APPLIED MICROBIOLOGY, Sept. 1972, p. 341-348
Vol. 24, No. 3

Bacillus cereus-Induced Fluid Accumulation in Rabbit Ileal Loops

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Received for publication 27 March 1972

The usefulness of the ligated rabbit ileal loop as an experimental model of *Bacillus cereus* food poisoning was investigated. Positive responses, as measured by fluid accumulation in the loop, were obtained from 19 of 22 strains of *B. cereus*. Four of six strains of *B. thuringiensis* also elicited fluid accumulation, but eight strains of other *Bacillus* spp. failed to evoke a response. The growth medium employed markedly affected the ability of a given strain of *B. cereus* to provoke a response. Brain heart infusion broth (BHI) (Difco) proved to be best for this purpose. Loop fluid-inducing activity was produced by exponentially growing cells and was present in cell-free culture filtrates and associated with washed vegetative cells. Intraluminal growth of *B. cereus* did not elicit fluid accumulation. Cultures grown at temperatures in the range of 18 C to 43 C were loop active. When BHI cultures of selected loop positive strains were injected intraluminally into the normal ileum of rabbits, they failed to elicit diarrhea.

Bacillus cereus has been recognized as a causative agent of food poisoning for over 20 years. For the most part, reports describing outbreaks of *B. cereus* poisoning have appeared in the European literature. It seems safe to say that the magnitude of the problem of *B. cereus* poisoning in the United States is unknown.

Even less is known about the mechanism of pathogenicity of these organisms. This has been due primarily to the lack of a suitable model in which to investigate this aspect. In the past, rodents challenged per os with cultures of *B. cereus* did not exhibit any unusual responses (8, 20). Cats (17, 18) and dogs (8, 13, 19) have suffered severe diarrhea after consuming food contaminated with large populations of *B. cereus*. For obvious reasons, this is not a practical method of determining or investigating pathogenicity.

The ligated loop of the rabbit small intestine has been used successfully in the study of various enteropathogenic bacteria. The injection of whole cultures (4), culture supernatant fluids (5), and cell extracts (3) of *Vibrio cholerae* caused dilation of the loop due to fluid accumulation. This investigative technique has also been used to study the enteropathogenicity of *Escherichia coli* (11, 15, 21), salmonellae and shigellae (24), *Pseudomonas aeruginosa* (12), *Clostridium perfringens* (6, 7), and, more recently, the nonagglutinable vibrios (*V. parahemolyticus* and *V. alcaligenes*) (1). We initiated the present study to evaluate the adaptability of the rabbit ileal loop test to the study of *B. cereus* food poisoning.

MATERIALS AND METHODS

Cultures. The strains of *B. cereus* and other *Bacillus* spp. used in this study are listed with their source in Table 1.

Stock cultures maintained on nutrient agar slants at room temperature were subcultured in Bacto-nutrient broth (Difco) for 12 hr at 32 C. A culture to be used for ileal loop challenge was prepared by adding 0.2 ml of this nutrient broth culture to a 125-ml Erlenmeyer flask containing 20 ml of the appropriate medium. The standard conditions for incubation were 32 C for 12 hr on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 84 cycles/min.

Spores of *B. cereus* were obtained from cultures that were grown in G medium (9) for 5 days at 32 C on a reciprocal shaker. The spores were harvested and washed twice with cold physiological saline and resuspended in saline or sterile brain heart infusion (BHI) broth (Difco) at 4 C. Heat shocking was carried out by heating spore suspensions at 75 C for 10 min.

Surgical procedure. New Zealand white rabbits

TABLE 1. *Sources of Bacillus spp.*

Strain	Source	Comments
B. cereus		
B-2ac, B-4ac, B-5ac, B-6ac, B-7ac, B-9ac	D.A.A. Mossel, Louvain, Belgium	Isolated from food poisoning outbreaks
F-42, F-66, F-77	C. V. Hall, Public Health Lab, Seattle, Wash.	Isolated from food poisoning outbreaks
5065	T. Midura, State Department Public Health, Berkeley, Calif.	Isolated from food poisoning outbreak
T	R. S. Hanson, Univ. of Wis., Madison, Wis.	
USDA 201	W. B. Sarles, Univ. of Wis., Madison, Wis.	
2280	G. W. Gould, Unilever Research, Bedford, England	Received as lecithinase negative strain
1979	G. W. Gould, Unilever Research, Bedford, England	
331	G. W. Gould, Unilever Research, Bedford, England	Asporogenic mutant
39 benz, D_1	U. de Barjac, Institut Pasteur, Paris, France	Received as lecithinase negative strains
A-1, A-13-1, D-1, A-16-4, F-19-2, H-4	Food Research Institute, Madison, Wis.	Isolated from food products
B. thuringiensis ATCC 10792	W. B. Sarles, Univ. of Wis., Madison, Wis.	
B. megaterium USDA 234	W. B. Sarles, Univ. of Wis., Madison, Wis.	
B. subtilis WBS 1958	W. B. Sarles, Univ. of Wis., Madison, Wis.	
B. licheniformis V-37	E. Kampelmacher, Utrecht, The Netherlands	Isolated from food poisoning outbreak
Other *Bacillus* spp.		
A-8s, B-15s2, D-12s1, G-13s2	Food Research Institute, Madison, Wis.	Possible *B. licheniformis*—isolated from foods
G-13L	Food Research Institute, Madison, Wis.	Member of *B. polymyxa-macerans* groups—isolated from food

