Effects of pH Shifts, Bile Salts, and Glucose on Sporulation of *Clostridium perfringens* NCTC 8798†

C. S. HICKEY‡ AND M. G JOHNSON§*

Departments of Food Science and Microbiology, Clemson University, Clemson, South Carolina 29631

The sporulation of *Clostridium perfringens* NCTC 8798 was studied after exposing vegetative cells to: pH values of 1.5 to 8.0 in fluid thioglycolate broth (for 2 h) and then transferring them to Duncan-Strong (DS) sporulation medium; sodium cholate or sodium deoxycholate (0.3 to 6.5 mM) in DS medium; or Rhia-Solberg medium with 0.4% (wt/wt) starch, glucose, or both added at 0 to 55 mM. At pH 1.5, no culturable heat-resistant spores were formed. For cells exposed to pH 3.0, 4.0, 5.0, or 6.0, increases in heat-resistant spores were not seen until after a lag of 12 to 13 h, whereas the lag was only 2 to 3 h for cells exposed to pH 7.0 or 8.0. Maximal spore crops were produced after only 6 to 8 h for cells exposed to pH 7 or 8, but 16 to 18 h was required for production of maximal spore crops by cells exposed to the lower-pH media. The addition of sodium cholate (3.5 to 6.5 mM) to DS medium only slightly reduced the culturable heat-resistant spore count from 1.9×10^7 to 3×10^6/ml. The addition of 1.8 mM or more sodium deoxycholate reduced the culturable heat-resistant spore count to less than 10/ml. When either starch or glucose alone was added to Rhia-Solberg medium there was no production of culturable heat-resistant spores, but a combination of 0.4% (wt/wt) starch and 4.4 mM glucose yielded 6×10^5 spores/ml. The spore production remained at this level for glucose concentrations of 6 to 22 mM, but then declined to about 3×10^3 spores per ml at higher concentrations.

Clostridium perfringens is one of the leading causes of bacterial foodborne illness of humans in the United States. Onset of illness is usually 12 to 14 h after ingesting food containing a large number of viable cells. When the cells sporulate in the small intestine (6, 7, 11, 14, 16), they produce an enterotoxin which is part of the spore coat protein. In vitro tests showed that the enterotoxin appears in about 10 to 12 h when sporangial lysis occurs (4).

The sporulation of *C. perfringens* cells in the intestinal tract should be influenced by the dynamics of the intestinal tract, i.e., shifts in pH and bile acid concentrations. The pH varies from a low of 1.5 in the stomach to a high of 8.0 in the small intestine, where bile salts are also present (2, 13, 20). Tsai and Riemann (32) reported that the level of spores produced in 24 h is not affected by exposing incubated vegetative cells to pH 2.0 to 3.0 for 30 min. Labbe and Duncan (16) showed that a pH of 7.0 was optimal for sporulation, a pH of 8.0 or 6.0 decreased sporulation, and a pH of 5.5 halted sporulation completely. Bile has an alkaline pH to neutralize stomach acid. The bile components cholic, deox-

ycholic, and chenodeoxycholic acid inhibit vegetative growth (9), but little work has been reported on the effects of these agents on sporulation of *C. perfringens*.

Sporulation by clostridia requires a complex medium which includes amino acids, minerals, and a carbon source; unlike bacilli, clostridia require carbon and energy sources throughout the sporulation cycle. A number of complex media have been developed (8, 9, 22), with Duncan-Strong (DS) medium (5) having the widest acceptance. Several defined media, based on Boyd et al. (1), have been developed for growth (12, 25, 28, 31) and sporulation (22, 26, 30). In both defined and complex media, a poorly utilized carbon source such as starch or raffinose appears to be critical for good sporulation (17–19, 30).

To produce spores in DS medium, the inoculum is the second serial transfer in fluid thioglycolate (FT) medium that is incubated for 4 h. Since the sporulation medium is inoculated with 10% (vol/wt) FT medium unutilized glucose in the FT medium can be carried over to the DS medium. This glucose in the inoculum may have a different influence on sporulation in a complex medium, such as DS medium (19), than in a defined medium, such as the RS medium of Rhia and Solberg (25).

This study was designed to elucidate the effects on sporulation when cells that are to be

inoculated into sporulation media are first exposed to different pH values, bile salts, or different glucose concentrations in the presence of 0.4% starch.

(A preliminary report of this study has been presented [C. S. Hickey and M. G. Johnson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, O4, p. 245].)

MATERIALS AND METHODS

Cultures. Strain NCTC 8798 *C. perfringens* type A was obtained from C. L. Duncan, University of Wisconsin, Madison. Stock cultures were grown at 36°C for 24 h in cooked meat medium (Difco Laboratories) and stored at 2°C.

Sporulation medium. DS medium (5) had 1.5% proteose peptone (Difco), 0.4% yeast extract (Difco), 0.1% sodium thioglycolate, and 0.4% soluble starch (Fisher Scientific Co.). The defined medium used was RS medium (25). All components of RS medium except the glucose and $FeSO_4 \cdot 7H_2O$ were dissolved in glass-distilled deionized water and autoclaved together with filter-sterilized $FeSO_4 \cdot 7H_2O$ added after autoclaving to make the basal RS medium. Glucose was autoclaved as a stock solution and added at various concentrations. The pH of this medium was adjusted to 7.4 before sterilization.

Growth conditions. All cultures were grown at 36°C. Air was dispelled from tubes of media by placing them in a boiling water bath for 5 min and then cooling before inoculation. Cultures were enumerated with the medium of Shahidi-Ferguson (SFP; 29) in petri plates protected from oxygen with the GasPak system (BBL Microbiology Systems). The GasPak generator was activated by adding 10 ml of a 25% (wt/vol) solution of KH_2PO_4 instead of water, as suggested by Ferguson et al. (10).

Preparation of inocula. A 0.1-ml amount of cooked meat stock culture was transferred into 19 ml of previously exhausted FT medium (Difco), heat shocked for 20 min at 75°C, and incubated for 16 h. Two additional serial transfers of culture were made into freshly exhausted FT medium, and each was incubated at 36°C for 4 h. The third culture (10%, vol/vol) was used to inoculate DS and RS media.

Before the exponential-phase cells were inoculated into RS medium, they were harvested by centrifugation at 10,000 × *g* for 20 min at 25°C. The resulting cell pellets were washed twice by suspension in 0.1 M phosphate buffer (pH 7.2), centrifuged as above, and finally resuspended in buffer to yield the original culture volumes.

Culturable heat-resistant spore cell counts. Culturable heat-resistant spore cell counts were made by heating 2-ml samples of undiluted cultures in tightly closed screw-capped test tubes (16 by 150 mm) in a mineral oil bath at 75°C for 20 min. A sample required 2 min to reach 75°C. After the heat shock treatment, decimal dilutions of cultures were made in 0.1 M phosphate buffer (pH 7.4) containing 0.1% peptone, and appropriate dilutions were pour plated with SFP medium. Total culturable counts were made by diluting unheated samples of culture in the above phosphate-peptone buffer and pour plating them with the same medium.

Total direct cell and spore counts. Microscopic counts of cells and spores were made with a Petroff-Hausser counting chamber and a phase-contrast microscope (Zeiss model RA), using wet-mount preparations under no. 1½ cover slips. At least two preparations were prepared for each sample to be counted, and at least four rows per preparation were examined. Culture samples were diluted to yield two to six cells or spores per square. At least 200 cells (or spores) were counted to calculate the count per milliliter. The average count obtained per row was multiplied by the dilution factor × 10 to obtain the count per milliliter.

