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Raffinose Increases Sporulation and Enterotoxin Production by *Clostridium perfringens* Type A†

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Replacement of starch with raffinose in Duncan and Strong sporulation medium improved percent sporulation in six of eight strains tested. Enterotoxin concentration in cell extracts was increased in the case of four of five known enterotoxin-positive strains. With strain NCTC 10240, levels of 0.3, 0.4, and 0.5% raffinose produced the highest enterotoxin concentration, 300 to 320 μg of enterotoxin per mg of cell extract protein. At a level of 0.4% raffinose the highest enterotoxin concentration in cell extracts of NCTC 10240 occurred after 8 h of growth in Duncan and Strong medium. Enterotoxin produced in the presence of starch or raffinose by three separate strains all migrated at similar R_m by polyacrylamide gel electrophoresis.

Clostridium perfringens is one of the leading causes of human food poisoning. Illness results from ingestion of large numbers of vegetative cells which sporulate in the intestine (3). The enterotoxin is associated with the bacterial spore coat as a structural protein (7). Toxin is only produced in laboratory media which promote sporulation of this organism. A number of complex media have been described for this purpose (2, 6, 8, 10, 14, 18, 23), but that of Duncan and Strong (DS medium [5]) or minor modifications of it (1, 13, 15) have gained the widest acceptance. The carbohydrate source influences sporulation in this medium (12, 13). Raffinose in particular was effective in promoting sporulation of *C. perfringens* (17). We have reinvestigated the ability of this carbohydrate to promote sporulation and in addition determined its effect on enterotoxin production. Results reported here demonstrate that raffinose increases sporulation of seven of eight strains of *C. perfringens* and increases the level of enterotoxin formation in four of five known enterotoxin-positive strains.

MATERIALS AND METHODS

Cultures. Cultures of *C. perfringens* type A were obtained from C. Duncan, Food Research Institute, University of Wisconsin, Madison. They are listed in Table 1. Three, ATCC 3624, F42, and FD1 do not produce enterotoxin. All others are enterotoxin positive. All were maintained at 4°C in cooked-meat medium (Difco).

Sporulation medium. DS medium was comprised of the following: 1.5% proteose peptone (Difco), 0.4% yeast extract (Difco), 0.1% sodium thioglycollate, 0.4% soluble starch (ICN; 13) or 0.4% (except as noted) raffinose (Sigma), and 1.0% sodium phosphate (dibasic heptahydrate).

Cultural methods. The inoculation sequence was as follows: two drops of a cooked-meat stock culture were added (and dispersed by gentle shaking) to 10 ml of fluid thioglycollate medium (FTG). The latter was heated for 10 min at 75°C, cooled, and incubated overnight (16 to 18 h) at 37°C. The screw caps of the culture tubes were kept loose to permit evolved gas to escape. A 1% inoculum from the FTG tube was then added to 100 ml of sporulation medium contained in a 125-ml Erlenmyer flask. No special precautions were followed to obtain anaerobic conditions other than inclusion of sodium thioglycollate and stationary incubation of the culture. Experiments were done at least in duplicate. In one experiment dealing with the time course of enterotoxin formation, the volume of the sporulation medium was increased to 2,000 ml (20-ml inoculum of FTG), and hourly 100-ml samples were removed for determination of enterotoxin and heat-resistant spore levels.

Enumeration of spores. Percentage of sporulating cells (cells that had reached morphological stage II of sporulation or beyond) was determined by phase-contrast microscopy; the number of sporulating cells out of a total of 300 cells was counted. The heat-resistant spore population was determined by heating 5 ml of a sporulating culture for 20 min at 75°C in a screw-capped tube followed by cooling and enumeration in a medium consisting of 1.0% yeast extract (Difco), 1.5% Trypticase (Baltimore Biological Laboratory), and 1.5% agar. Plates were counted after 48 h of incubation at 37°C in a GasPak system (Baltimore Biological Laboratory). Except as noted, both percent sporulation and heat-resistant spore levels were determined after 7 h of incubation in the sporulation medium.

Preparation of cell extract. Cultures growing in sporulation medium were harvested at 7 h by centrifugation at 10,000 × *g* for 15 min. The pellet was resuspended in 4 ml of distilled water and placed in a

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polycarbonate centrifuge tube (16 by 100 mm). The tube was placed in an ice-water bath, and the cells were disrupted with a Branson Sonifier (Branson Sonic Power, Danbury, Conn.). Sonication was continued until 90 to 95% of the spores were free. The disrupted cells were then centrifuged at 10,000 × *g* for 15 min at 4°C. About 2 ml of the supernatant fluid was removed and frozen until assayed for enterotoxin.

Enterotoxin determination. Quantitation of enterotoxin in cell extracts was by electroimmunodiffusion (4) with a sporulating cell extract of NCTC 8798 as a standard. The latter had been previously standardized against purified enterotoxin. Protein concentrations were determined by the method of Lowry et al. (16). All determinations were done in duplicate. Concentration of toxin is expressed as micrograms of enterotoxin per milligram of cell extract protein.

