Sporulation and Enterotoxin Production by *Clostridium perfringens* Type A at 37 and 43°C

JOSE S. GARCIA-ALVARADO,1,2 RONALD G. LABBÉ,3* AND MANUEL A. RODRIGUEZ1

Departamento de Microbiología, Facultad de Medicina,1 and Departamento de Microbiología e Immunología, Facultad de Ciencias Biológicas,2 Universidad Autónoma de Nuevo León, Monterrey, Nuevo León 66450, Mexico, and Food Microbiology Laboratory, Department of Food Science, University of Massachusetts, Amherst, Massachusetts 010033

Received 12 November 1991/Accepted 4 February 1992

Enterotoxin-positive strains of *Clostridium perfringens* were grown in Duncan-Strong sporulation medium in the presence of 0.4% (7.9 mM) raffinose at 37 and 43°C. Enterotoxin- and heat-resistant spores were produced at similar concentrations but sooner at 43°C than at 37°C. There was a direct relationship between spore heat resistance and sporulation temperature (32, 37, and 43°C).

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*Clostridium perfringens* is a spore-forming bacterium that is perhaps the most widely distributed pathogenic bacterium in the environment. It has an optimal growth temperature of 43 to 45°C (14), at which it has one of the shortest generation times shown by any microorganism (14, 29).

Human food poisoning produced by *C. perfringens* is due to an enterotoxin which is produced during sporulation of the organism in the small intestine following ingestion of large numbers of vegetative cells (14). Although many *Bacillus* and *Clostridium* species sporulate satisfactorily at their optimal growth temperatures, two reports have indicated that *C. perfringens* is unable to sporulate at its optimal growth temperature (13, 15). Those experiments were carried out using sporulation media with starch as the main carbohydrate source. However, recently we found that many enterotoxin-positive (ENT+) strains were not able to hydrolyze starch at temperatures above 43°C and that the organism could not grow well under these conditions (7).

Several conditions during sporulation can influence the resistance of spores (18, 24, 28). Craven recently reported that during the sporulation of *C. perfringens* the pH of the environment can alter the heat resistance of the spores (3). Also, it has been shown for other spore-forming bacteria that the heat resistance of spores is enhanced as sporulation temperature is increased within a specific range (12, 19, 20, 30). For *C. perfringens*, Rey et al. (23) reported that incubation temperature did not alter the heat resistance of spores when they were produced and heated in cooked meat medium.

In addition to starch, several other carbohydrates and substances have been shown to support sporulation and enterotoxin production of *C. perfringens* at 37°C (10, 16, 17, 26). In this work we investigated the sporulation, heat tolerance, and enterotoxin production of *C. perfringens* type A at 43°C cultured in a sporulation medium containing raffinose as the principal carbohydrate.

**Cultural conditions.** The following *C. perfringens* strains were used: NCTC 10240, FD-1, ATCC 3624, FD-1041, and FD-884. Strains FD-1041, FD-884, and NCTC 10240 are ENT+. Strains ATCC 3624 and FD-1 are enterotoxin negative (ENT−). All strains were maintained as sporulated stock cultures in Robertson cooked meat broth (31) at −20°C. Inocula were prepared as previously described (7) and used to inoculate (0.5% inoculum) Duncan-Strong sporulation medium (5) in which 0.4% (7.9 mM) raffinose replaced starch (17). The medium also contained 1.2% 0.66 M sodium carbonate to enhance sporulation (9). Sporulating cell cultures were incubated at 32, 37, or 43°C. Heat-resistant spore levels were determined and cell extracts were prepared as previously described (7). Enterotoxin concentration was determined from cell extracts by counterimmunoelectrophoresis as described by Naik and Duncan (22). Enterotoxin was produced and purified as described by Granum and Whitaker (8). Antienterotoxin serum was produced as reported by Bartholomew and Stringer (1).

Sporulated cultures were centrifuged at 3,000 × g at 4°C after 10 h for those cultures incubated at 37 and 46°C and after 14 h for those incubated at 32°C. Spores were purified after repeated low-speed centrifugation (500 × g for 15 min) at 4°C and subsequent washing (usually 10 to 15 times) with ice-cold purified (Milli-Q water system; Millipore) water. Cleaned spores were suspended in water, kept at 2°C, and used within 8 days.

**Inactivation of spores.** To measure D values (the number of minutes required to decrease the viable spore number by a factor of 10 at the specified temperature), spore suspensions were adjusted to an *A*$_{260}$ of 0.5. Glass tubes (0.6 cm in diameter) were filled with 1.6 ml of a spore suspension and heat sealed. The tubes were heated at 75°C for 15 min to activate spores, submerged in a water bath at 85 or 95°C for selected times, cooled, and aseptically opened. The contents were briefly sonicated (15 s) to disrupt spore clumps. This suspension was then diluted 10-fold in 1% peptone water, and 0.1-ml aliquots were plated by using the medium and conditions described above. Thermal death curves of spores of the different treatments were obtained by the least-squares regression method. The D value was determined from the slope of the linear portion of the thermal survival curves (11). Sporulating cultures were examined by electron microscopy as previously described (25). The method of Bradford (2) was employed to determine the protein concentration, with bovine serum albumin as a standard. Ouchterlony immunodiffusion was carried out as reported by Stark and Duncan (27).