Sporulation after pH exposure. The starting inocula were 34 ml of FT culture grown for 16 h. These cultures were centrifuged at 2,000 × *g* for 15 min at 25°C; then the cell pellets were suspended in 35 ml of freshly exhausted, sterile tempered (36°C) FT medium and incubated at 36°C for 4 h. These secondary cultures were then centrifuged at 10,000 × *g* for 20 min at 25°C. The supernatants from these cultures were decanted, and the pellets were resuspended with 35 ml of freshly exhausted, sterile tempered (36°C) FT medium which, before autoclaving, had been adjusted with 5 N HCl to pH 1.5, 3, 4, 5, 6, or 7 or with 5 N NaOH to pH 8. The cells were incubated for 2 h at 36°C in these media before they were harvested by centrifugation as above. The resulting cell pellets were suspended in 35-ml amounts of DS medium in screw-capped centrifuge tubes (25 by 125 mm) which were then incubated at 36°C. Two-milliliter samples were removed periodically from the tubes of DS medium and assayed for direct microscopic cell and spore counts, culturable cell counts, and heat-resistant spore counts as detailed above.

The water-soluble sodium cholate and sodium deoxycholate were compared with ethylenediaminetetraacetic acid (EDTA) and sodium lauryl sulfate (SLS) for effect on sporulation and were added to give final concentrations of 0.3, 0.6, 1.2, 1.8, 2.5, 3.5, or 6.5 mM in 50-ml amounts of DS medium. Inocula were prepared by the procedure outlined above. Samples were removed at 24 h and assayed for direct microscopic and culturable heat-resistant spore counts.

Glucose utilization in FT medium. Twenty-seven milliliters of a freshly exhausted, tempered (36°C) FT medium was inoculated with 3.0 ml of a 16-h-old culture of cells grown in FT medium and incubated at 36°C for 4 h. Five duplicate tubes containing 10 ml of freshly exhausted, tempered (36°C) FT broth were inoculated with 1-ml volumes from the previous 4-h-old FT culture. At 1-h intervals, duplicate tubes were removed and placed in an ethanol-ice bath (−15°C) to stop growth. Residual reducing sugars in the medium were determined by the method of Nelson (24), and residual glucose was determined by the Glucostat method (Worthington Biochemicals Corp.), using appropriate dilutions of media and known glucose solutions for standards.

RESULTS

Effect of pH shifts on sporulation. The influence of pH on initiation of sporulation by *C. perfringens* cells is shown in Fig. 1 for pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. At pH 1.5, the lowest pH tested, no spores were produced (data not
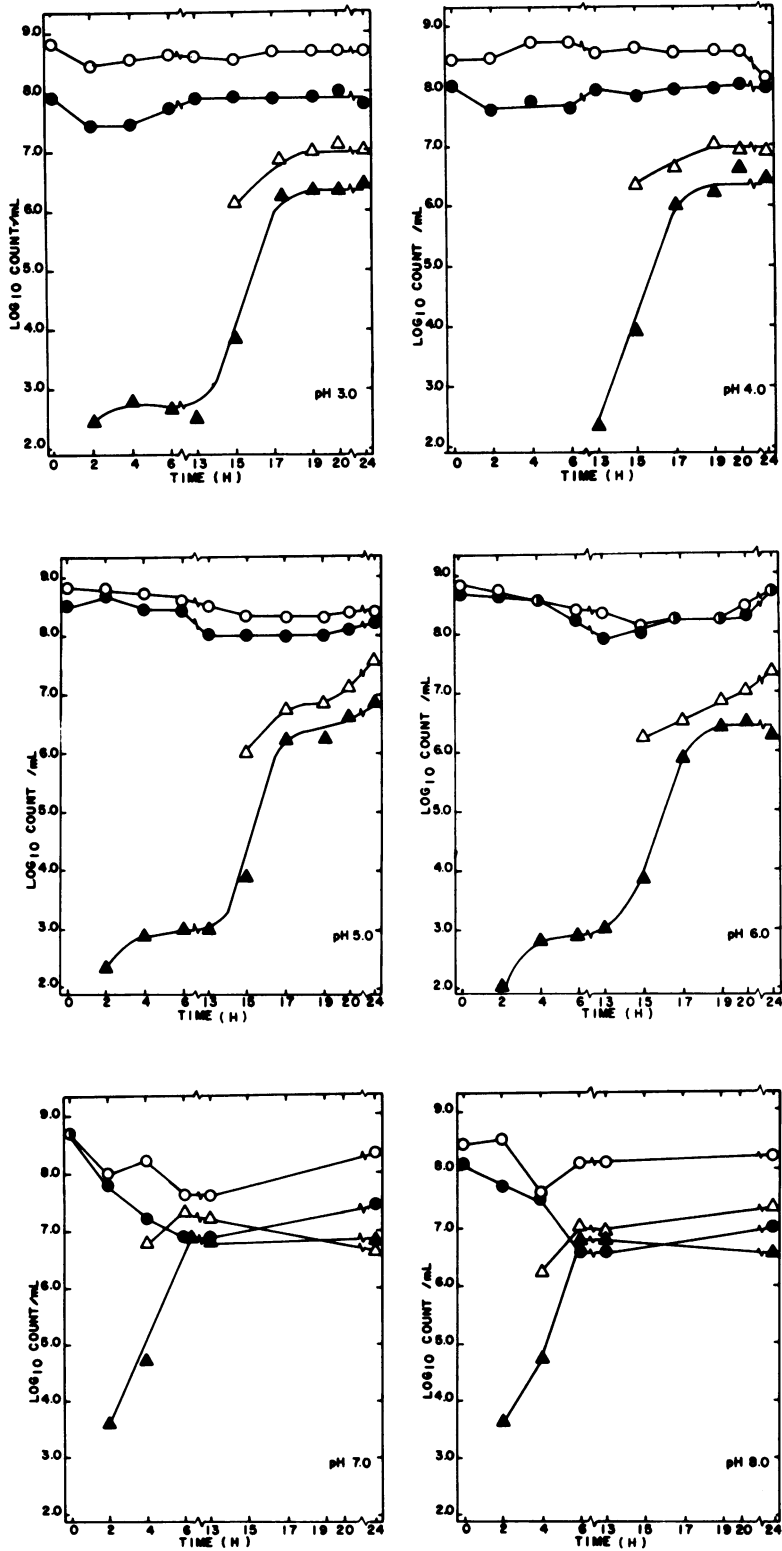
FIG. 1. *Time study of sporulation in DS medium by cells of C. perfringens NCTC 8798 previously subjected to different pH values in FT medium for 2 h. Symbols:* ○, *direct microscopic cell count;* ●, *culturable cell count;* △, *phase-bright spore count;* ▲, *culturable heat-resistant spore count.*

shown). At pH 7.0 or 8.0, microscopic spore and culturable heat-resistant spore counts began to increase after only 2 to 4 h and reached maximal values of about 10^7/ml by 6 to 7 h. Cells exposed to intermediate pH values of 3.0, 4.0, 5.0, or 6.0 showed a lag period of about 13 to 15 h before culturable heat-resistant spore counts began to increase. Maximal cultural heat-resistant spore counts of 10^6 to 5×10^6/ml were not produced by these cells until about 17 to 20 h.

Effect of bile salts on sporulation. Direct microscopic spore counts declined no more than 0.5 log for cells exposed to sodium cholate concentrations of 3.5 to 6.5 mM (Table 1). However, a sodium deoxycholate concentration of 1.2 mM reduced the heat-resistant spore count by 1.5 log cycles, whereas cells exposed to a concentration of 1.8 mM or greater produced no detectable spores. Concentrations of 0.3 and 1.2 mM EDTA, a chelating agent, reduced heat-resistant spore production by 2.9 and 3.9 logs, respectively, and at concentrations of 1.8 mM or greater no spores were detected. An SLS concentration of 1.8 mM reduced heat-resistant spores by 6.2 log cycles, and at concentrations of 2.5 mM or greater no spores were detected.

Effect of sporulation in RS medium containing glucose and starch. In preliminary experiments, basal RS medium containing 28 mM glucose inoculated with 10% (vol/vol) unwashed or washed cells from FT medium produced only 5×10^2 heat-resistant spores per ml in 24 h. Conversely, the basal RS medium with 0.4% (wt/vol) starch yielded culturable heat-resistant spores of 2.5×10^6 and <10/ml with unwashed and washed inocula from FT medium, respectively (data not shown). These results indicated that something carried over with the inocula from the FT medium positively or negatively affected sporulation in RS medium.

Therefore, residual glucose in FT cultures was assayed at 1-h intervals over the 4-h cultivation period normally used before inoculating the sporulation medium (Fig. 2). The assays indicated that about 4 mM glucose or 7 mM total reducing sugars was carried over to the RS medium with the unwashed inocula from 4-h-old cultures of FT medium.

The effect on sporulation of adding increased amounts of glucose to basal RS medium containing 0.4% starch was then studied with buffer-washed inocula (Fig. 3). As the glucose concentration was increased from 0 to 4.4 mM, the culturable heat-resistant and phase-bright spore count increased from none to 4×10^5 and 4.5×10^7/ml, respectively. The heat-resistant and phase-bright spore count production remained constant at about 10^5 and 5×10^7/ml, respectively, for glucose concentrations of 4.4 to 22 mM. At 22 to 55 mM glucose, the heat-resistant
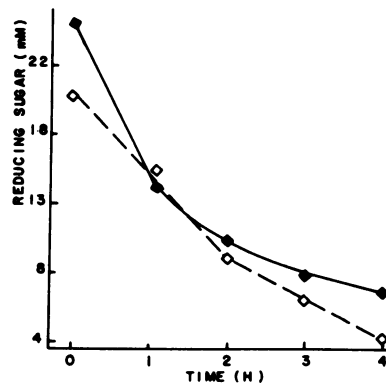

FIG. 2. *Residual glucose in FT medium after growth of C. perfringens NCTC 8798 for 0 to 4 h. Symbols:* ◆, *reducing sugar (Nelson's assay);* ◇, *glucostat assay.*

TABLE 1. *Effect of adding sodium cholate (Na-CA), sodium deoxycholate (Na-DCA), EDTA, and SLS on culturable heat-resistant spores and direct microscopic spore counts produced by C. perfringens NCTC 8798 in DS medium, initial pH 7.4, after 24 h at 36°C*

Material added (mM)	Log₁₀ counts/ml							
	Na-CA		Na-DCA		EDTA		SLS	
	DMSC[a]	HRSC[b]	DMSC	HRSC	DMSC	HRSC	DMSC	HRSC
0.0	8.0	7.1	7.9	7.3	7.9	7.4	7.9	7.3
0.3	7.8	7.4	7.5	6.5	7.5	4.5	7.5	7.1
0.6	7.8	7.0	6.0	5.9	5.0	3.8	7.5	7.1
1.2	7.9	7.1	6.5	5.8	5.0	3.5	5.7	5.6
1.8	7.8	6.9	NT[c]	NT	NT	NT	5.1	1.1
2.5	7.5	7.0	NT	NT	NT	NT	NT	NT
3.5	7.4	6.5	NT	NT	NT	NT	NT	NT
6.5	6.7	6.5	NT	NT	NT	NT	NT	NT

[a] DMSC, Direct microscopic spore count; derived from a total of at least 200 spores.
[b] HRSC, Culturable heat-resistant spore counts (75°C, 15 min); means for duplicate platings.
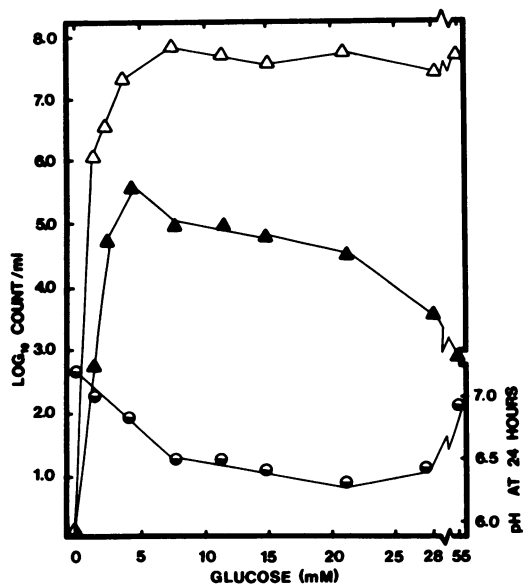[c] NT, No detectable spores.

FIG. 3. *Effect of initial glucose level in RS medium (containing 4.0 g of starch per liter) after 24 h at 36°C. Symbols: △, phase-bright spore count; ▲, culturable heat-resistant spore count; ⊖, final pH.*

spore count dropped to 8×10^2/ml, but the phase-bright spores remained at about 5×10^7/ml. The final pH of cultures in RS medium, initially pH 7.4, fluctuated from a value of 6.5 to 7.7 through 28 mM to 6.9 at 55 mM glucose (Fig. 3).

DISCUSSION

The pH in the stomach region may be as low as 1.5. However, in the duodenum the acidic chyme from the stomach is neutralized by the bile to yield a pH of about 7.0. Therefore, during digestion bacterial cells in consumed food are normally exposed to a low pH for only 2 to 3 h (3, 13). Tsai and Rieman (33) reported that vegetative cells of *C. perfringens* exposed for 30 min to acid environments as low as pH 2.0 sporulated normally. However, they measured sporulation only after 24 h. The present sporulation study showed a lag of about 13 to 15 h before sporulation was initiated in DS medium by cells previously exposed for 2 h to pH 3.0, 4.0, 5.0, or 6.0, in FT medium. The delay of 13 h in the initiation of sporulation by cells exposed to an acidic environment is in agreement with the generally observed incubation period of 10 to 14 h for the onset of gastroenteritis symptoms associated with ingestion of *C. perfringens* cells (4).

Inhibition of *C. perfringens* cell growth by bile acids was reported by Floch et al. (9). However,

to our knowledge no data have been reported about the effect of bile acids on sporulation of this organism. Our present data indicate that sporulation was reduced very little by increasing the sodium cholate concentration from 3.5 to 6.5 mM, and even at 6.5 mM the heat-resistant spore level was only 0.6 log lower than for the control culture (Table 1). Conversely, a sodium deoxycholate concentration of 1.8 mM prevented production of any heat-resistant spores. This depressing effect of sodium deoxycholate on sporulation may help explain how persons who contract a case of *C. perfringens* gastroenteritis, although they may continue to harbor the organism (34) in their intestinal tract, generally do not continue to show symptoms presumably because sporulation or enterotoxigenesis (4, 14) does not occur as readily or extensively with bile present. During normal digestion of a meal, the bile salt concentration in the duodenum of humans rises to 6 to 10 mM, high enough to cause the same kind of depression of sporulation in vivo as reported here for in vitro tests.

Bile acids can act chemically both as surface tension-reducing materials and as chelating agents. Therefore, effects on sporulation of a surface tension-reducing agent, SLS, and a chelating agent, EDTA, were tested. The fact that 1.2 to 1.8 mM sodium deoxycholate or SLS reduced sporulation appreciably may suggest that sodium deoxycholate inhibited sporulation by a surface tension depression rather than by a chelation mechanism. Since lauric acid has some antimicrobial characteristics (21), further study of the surface tension effect on sporulation should be undertaken.

Spore production can be reduced by the mechanism of catabolite repression which occurs when a readily utilized energy source is supplied to cells. It is hypothesized that catabolites derived from, and dependent upon, the nature of both the carbon and the nitrogen sources in media can act as repressors of one or more sporulation-specific genes (15, 27). Labbe and Duncan (17) and Labbe and Rey (18) mentioned that glucose appeared to repress sporulation of *C. perfringens* but did not report the glucose concentrations at which repression occurred. In our tests both glucose and starch were needed to foster good sporulation of strain NCTC 8798 in the defined RS medium. A combination of 4.4 mM glucose and 0.4% starch in defined RS medium led to maximal direct microscopic and culturable heat-resistant spore counts of 4.5×10^7 and 4×10^5/ml, respectively (Fig. 3). Starch alone in RS medium initially produced 3×10^3 heat-resistant spores per ml, declining to <10/ml in 24 h, but with no glucose in the RS medium

no heat-resistant spores were produced (Fig. 3). This indicated that about 4 to 5 mM glucose is essential to sporulation and verified the general claim that clostridia cannot form spores endotropically (17, 23). The 1,000- to 100,000-fold-lower heat-resistant spore counts compared with phase-bright refractile spore counts obtained at 22 to 55 mM glucose (Fig. 3) point out the importance of directly monitoring spore heat resistance in sporulation studies of this organism.

Our results further indicate that glucose-induced catabolite repression of sporulation by this organism can be circumvented if the initial glucose concentration is held sufficiently low, below about 20 mM (Fig. 3), and starch is also present. Starch, besides slowly providing glucose units via bacterial amylase action, may promote sporulation by adsorbing from the culture medium small molecules that are inhibitory to sporulation, in a manner similar to that postulated for resins (2) and charcoal (7).