Disc gel immunodiffusion. Disc gel immunodiffusion was performed as described by Skjelkvåle and Duncan (21).

RESULTS

Effect of raffinose versus starch on sporulation and enterotoxin formation. The influence of starch versus raffinose on sporulation and enterotoxin production by eight strains of *C. perfringens* is shown in Table 1. The results confirm a previous report (7) in that three strains (ATCC 3624, F42, and FD1) did not produce detectable enterotoxin. Likewise, those strains that produced enterotoxin were previously reported to be enterotoxin positive (19). Compared to starch, raffinose increased percent sporulation in seven of the eight strains. This parameter varied with the strain tested and ranged from 2% (NCTC 8238, starch) to 93% (ATCC 3624, raffinose). Such variation among strains is a well-known characteristic of this organism. The level of heat-resistant spore formation was also strain dependent; the range was from 1.8×10^3/ml (NCTC 8238, raffinose) to 2.8×10^7/ml (FD1, raffinose). With regard to heat-resistant spore formation, raffinose was an improvement for only four of eight strains. These results indicate that, although raffinose may promote sporulation in the early stages, its use does not insure that later (heat-resistant) stages of sporulation will be completed. This was most dramatic in the case of ATCC 3624 which sporulated at a level of 93% in the presence of raffinose versus 70% with starch, but which yielded almost 10,000-fold fewer heat-resistant spores per ml when grown with raffinose. It is not known whether this is because raffinose is a poor carbohydrate for sporulation by this strain or whether the level of raffinose is exhausted early during sporulation.

In the case of enterotoxin formation, the use of raffinose increased its concentration in four of five cases. The most dramatic increase occurred with NCTC 10240. With this strain the use of raffinose in place of starch produced a 10-fold increase. This amount, 330 μg of enterotoxin per mg of cell extract protein, was also the greatest produced by any of the strains tested.

Use of a mixture of raffinose and starch resulted in enterotoxin levels intermediate between those obtained by using each carbohydrate alone (data not shown).

Effect of raffinose concentration on sporulation and enterotoxin production by NCTC 10240. We next sought to determine at what level raffinose would be most effective in

TABLE 1. *Effect of starch and raffinose on sporulation and enterotoxin production by C. perfringens in DS medium*

Strain	Hobb serotype	Carbohydrate	% Sporulation	Heat-resistant spores/ml	Enterotoxin concn (μg/mg of cell extract protein)
NCTC 8238	2	Starch	2	1.9×10^4	3.0
		Raffinose	13	1.8×10^3	6.6
NCTC 8239	3	Starch	16	6.5×10^6	91.0
		Raffinose	25	2.2×10^3	11.0
NCTC 8679	6	Starch	4	3.4×10^4	4.3
		Raffinose	4	4.1×10^4	7.6
NCTC 8798	9	Starch	70	1.1×10^7	72.0
		Raffinose	86	2.6×10^7	160.0
NCTC 10240	13	Starch	59	2.6×10^6	24.0
		Raffinose	90	1.0×10^7	330.0
ATCC 3624		Starch	70	1.0×10^7	ND[a]
		Raffinose	93	2.8×10^3	ND
F42		Starch	62	1.9×10^7	ND
		Raffinose	73	4.8×10^6	ND
FD1		Starch	50	1.4×10^7	ND
		Raffinose	62	2.8×10^7	ND

[a] ND, Enterotoxin not detectable.

promoting sporulation and enterotoxin formation by strain NCTC 10240. The results (Table 2) show that of the levels tested 0.3 and 0.5% produced slightly higher concentrations of enterotoxin than 0.4%. Raffinose at 0.4% yielded the largest number of heat-resistant spores per milliliter. However, in view of the range of values obtained, either 0.3 or 0.4% raffinose appears to be sufficient for routine work.

Time course of enterotoxin synthesis by strain 10240. We then followed the production of heat-resistant spores and enterotoxin synthesis by strain 10240 in DS medium containing 0.4% raffinose. Figure 1 shows that enterotoxin concentration was highest after 8 h of growth, reaching a level of 355. Heat-resistant spore levels had reached a plateau by 7 h. The decrease in enterotoxin concentration at 9 h was due to an increase in cell extract protein rather than a decrease in enterotoxin. This was presumably due to renewed vegetative cell growth after 7 h, a situation which has been previously reported to occur with this organism (11).

TABLE 2. *Effect of raffinose concentration on sporulation and enterotoxin production by NCTC 10240*

% Raffinose	% Sporulation	Heat-resistant spores/ml	Enterotoxin concn (μg/mg of cell extract protein)
0	86	$2.2 \times 10^3 \pm 0.5$	29.0 ± 1.3
0.20	90	$1.5 \times 10^6 \pm 1.0$	190 ± 12.0
0.30	90	$1.5 \times 10^7 \pm 0.1$	320 ± 10.0
0.40	89	$2.6 \times 10^7 \pm 1.0$	300 ± 20.0
0.50	87	$1.1 \times 10^7 \pm 0.7$	320 ± 20.0