were acclimatized to the laboratory for at least 1 week before testing. They were maintained on commercially available rabbit pellets. Water was supplied ad libitum at all times, but the rabbits were fasted for 48 hr prior to externalizing the ileum.

The surgical procedure was basically the same as described by Duncan, Sugiyama, and Strong (7). Segments of the ileum were tied off to form six test loops each approximately 10 cm long, separated from one another by 5-cm blank loops.

Intraluminal injections of 2 ml of test material were made into each of the six test loops. In each rabbit, one loop was injected with sterile medium as a control. After the ileum was returned to the abdominal cavity, the incision was closed. Following recovery from anaesthesia, the rabbit was provided with water but not food.

Postmortem examination. After the holding period, test animals were killed and opened immediately for examination. The gross appearance of the loops was noted, and, if either the control loop or any of the blank loops contained fluid, all tests in that rabbit were considered invalid. The length and fluid volume of each test loop was measured. To compensate for variations in the length of the test loops, the ratio of the fluid volume (milliliters) to the loop length (centimeters) was employed (3).

To enumerate *B. cereus*, each test loop and its contents were transferred to a test tube and the volume was adjusted to 10 ml by the addition of sterile 0.1% peptone-water. If the fluid volume was greater than 10 ml, no peptone-water was added. The sample was then agitated on a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.) for

1.5 min. Viable cell counts in these samples were determined by surface plating appropriate dilutions (made in 0.1% peptone-water) on duplicate plates of nutrient agar. Spore counts were obtained by heating samples for 15 min at 75 C before plating on nutrient agar. Plates were incubated at 32 C for 24 hr before enumerating the colonies.

RESULTS

Standardization of the rabbit ileal loop test. A positive loop response consisted of the accumulation of 3 to 20 ml of straw-colored, often bloody fluid. Thus, the gross appearance of the loops resembled that reported for *C. perfringens* (7), *V. cholerae* (4), and *E. coli* (21). But unlike the case with *C. perfringens* (7), distension of the loop due to gas formation was not observed in any trials. Though the amount of fluid elicited by a given strain varied from one test to another, the average fluid volume to loop length ratio from three tests proved to be a reasonably accurate indication of the fluid-inducing ability of a particular strain. This variation in individual responses was also noted for *V. cholerae* by Burrows and Musteikis (3), who reported that the average of at least four tests was relatively constant.

Early in the course of this study we noted that the loop response in older (larger) rabbits was sporadic and rarely reproducible. In contrast, the loop response in smaller rabbits was consistent. To determine if the age of test animals was a factor in the observed disparity of response, animals from 4 to 16 weeks old, weighing from 800 to 2,500 g were challenged with 12-hr BHI or skim milk (SM) cultures of B-4ac. These challenge inocula were chosen because they had previously elicited consistent fluid accumulation in young rabbits and occasional positive responses in older animals. In virtually every test, animals weighing less than 1,200 g gave consistently positive responses, but results with larger rabbits given the same challenge doses were sporadic or negative. The age of the test animal was thus an important consideration and all subsequent work was done with rabbits eight weeks old or younger, weighing less than 1 kg. Similar results were obtained by Moon and Whipp (14) working with *E. coli* in piglets. The age of the piglets was an important factor since some strains of *E. coli* were able to elicit a loop response in pigs of all ages whereas others were positive in pigs less than 2 weeks old but not over 6 weeks old. It would appear that these test animals are able to develop resistance as they grow older.

Duncan, Sugiyama, and Strong (7) reported that the culture medium influenced the ability of *C. perfringens* to elicit fluid accumulation. The influence of growth medium on the fluid-inducing activity of *B. cereus* B-4ac was investigated. This strain of *B. cereus* is lecithinase positive and hemolytic and was isolated from a food poisoning outbreak.

The culture was grown under standard conditions in BHI, SM, Trypticase soy broth (TSB), nutrient broth (NB), and beef infusion (BI) prepared from fresh beef. B-4ac grew well, but sporulated (i.e., formed heat-resistant spores) poorly in all media tested. Ileal loop responses obtained with B-4ac grown in the various media are shown in Table 2. Only cultures grown in NB failed to evoke a loop response. Because growth in BHI engendered the greatest loop response, it was chosen as the standard growth medium for subsequent investigations.

Taylor, Maltby, and Paine (23) reported that ileal loops nearer the stomach were more sensitive to *E. coli* enterotoxin than those toward the colon. In our investigations, variations in loop response were not significantly affected by the position of the test loop. When challenged with cultures of *B. cereus*, the loops nearest the colon were somewhat more sensitive than those nearer the stomach. But the maximum difference in the average indexes of fluid accumulation (fluid volume to loop length ratio) was only 0.13. Since the position of the loop used for a given strain was deliberately varied each time, even this small bias

TABLE 2. *Effect of growth medium on fluid accumulation response to cultures of Bacillus cereus B-4ac*

Medium	Total viable organisms ($\times 10^{-9}$)	Challenge spores[a, b] ($\times 10^{-2}$)	Number of tests	Average fluid volume to length ratio[b]
Fresh beef infusion	1.0	0.4	4	0.46
Brain heart infusion	0.72	0.2	5	0.97
Trypticase soy broth	0.40	0.4	2	0.60
Skim milk	0.14	10.0	4	0.45
Nutrient broth	0.17	0.6	5	Neg.

[a] Two milliliters of 12-hr cultures grown at 32 C injected per loop.

[b] The variation between media was greater than the variation between tests employing the same medium ($P = 0.05$).

was negated.

Originally, we had intended to hold test animals for 24 hr following challenge before examining the ileal loops for fluid accumulation. However, most of the rabbits in which one or more of the test loops were strongly positive died within 10 hr. The nature of this lethal activity has not yet been fully investigated, but its presence necessitated a markedly shorter holding time. Since no rabbits had died during the first 7 hr, this was selected as the standard holding period. The amount of fluid accumulated in loops injected with 12-hr BHI cultures of *B. cereus* was measured at various times during these 7 hr (Fig. 1). The accumulation of fluid in loops challenged with cultures of 331 and B-4ac was relatively linear with respect to time but that for strain 5065 was somewhat inconsistent. However, the data presented demonstrate a sufficient development and differentiation of loop responses to the various challenge inocula to support the choice of a 7-hr holding period.

The time-course of the appearance of fluid in ileal loops injected with *B. cereus* is extremely rapid compared to that achieved with *C. perfringens* (7). After 7 hr, strain 331 elicited over twice as much fluid accumulation as did the most active strain of *C. perfringens* (NCTC 8798) tested (7). Rabbits used in both studies were of the same breed, were obtained from the same colony, and were maintained under similar conditions, so a difference in the sensitivity of test animals is not likely. Unfortunately, the amount of fluid elicited in rabbits held for 24 hr (the standard holding period in studies involving *C. perfringens*) cannot be compared until loop fluid-inducing activity is separated from lethal activity in cultures of *B. cereus*.

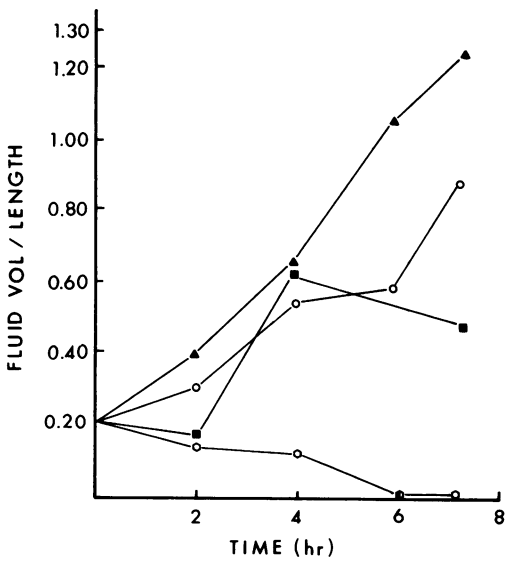

FIG. 1. *Fluid accumulation in rabbit ileal loops injected with Bacillus cereus. Cultures were incubated 12 hr at 32 C on a reciprocal shaker. A 2-ml amount was injected into each test loop. Each point is the average of results from two to four different rabbits. Symbols: Sterile brain heart infusion (BHI)* (○——○), *strain B-4ac* (○——○), *strain 331* (▲——▲), *and strain 5065* (■——■).

Animal response to various strains of B. cereus. The results of a survey of rabbit ileal loop response to BHI cultures of *B. cereus* and other *Bacillus* spp. grown under standard conditions are presented in Table 3. One loop in each test animal was injected with 2 ml of a BHI culture of B-4ac as a positive control. Only one rabbit failed to respond to the positive control and was discarded.

