than has been reported for the aerobic sporeformers. This, together with its complex nutritional requirements for sporulation, suggests that C. perfringens has relatively poor biosynthetic capabilities, and that relatively little degradation of pre-existing proteins occurs during sporulation. However, it should be noted that the accuracy of determining protein breakdown by methods reported here is limited by the equilibration of external amino acids and internal pools, i.e., the efficiency of trapping of degraded and excreted labeled compounds. In fact a decrease in the net efflux system during sporulation of B. licheniformis has been reported (3).

Protein breakdown appears to be an important biochemical event in sporulation and allows the cell to significantly change its complement of proteins. Mutants of B. subtilis that do not show protein turnover are usually asporoge-
Fig. 5. Percentage of sporulation, protein synthesis, and protein breakdown during growth in DS medium. Sup., counts per minute per milliliter in supernatant fluid.
Fig. 6. Effect of chloramphenicol (60 μg/ml) on turbidity and protein breakdown during growth in DS medium. Arrows indicate times of antibiotic addition. Cam., Chloramphenicol.

ous (13, 25). Turnover itself may be necessary but is not sufficient for sporulation, since asporogenic mutants do turn over their protein components (5), and turnover is observed during vegetative cell growth, although at a reduced rate.

The time of appearance of protease activity correlates well with an increase in the rate of protein turnover and sporulation (1, 18). There have been many and conflicting reports regarding the role of proteases during sporulation. Indeed, synthesis of a protease is thought to be one of the early steps required for sporulation (11). However, mutants capable of sporulating but producing low or no levels of exoprotease have been isolated (1, 14, 22). It is therefore possible that protease production coincides with sporulation because the same process that triggers sporulation also signals the increased biosynthesis of the enzyme.

Most reports of protease activity during sporulation have dealt with extracellular proteases. Setlow (19) failed to detect extracellular proteases during sporulation of B. megaterium, although the level of an intracellular protease was 50 times higher in sporulating cells than in vegetative cells. Schaeffer (18) concluded that extracellular protease activity is not responsible for sporulation but that the intracellular enzyme may be involved. Szulmajster et al. (25) recently reported that a temperature-sensitive mutant of B. subtilis, which lacked an intracellular protease, exhibited no protein turnover and was asporogenic. An extracellular protease was produced at both the restrictive and permissive temperature. It is clear that the exact
relationship between protease biosynthesis and sporulation remains to be established.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison; by research grant FD-00205-05 from the Food and Drug Administration; by Public Health Service research grant AI-11865-05 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-70721-02 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED