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Influence of Carbohydrates on Growth and Sporulation of *Clostridium perfringens* Type A

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Growth and sporulation of *Clostridium perfringens* type A in Duncan and Strong (DS) sporulation medium was investigated. A biphasic growth response was found to be dependent on starch concentration. Maximal levels of heat-resistant spores were formed at a starch concentration of 0.40%. Addition of glucose, maltose, or maltotriose to a sporulating culture resulted in an immediate turbidity increase, indicating that biphasic growth in DS medium may be due to such starch degradation products. Amylose and, to a lesser extent, amylopectin resulted in biphasic growth when each replaced starch in the sporulation medium. A level of heat-resistant spores approximately equal to the control was produced with amylopectin but not amylose as the added carbohydrate. Addition of glucose or maltose to a DS medium without starch at stage II or III of sporulation did not alter the level of heat-resistant spores as compared with the level obtained in DS medium with starch. Omission of starch or glucose or maltose resulted in an approximately 100-fold decrease in the number of heat-resistant spores, although the percentage of sporulation (90%) was unaffected. The role of starch and amylopectin in the formation of heat-resistant spores probably involves the amylolytic production of utilizable short-chain glucose polymers that provide an energy source for the completion of sporulation.

In the course of a recent investigation of the influence of pH and temperature on sporulation and enterotoxin production by *Clostridium perfringens* type A, a biphasic growth pattern was observed. The secondary turbidity increase occurred 11 or 12 h after inoculation of Duncan and Strong (DS) sporulation medium either when the pH was controlled at 7.0 or not externally controlled by addition of NaOH (4). In spite of the renewed growth, no increase in the number of heat-resistant spores after 6 h was noted. Microscopic examination showed that vegetative cells were responsible for the turbidity increase. In conjunction with the release of free spores, which occurred at 7 to 8 h, the net affect was a drastic reduction in the specific activity of intracellular enterotoxin protein. In this report we attempt to elucidate the factors involved in this biphasic growth and their relationship to the sporulation process.

MATERIALS AND METHODS

General. *C. perfringens* NCTC 8798 (Hobbs serotype 9) was used throughout. Cultural conditions, measurement of the levels of sporulation, heat-resistant spores, and turbidity were as previously described (4) except that 1 liter of DS sporulation medium was used and the incubation temperature was 40 C. No attempt was made to externally control the pH. Total cell counts were determined by using a Petroff-Hauser counting chamber. Vegetative cell numbers were obtained by multiplying the total cell counts by the percentage of the nonsporulating population.

Chemicals. Amylose was purchased from Sigma Chemical Co., maltotriose and amylopectin from Calbiochem, and amylase from Worthington Biochemical Corp. One unit of amylase activity is the amount that liberates from starch 1 mmol of reducing groups, calculated as maltose, per minute at 25 C.

Starch-iodine reaction. The levels of starch were determined as follows. A stock iodine solution was composed of 5 g of KI and 0.5 g of I_2. Five milliliters of culture was centrifuged at 27,000 × *g* for 10 min. To 0.5 ml of the supernatant fluid was added 5 ml of the diluted (1:10) stock iodine solution. The optical density (620 nm) of the resulting blue solution was immediately read by using a Perkin-Elmer double-beam spectrophotometer.

RESULTS

Growth of NCTC 8798 in DS sporulation medium. When an 11- to 13-h fluid thioglycolate culture of NCTC 8798 was inoculated into DS sporulation medium, vegetative cell multiplication occurred for 2 h (Fig. 1). Vegetative cell numbers are actually estimates since the earliest stage of sporulation that could be de-

tected by light microscopy was stage II. By 4 h the number of vegetative cells decreased concomitant with the onset of sporulation. The vegetative cell level remained approximately constant for the next 4 h, while most (about 90%) of the population sporulated. After approximately 8 h vegetative cell multiplication resumed, resulting in the characteristic biphasic pattern. An increase in Klett units was not seen until 2 h later because sporangial lysis also occurred at 8 h, as indicated by the initial appearance of spores free of their sporangium.

The level of heat-resistant spores increased between 3 and 4 h and usually remained stable for the next several hours. Little decrease in the starch-iodine reaction occurred until the onset of sporulation. The achroic point (point where starch and iodine no longer give a color reaction) was reached in 14 to 15 h.

Influence of starch on biphasic growth. The influence of starch concentration on growth of *C. perfringens* in DS sporulation medium is shown in Fig. 2. The concentration of starch routinely used in the sporulation medium is 0.4%. This formulation will subsequently be referred to as DS control. The plateau at 8 h and subsequent biphasic growth in control DS were also observed at a starch concentration of 0.3%, though the secondary turbidity increase was somewhat less pronounced. Nevertheless, the dependence of biphasic growth on starch concentration was evident. No biphasic pattern was observed at starch concentrations of 0.1 or 0.2% (not shown). Given such a concentration dependence, it seemed that the observed biphasic growth might be due to products of starch hydrolysis. During the first 2 h of incubation, there was little decrease in optical density of the starch-iodine reaction (Fig. 1). Energy for vegetative cell growth during this period probably came from proteose peptone and yeast extract. After commencement of sporulation (2 to 2.5 h), there was continued decrease in the iodine staining ability until about 15 h, just before the end of diauxic growth (Fig. 1). This suggested that the latter was due to a sudden increase in utilizable carbohydrates resulting from amylolysis.

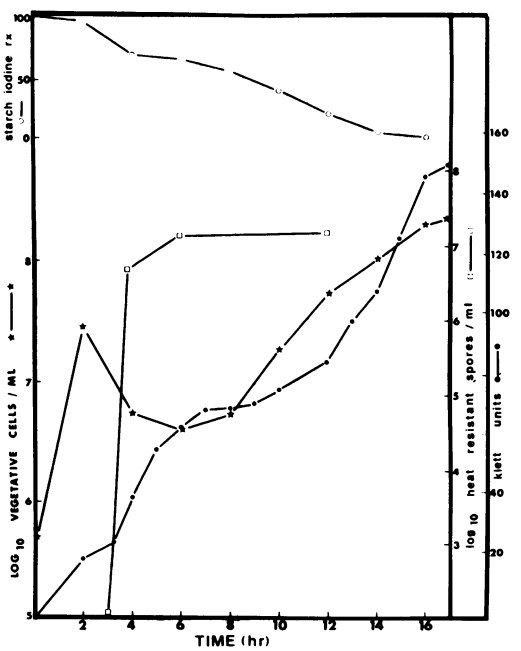

FIG. 1. *Turbidity, starch-iodine reaction, levels of vegetative, and heat-resistant spore population during growth of C. perfringens NCTC 8798 in DS medium. Values for starch-iodine reaction are percentage of original level.*

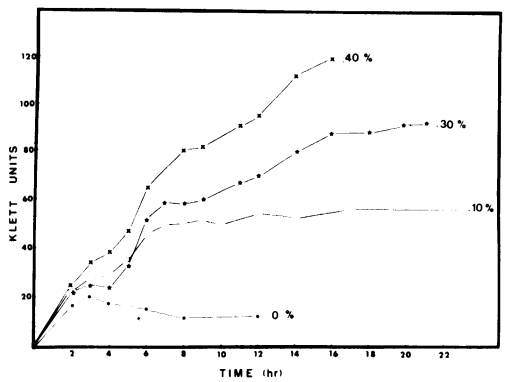

FIG. 2. *Effect of starch concentration on growth of C. perfringens NCTC 8798 in DS medium.*

If short-chain carbohydrates produced as a result of starch hydrolysis were responsible for biphasic growth at 10 to 11 h, then addition of maltose, for example, should also result in an immediate turbidity increase. This, in fact, did occur when maltose, glucose, or maltotriose was added at 7 h (Fig. 3a). Similar results were obtained when these carbohydrates were added at 4 h (Fig. 3b). However, subsequent biphasic growth also resulted, probably due to initial utilization of the added carbohydrates and subsequent starch hydrolysis. Further evidence for the involvement of amylase in the biphasic growth pattern is shown in Fig. 4. Addition of amylase to a 7-h culture resulted in an immediate decrease in the starch-iodine color reaction and concomitantly a dramatic turbidity increase.