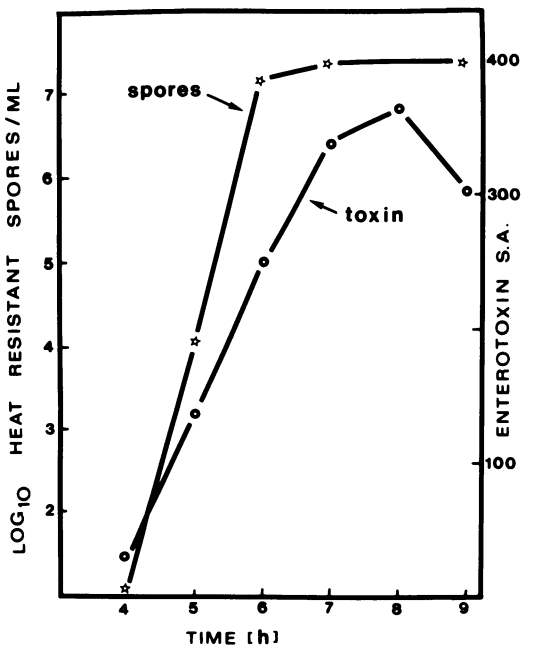

FIG. 1. *Time course of production of heat-resistant spores and enterotoxin by C. perfringens type A strain 10240 in DS medium with 0.4% raffinose in place of starch.*

Identification of enterotoxin by disc gel immunodiffusion. As mentioned above, the level of enterotoxin concentration produced by *C. perfringens* in DS medium was dependent on the strain and carbohydrate employed. To assure that the enterotoxin produced by each of three strains was serologically similar, the cell extracts were subjected to disc gel immunodiffusion. An example of the results obtained (strain 10240, raffinose) is shown in Fig. 2. In this instance the precipitin arc corresponded to an R_m (relative mobility) of enterotoxin of 0.54. Similar experiments with three different strains and two different carbohydrates yielded R_m values of 0.55 ± 0.1 in each case (data not shown). This suggests that the enterotoxin produced by these strains in the presence of raffinose or starch was serologically similar.

DISCUSSION

Sporulation has been the nemesis of many who have worked with *C. perfringens*. No one sporulation medium is satisfactory for all strains, although DS medium (5) has been more widely accepted than any other. Results reported here show that replacing the starch component of DS medium with raffinose increases sporulation and enterotoxin production of some strains. The reason that raffinose improves sporulation of certain strains is not known. It is possible that the rate of extracellular hydrolysis of raffinose and starch to release readily fermentable carbohydrates plays an important role. Periodic additions of low levels of glucose or maltose were shown to promote high numbers of heat-resistant spores in DS medium which lacked starch (11). Conceivably the breakdown of raffinose is

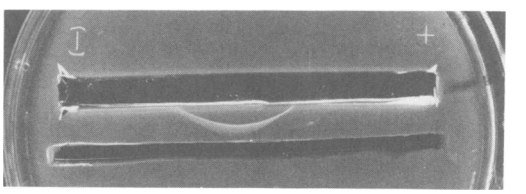

FIG. 2. *Acrylamide disc gel immunodiffusion of cell extract from C. perfringens type A strain 10240, grown in DS medium with raffinose in place of starch. The arc corresponds to a relative mobility of 0.54.*

at a rate more favorable for the sporulation process compared with the rate of starch breakdown.

We noticed that the use of raffinose resulted in large amounts of gas. This could interfere with continuous-flow centrifugation procedures if large spore crops were desired. However, we found that simple agitation of the contents of the flasks before harvesting caused most of the gas to evolve.

A rather important consideration in obtaining a high percentage of sporulation was the degree of vegetative cell growth in FTG medium. Poor growth in the latter usually resulted in poor sporulation in DS medium. Presumably this was due to transfer of unutilized glucose from the FTG to DS medium. Glucose represses sporulation of *C. perfringens* in DS medium (Labbe and Duncan, unpublished data). This inhibitory effect could be minimized by assuring that growth had occurred throughout the FTG tube before transfer (visual inspection). Also helpful in this regard was the use of FTG medium prepared less than 1 week in advance.

One of the most surprising results of this study was the high level of enterotoxin produced by strain NCTC 10240 with raffinose as the carbohydrate. The strain of choice for enterotoxin production over the years has been NCTC 8239 (9, 20–22, 24). Expressing enterotoxin concentration as per milliliter of cell extract, Nilo (19) reported that strain NCTC 8239 produced more enterotoxin than any of 15 strains tested. DS medium (starch) was used as the sporulation medium. Our results, however, suggest that with raffinose as the carbohydrate strain NCTC 10240 is the better strain for enterotoxin production in DS medium. A level of 0.4% was found to be best for both enterotoxin formation and production of heat-resistant spores. Based on its migration in polyacrylamide gels, the enterotoxin produced by NCTC 10240 seems serologically identical to that produced by NCTC 8239. Thus, the former seems to be the strain of choice for those workers interested in a high level of enterotoxin, although not necessarily for the production of high numbers of heat-resistant spores. However, we have found that the high percentage of sporulating cells produced by this strain facilitates cleaning of spores. Such consideration may override the use of other media or strains when large numbers of clean spores are the objective.

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