**Growth and sporulation.** All strains grew well at each temperature. At 43°C strain FD-1041 grew to a final absorbance similar to that observed at 37°C (Fig. 1). Growth at 43°C, however, was more rapid than that at 37°C. Similar results were obtained with strains NCTC 10240, FD-884,
FD-1, and ATCC 3624 (data not shown). Heat-resistant spore levels were also comparable at 37 and 43°C, although at 43°C they were detected sooner than at 37°C (3 versus 5 h) (Fig. 1). Similar results were observed with strains NCTC 10240, FD-884, and FD-1 (Table 1). The ENT− strain ATCC 3624 produced fewer spores at 43°C than at 37°C.

Enterotoxin formation. Contrary to previous reports (13, 15) in which starch was used in the culture medium, enterotoxin was produced by all strains at 43°C. Toxin levels were comparable to those obtained at 37°C (Table 1). However, toxin was detected sooner (3 versus 4 h) at 43°C than at 37°C in the case of FD-1041 (Fig. 2). The enterotoxin concentration in cell extracts of strain FD-1041 was considerably higher than was previously reported for other strains of this organism (16, 17). Enterotoxin-containing extracts of cells grown at 43°C produced a line of complete identity in Ouchterlony gel diffusion with extracts of cells grown at 37°C when reacted with antienterotoxin serum (data not shown). This indicates that enterotoxins produced at 37 and 43°C are structurally similar. No intracellular enterotoxin was detected in the extracts of sporulating cells of the ENT− strains ATCC 3624 and FD-1 when cultured at 37 or 43°C.

Ultrastructure of sporulating cells. Thin sections of sporulating cells of FD-1041 incubated at 43°C revealed spores of normal appearance within sporangia which also contained a round inclusion body surrounded by vacuoles (Fig. 3). This inclusion, when observed by phase-contrast microscopy, appeared as a nonrefractile body. Strain FD-884 also produced this inclusion. Some cells of these strains also produced the bar-shaped inclusion body commonly seen in ENT+ sporulating cells (4, 21). Strain NCTC 10240 produced mainly the bar-shaped inclusion. No inclusions were observed in the ENT− strains ATCC 3624 and FD-1. Vegetative cells of C. perfringens also produce an inclusion body at temperatures above 40°C (6). It is composed of non-enterotoxin-related proteins. It differs from the round inclusion body referred to here in that it is refractile (as viewed by phase-contrast microscopy), is not surrounded by vacuoles, and is produced by both ENT+ and ENT− strains.

D values. Heat tolerance of the spores varied depending on the strain used. D values are summarized in Table 2. Spores of the ENT+ strain FD-1041 were more heat resistant than those of the ENT− strains FD-1 and ATCC 3624. When heated at 85°C, spores of strains FD-1 and ATCC 3624

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**TABLE 1.** Sporulation and enterotoxin production by C. perfringens in Duncan-Strong medium containing 0.4% (7.9 mM) raffinose

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temperature (°C)</th>
<th>Maximum no. of heat-resistant spores/mla</th>
<th>Enterotoxin concb</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD-1041</td>
<td>37</td>
<td>(6.5 ± 0.1) × 10⁶</td>
<td>536 ± 15</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>(1.3 ± 0.1) × 10⁷</td>
<td>513 ± 32</td>
</tr>
<tr>
<td>FD-884</td>
<td>37</td>
<td>(2.6 ± 0.6) × 10⁶</td>
<td>394 ± 10</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>(9.1 ± 0.4) × 10⁶</td>
<td>392 ± 11</td>
</tr>
<tr>
<td>NCTC 10240</td>
<td>37</td>
<td>(2.4 ± 0.1) × 10⁶</td>
<td>337 ± 72</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>(9.8 ± 1.5) × 10⁶</td>
<td>301 ± 13</td>
</tr>
<tr>
<td>FD-1</td>
<td>37</td>
<td>(2.6 ± 0.6) × 10⁶</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>(1.1 ± 0.1) × 10⁶</td>
<td>ND</td>
</tr>
<tr>
<td>ATCC 3624</td>
<td>37</td>
<td>(3.5 ± 0.5) × 10⁷</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>(8.7 ± 1.1) × 10⁷</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a Numbers are means ± average deviations.

*b Micrograms of enterotoxin per milligram of cell extract. ND, not detectable.

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**TABLE 2.** D values of spores of C. perfringens produced at 32, 37, and 43°C

<table>
<thead>
<tr>
<th>Sporulation temp (°C)</th>
<th>D₉₅ (min) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FD-1</td>
</tr>
<tr>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>37</td>
<td>64</td>
</tr>
<tr>
<td>43</td>
<td>85</td>
</tr>
</tbody>
</table>

₅₀ and D₉₅, D values for spores heated at 85 and 95°C, respectively.
produced at 32, 37, and 43°C showed a direct relationship between heat tolerance and sporulation temperature. A similar behavior was showed by spores of strain FD-1041 when heated at 95°C; i.e., the $D$ values for spores produced at 32, 37, and 43°C and heated at 95°C were 22, 116, and 200 min, respectively. Most of the spores produced at 32°C exhibited low resistance compared with that of those produced at 37 and 43°C. In the case of spores of this strain a biphase heat inactivation pattern was observed for spores produced at 32°C (Fig. 4).

Lindsay et al. (20) also found relatively low heat resistance for spores of Bacillus subtilis A produced near their lower temperature limit of sporulation. Contrary to our findings, Rey et al. (23) found no relationship between sporulation temperature and spore heat resistance of C. perfringens. This could be due to differences in methodology, particularly their use of cooked meat medium as the sporulation and heating medium. It has been reported that the composition of the heating menstruum can modify the thermostolerance of spores (11, 24).

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REFERENCES
5. Duncan, C. L., and D. H. Strong. 1968. Improved medium for