An attempt was made to determine whether the spore population contributed (after possible

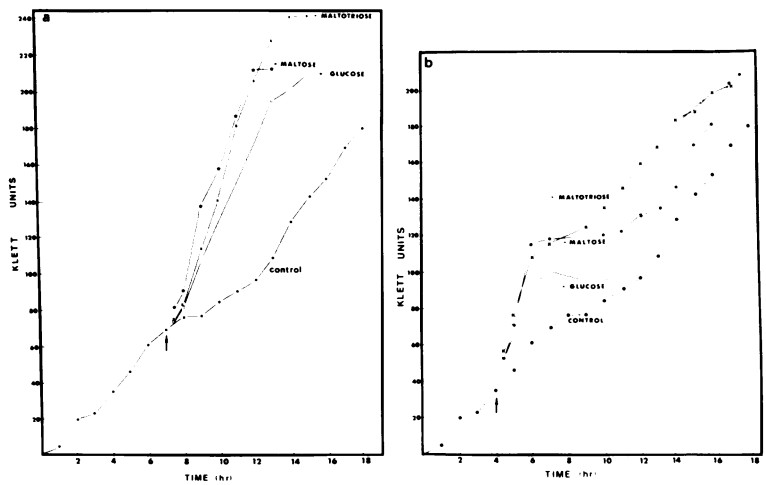

FIG. 3. *Effect of 7-h (a) or 4-h (b) addition (arrow) of 0.1% glucose, maltose, and maltotriose on growth of C. perfringens NCTC 8798 in DS medium.*

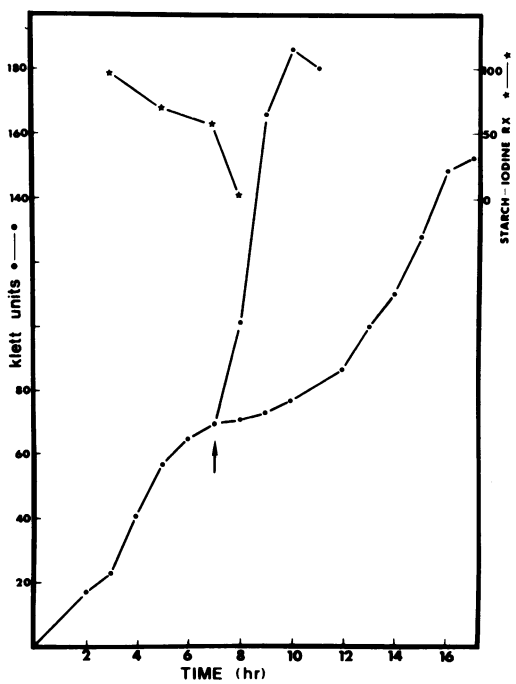

FIG. 4. *Effect of 7-h addition (arrow) of amylase (0.28 units/ml) on growth of C. perfringens NCTC 8798 in DS medium.*

germination and subsequent outgrowth) to the renewed vegetative growth characteristic of the secondary turbidity increase. Cleaned NCTC 8798 spores were incubated in the presence of 0.2% maltose. Germination, measured by a decrease in the optical density of the spore suspension, did not occur even if sterile culture filtrate from a 10-h sporulating culture was included. Thus it appears that germination and subsequent outgrowth of the spore population is not responsible for biphasic growth.