LITERATURE CITED

1. **Boyd, J. M., M. A. Logan, and A. A. Tytell.** 1948. Growth requirements of *Clostridium perfringens* BP6K. J. Biol. Chem. **174**:1013-1025.
2. **Clifford, W. J., and A. Anellis.** 1971. Sporulation in a biphasic glucose-ion-exchange resin medium. Appl. Microbiol. **22**:856-861.
3. **Davenport, H. W.** 1968. Physiology of the digestive tract. Yearbook Publishers Inc., Chicago.
4. **Duncan, C. L.** 1973. Time of enterotoxin formation and release during sporulation of *Clostridium perfringens* type A. J. Bacteriol. **113**:932-936.
5. **Duncan, D. L., and D. H. Strong.** 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16**:82-89.
6. **Duncan, C. L., and D. H. Strong.** 1969. Experimental production of diarrhea in rabbits with *Clostridium perfringens*. Can. J. Microbiol. **15**:765-770.
7. **Duncan, C. L., and D. H. Strong.** 1969. Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of *Clostridium perfringens*. J. Bacteriol. **100**:86-94.
8. **Ellner, P. D.** 1956. A medium promoting rapid quantitative sporulation in *Clostridium perfringens*. J. Bacteriol. **71**:495-496.
9. **Floch, M. H., H. S. Binder, B. Filburn, and W. Gershengaren.** 1972. The effect of bile acids on intestinal microflora. Am. J. Clin. Nutr. **25**:1418-1426.
10. **Ferguson, I. R., K. D. Phillips, and P. V. Tearhe.** 1975. An evaluation of the CO_2 component in the gaspak anaerobic system. J. Appl. Bacteriol. **39**:167-173.
11. **Frieben, W. R., and C. L. Duncan.** 1973. Homology between enterotoxin protein on spore structural protein in *Clostridium perfringens*. Eur. J. Biochem. **39**:393-401.
12. **Fuchs, A. R., and G. J. Bonde.** 1957. The nutritional requirements of *Clostridium perfringens*. J. Gen. Microbiol. **16**:317-329.
13. **Guyton, A. C.** 1971. Textbook of medical physiology. The W. B. Saunders Co., Philadelphia.
14. **Hauschild, A. H. W., L. Nilo, and W. J. Dorward.** 1970. Enteropathogenic factors of food poisoning *Clostridium perfringens* Type A. Can. J. Microbiol. **16**:311-338.
15. **Hsu, E. J., and E. J. Ordal.** 1969. Sporulation of *Clostridium thermosaccharolyticum* under conditions of restricted growth. J. Bacteriol. **97**:1511-1512.
16. **Labbe, R. G., and C. L. Duncan.** 1974. Sporulation and enterotoxin production by *Clostridium perfringens* Type A under conditions of controlled pH and temperature. Can. J. Microbiol. **20**:1493-1502.
17. **Labbe, R. G., and C. L. Duncan.** 1975. Influence of carbohydrates on growth and sporulation of *Clostridium perfringens* type A. Appl. Microbiol. **29**:345-351.
18. **Labbe, R. G., and D. K. Rey.** 1979. Raffinose increases sporulation and enterotoxin production by *Clostridium perfringens* Type A. Appl. Environ. Microbiol. **37**:1196-1200.
19. **Labbe, R. F., E. Somers, and C. L. Duncan.** 1976. Influence of starch source on sporulation and enterotoxin production by *Clostridium perfringens* type A. Appl. Environ. Microbiol. **31**:455-457.
20. **Luckey, T. D.** 1974. Introduction: the villus in chemostat man. Am. J. Clin. Nutr. **27**:1266-1276.
21. **Kabara, J. J., and R. V. Kable.** 1977. Antimicrobial lipids: natural and synthetic fatty acids and monoglycerides. Lipids **12**:753-759.
22. **Muhammed, S. I., S. M. Morrison, and W. L. Boyd.** 1975. Nutritional requirements for growth and sporulation of *Clostridium perfringens*. J. Appl. Bacteriol. **38**:245-253.
23. **Murrell, W. G.** 1967. The biochemistry of the bacterial endospore, p. 133-251. *In* A. H. Rose (ed.), Advances in microbial physiology. Academic Press, Inc., New York.
24. **Nelson, N.** 1944. A photometric adaption of the somogyi method for the determination of glucose. J. Biol. Chem. **153**:375-380.
25. **Rhia, W. E., and M. Solberg.** 1971. Chemically defined medium for the growth of *Clostridium perfringens*. Appl. Microbiol. **22**:738-739.
26. **Sacks, L. E., and P. A. Thompson.** 1978. Clear defined medium for the sporulation of *Clostridium perfringens*. Appl. Environ. Microbiol. **32**:405-410.
27. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. U.S.A. **54**:704-711.
28. **Sebald, M., and R. N. Costilow.** 1975. Minimal growth requirements for *Clostridium perfringens* and isolation of auxotrophic mutants. Appl. Microbiol. **29**:1-6.
29. **Shahidi, S. A., and A. R. Ferguson.** 1971. New quantitative, qualitative, and confirmatory media for rapid analysis of food for *Clostridium perfringens*. Appl. Microbiol. **21**:500-506.
30. **Strong, D. H., C. L. Duncan, and G. Perna.** 1971. *Clostridium perfringens* type A food poisoning. II. Response of the rabbit ileum as an indication of enteropathogenicity of strains of *Clostridium perfringens* in human beings. Infect. Immun. **3**:171-178.
31. **Ting, M. N., and D. Y. C. Fung.** 1972. Chemically defined medium for growth. Appl. Microbiol. **25**:755-759.
32. **Tsai, C. C., and H. P. Riemann.** 1974. Improved culture techniques and sporulation medium for enterotoxin production by *Clostridium perfringens* Type A. J. Formosan Med. Assoc. **73**:404-409.
33. **Tsai, C. C., and H. D. Riemann.** 1974. Relation of enterotoxigenic *Clostridium perfringens* Type A to food poisoning. II. Acid exposure and storage conditions affecting enterotoxigenesis of *Clostridium perfringens*. J. Formosan Med. Assoc. **73**:703-708.
34. **Uemura, R., C. Genigeorgis, H. P. Riemann, and C. E. Franty.** 1974. Antibody against *Clostridium perfringens* type A enterotoxin in human sera. Infect. Immun. **9**:470-471.