Influence of amylose and amylopectin on growth and sporulation. Since starch was responsible for biphasic growth in DS medium, we sought to determine which starch fraction, amylose or amylopectin, was the responsible component (Fig. 5). When each fraction replaced starch in the sporulation medium, two distinct results were obtained. Addition of both 0.8 and 1.6% amylose resulted in secondary turbidity increases whose onset was, respectively, 10 and 3 h later than that occurring in control DS. A concentration of 0.4% amylose resulted in a slight but noticeable turbidity increase between 19 and 41 h. Incorporation of 0.30% amylopectin in DS also yielded a slight secondary turbidity increase at 15 h. However, the extent of the latter (60 Klett units) was considerably less than that occurring in the control DS (about 150 Klett units). Addition of greater than 0.30% amylopectin into DS medium was precluded by solubility difficulties. Only a slight turbidity increase occurred between 24 and 36 h, when 0.075% amylopectin replaced starch in DS medium. Although 1.6% amylose used in the above experiments is four times the starch concentration in DS medium, the growth response of *C. perfringens* NCTC 8798 in the presence of this carbohydrate more nearly mimicked the biphasic response of this organism in control DS than did amylopectin.

To determine the function of starch during sporulation in DS, the effect of varying concentrations of soluble starch on sporulation was determined (Table 1). The level of sporulation

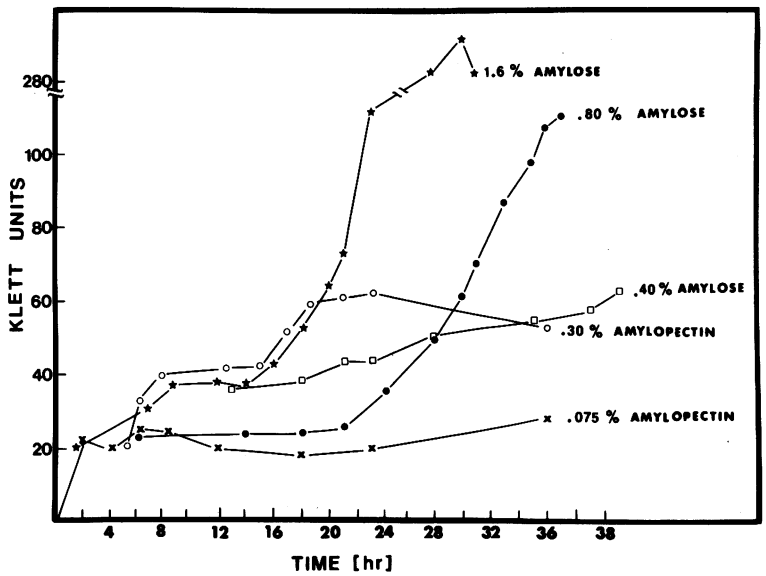

FIG. 5. *Growth of C. perfringens NCTC 8798 in DS medium without starch but with amylose or amylopectin. Turbidity during the first 4 h was approximately the same. For clarity, some points during this time are omitted.*

(80 to 90%) was quite high regardless of the level of starch. Up to 10^5 heat-resistant spores per ml was produced even if starch was omitted. However, most spores produced under these conditions were not fully refractile. The results in Table 1 do show that the formation of heat-resistant spores was dependent on starch concentration up to 0.4% added starch. Increasing the starch concentration to 0.6% failed to increase the heat-resistant spore level.

We attempted to determine whether the effect of starch on sporulation was due to the amylose or amylopectin fraction (Table 2). Although amylose increased the level of heat-resistant spores above that of DS without added carbohydrate (Table 1), the 12-h level was only about 2% of the control culture containing

TABLE 1. *Effect of starch concentrations on sporulation and formation of heat-resistant spores by C. perfringens NCTC 8798*

Time (h)	Spores/ml at starch concn of:			
	0	0.1%	0.2%	0.4%
3	<30	<30	<30	<30
4	3.3×10^8 (82)[a]	1.7×10^8 (90)	1.4×10^8 (90)	2.5×10^8 (89)
6	9.2×10^4	4.8×10^5	5.6×10^6	2.7×10^7
12	1.4×10^5	4.3×10^5	7.0×10^6	2.3×10^7

[a] Percentage of sporulation (in parentheses) included both mature heat-resistant and immature non-heat-resistant spores.

starch. However, the 4-h spore levels were the same. The formation of heat-resistant spores in the presence of amylopectin is, as in the case of starch, concentration dependent. Inclusion of 0.3% amylopectin in DS medium resulted in heat-resistant spore population levels almost equal to that of the controls. Percentage of sporulation was again quite high regardless of the added carbohydrates. Considering the 4-h level (80 to 90%) of sporulating cells in DS without starch, and considering that these were only at stage II or III (Table 3) of sporulation, the influence of starch, amylose, or amylopectin on the early sporulation process in this medium was minimal. An added carbohydrate in this medium became important after sporulating cells had reached stage II or III. This was not an absolute requirement, however, since about 10^5 spores per ml of culture (between 0.1 to 1% of the control level) was formed without starch, amylose, or amylopectin. At 6 h about 98% of sporulating cells in DS control had proceeded beyond stage III as compared with 72% of sporulating cells in DS without starch (Table 3). Since there was a greater than 2-log difference in the level of heat-resistant spores even at 12 h (Table 1), it is obvious that a substantial number of sporulating cells that proceeded beyond stage III in DS without starch did not become heat resistant.

Effect of periodic glucose or maltose addition on sporulation in DS medium without starch. One likely role of starch would involve

TABLE 2. *Effect of amylose and amylopectin on sporulation and the formation of heat-resistant spores by C. perfringens NCTC 8798*

Time (h)	Spores/ml at given starch concn						
	Amylose			Amylopectin			
	0.8%	1.6%	Control	0.075%	0.15%	0.3%	Control
3	<30	<30	<30	<30	<30	<30	<30
4	1.1×10^8 (88)[a]	1.0×10^8 (85)	1.3×10^8	—[b] (87)	— (91)	7.5×10^8 (89)	7.2×10^8 (88)
6	3.8×10^8	4.1×10^8	1.1×10^7	1.8×10^8	2.7×10^7	9.0×10^6	1.2×10^7
12	3.3×10^8	3.4×10^8	1.5×10^7	9.4×10^8	1.0×10^6	8.2×10^6	9.1×10^6

[a] Percentage of sporulation (in parentheses) included both mature heat-resistant and immature non-heat-resistant spores.
[b] —, Not determined.

TABLE 3. *Sporulation in DS medium and DS medium without starch*

Medium	Sporulation (%)			% at stage II or III			Heat-resistant spores/ml[a]		
	3 h	4 h	6 h	3 h	4 h	6 h	3 h	4 h	6 h
DS control	75	84	92	75	77	2	<30	2.5×10^8	2.7×10^7
DS without starch	74	83	87	74	81	25	<30	3.3×10^8	9.2×10^4

[a] Data reproduced from Table 1.

TABLE 4. *Formation of heat-resistant spores in DS medium without starch by C. perfringens NCTC 8798 by periodic[a] addition of glucose or maltose*

Time (h)	DS without starch	DS without starch plus glucose	DS without starch plus maltose	Control
3	<30[b]	<30 (72)[c]	<30	<30
4	—[d]	1.6×10^8	1.3×10^8	1.1×10^8
6	1.2×10^4	2.1×10^7 (88)	2.9×10^7	2.2×10^7
12	2.2×10^4	1.8×10^7	2.6×10^7	1.7×10^7

[a] Glucose or maltose added at 2.5 h and replenished at 0.5-h intervals from 3 through 5.5 h after inoculation of the sporulation medium.
[b] Spores per milliliter.
[c] Percentage of sporulation in parentheses.
[d] —, Not determined.

the amylolytic production of utilizable carbohydrates that could be used for energy during completion of the sporulation cycle. To test this possibility, 0.01% glucose or 0.01% maltose was added 2.5 h after inoculation of DS medium without starch and replenished at 0.5-h intervals thru 5.5 h (Table 4). A number of heat-resistant spores essentially equivalent to the control was produced under these conditions. Since there was no decrease in percentage of sporulation between 3 and 6 h, the added carbohydrates were apparently preferentially utilized by the sporulating population.

DISCUSSION

When *C. perfringens* NCTC 8798 is grown in DS sporulation medium, a biphasic growth response is observed. Results reported here indicate that this is due to the production of utilizable carbohydrates resulting from amylolytic reactions. Amylase is known to be produced by bacilli and clostridia at the end of exponential growth (9). The almost negligible decrease in the starch-iodine reaction during the first 2 h when vegetative cell multiplication occurred also implicates the involvement of amylase at the end of exponential growth. The appearance of new energy sources resulting from amylolysis allows the nonsporulating population to renew vegetative cell division. The dependence of heat-resistant spore levels on starch concentration suggests that starch also plays a role in the sporulation process.

Data reported here suggest that biphasic growth and sporulation are separate functions of the two starch components amylose and amylopectin. The rates of hydrolysis of these two components probably determine their role in the two processes. Conceivably, early fission products of amylose are oligosaccharides that are only slowly hydrolyzed to metabolizable G_1, G_2, or G_3 units (glucose, maltose, or maltotriose). Such a mechanism of action of amylase on amylose has been proposed for some time (1). Since the 4-h level of heat-resistant spores in

the presence of amylose was equal to the control, early hydrolysis products of amylose may also include a limited amount of G_1, G_2, or G_3 units. With regard to amylopectin, such short-chain glucose polymers could be early and major amylolytic products that could provide an energy source for the completion of sporulation. Differences in early hydrolysis products are likely related to differences in molecular structure between amylose and amylopectin. One (amylose) is a straight-chain polymer of glucose units joined α-1,4. The other (amylopectin) is a polymer of glucose units linked α-1,4, but with branch points linked α-1,6. In the case of both carbohydrates, resistant oligomers could be further hydrolyzed to G_1, G_2, or G_3 units at a time corresponding to the onset of biphasic growth. Such a two-phase reaction occurs in the case of malt alpha-amylase hydrolysis of amylose and corn starch (5) and by the action of *Bacillus subtilis* alpha-amylase on amylose and amylopectin (8). In the latter case early fission products of amylose and amylopectin included G_2 or G_3 units. The increased level of heat-resistant spores using amylopectin as the carbohydrate source and the similar effects of maltose when added early during the sporulation process indicates that hydrolysis of the amylopectin moiety of starch releases G_1, G_2, or G_3 units at about stage III of sporulation of *C. perfringens* in DS medium. Furthermore, since the replacement of starch with 0.4% amylose resulted in only minor biphasic growth, amylopectin probably also functions in this phenomenon.

Attempts to demonstrate the levels of glucose or reducing sugars were only partially successful due to the complex nature of the sporulation medium, which gave irreproducible results, high blank values, or blocked color reactions of such assays.

A complicating factor in these experiments is that the starch we used had been commercially solubilized by hydrolysis with dilute acid (soluble starch). This could account for the longer lag before biphasic growth when pure amylose was the substrate. Nevertheless, we conclude that energy for the completion of sporulation is derived mainly from amylopectin but that both amylopectin and amylose contribute to a secondary turbidity increase, which probably results from the slow amylolysis of oligomers produced during early starch hydrolysis.

Although little work has been done on detailed action patterns of bacterial amylases, it is known that the modes of attack of plant alpha-amylases depend on the source of the enzyme and substrate. Clearly a more detailed account of the action of *C. perfringens* amylase, including its designation as alpha or beta, await its purification.

Both carbohydrates from yeast extract and the utilization of proteose peptone may provide energy for the brief vegetative growth phase in DS medium. Exhaustion of either energy source would result in the end of vegetative growth and signal the onset of sporulation and production of amylase. As mentioned above, the latter would result in G_1 to G_3 units for sporulation beyond stage III. Energy for stages I to III could come from amino acid utilization via the Stickland reaction (6) or utilization or organic acid intermediates produced during vegetative growth. The latter mechanism has been reported to occur during sporulation of clostridia (2, 7) and bacilli (3). However, in a recent report (4) we failed to detect an increase in pH, which is characteristic of secondary metabolism of such compounds. The requirements for an exogenous energy source during sporulation by this organism are in sharp contrast to the aerobic bacilli, in which endotropic sporulation can occur.

We conclude that the function of starch and amylopectin and, to a lesser extent, amylose is to provide utilizable carbohydrates so that the majority of the sporulating fraction can become heat resistant.

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