Nineteen of 22 strains of *B. cereus* were capable of eliciting a positive loop response. In contrast, only one of nine members of other *Bacillus* spp. elicited fluid accumulation. The

TABLE 3. *Response of rabbit ileal loop to challenge by cultures of various Bacillus spp.*[a]

Strains of *Bacillus cereus*	Fluid volume to length[b]	Strains of other *Bacillus* spp.	Fluid volume to length[b]
39 benz	1.39	ATCC 10792	0.79
331	1.22	USDA 234	Neg.
F-42[c]	1.00	WBS 1958	Neg.
A-1	0.99	V-37[c]	Neg.
B-6ac[c]	0.94	G-13L	Neg.
B-4ac[c]	0.91	G-13s2	Neg.
USDA 201	0.88	D-12sl	Neg.
F-77[c]	0.75	D-15s2	Neg.
F-66[c]	0.68	A-8s	Neg.
F-19-2	0.62		
B-5ac[c]	0.62		
A-16-4	0.60		
1979	0.57		
B-2ac	0.53		
D_1	0.51		
A-13-1	0.47		
T	0.44		
H-4	0.32		
2280	Neg.		
B-9ac[c]	Neg.		
D-1	Neg.		

[a] Two milliliters of 12-hr brain heart infusion (BHI) cultures grown in 32 C injected per loop.

[b] Average of tests in at least three different rabbits.

[c] Strains isolated from food poisoning outbreaks.

exception, *B. thuringiensis* ATCC 10792, closely resembles *B. cereus* and has, in fact, been considered a variety of it by some investigators (2, 22). In a later survey of five additional strains of *B. thuringiensis*, three proved capable of eliciting fluid accumulation.

Strains which failed to elicit fluid accumulation when grown in BHI were grown under the same conditions in SM, BI, and TSB and were tested again. All such retests were negative.

Attempts were then made to induce diarrhea in rabbits by administering strain B-4ac by various routes. Quantities (2 to 20 ml) of 12-hr BHI cultures of B-4ac were injected into the ileum of unfasted 6-week-old rabbits. The standard surgical procedure was followed, except that no ligatures were made and the intestinal contents were not washed down with saline. The ileum was injected at a point 90 cm anterior to the meso-appendix. Test animals were allowed food and water following the operation. Diarrhea did not occur in any of the rabbits.

Unfasted 6-week-old rabbits were also challenged by stomach catheter with 10 to 20 ml of 12-hr BHI cultures of strains B-4ac, 331, 39 benz, and A-1. Test animals were allowed food and water after challenge. This procedure also failed to elicit diarrhea.

Correlation of numbers of organisms with loop response. Plate counts made on nutrient agar proved to be a completely satisfactory means of enumerating the strains surveyed. Comparative platings made on Trypticase soy agar (BBL) and on egg yolk–polymixin agar (16), a differential and semiselective medium for *B. cereus*, gave results substantially identical to those obtained when nutrient agar was used. Contamination by the normal intestinal flora was not a problem. Total numbers of *B. cereus* in uninjected loops and in loops injected with sterile BHI never exceeded one million.

Among the strains of *Bacillus* surveyed, neither the number of cells in the challenge dose nor the development of spores in test loops had a significant effect on loop response (Table 4). However, the relationship between the increase in cell numbers in a test loop and the loop response proved to be significant at the 95% confidence level. In virtually every individual test in which the ligated loop failed to respond, the total count of *B. cereus* in the loop at the postmortem examination was lower than the number of organisms present in the challenge dose.

Cell-free supernatant fluids obtained from 12-hr BHI cultures of B-4ac elicited the accumulation of a large volume of fluid in the ileal loop (Table 5). Presumably, most of the fluid elicited in a positive loop response was a result of this activity. In addition, some fluid-inducing activity was also associated with washed vegetative cells. But the initial number of washed cells necessary to provoke a loop response equivalent to that obtained with cell-free supernatants was so high (9.0×10^9/ml) that the possibility of further growth within the test loop was precluded. Therefore, it appears that the increase in cell numbers in positive test loops is an effect rather than a cause of fluid accumulation.

In order to demonstrate directly that intraluminal growth of B-4ac played no role in eliciting fluid accumulation, cells were grown in BHI and in NB. After 12 hr, the cells were harvested by centrifugation and washed twice with physiological saline. Both the NB- and BHI-grown cells were resuspended in fresh BHI at a level of 3.0×10^7/ml and injected into ileal loops. A fivefold increase in cell numbers was observed in both test loops at the postmortem examination. However, even though cells from both sources grew well within their respective test loops, no fluid was present in the loop containing the NB-derived cells, whereas that containing the BHI-derived cells registered a fluid volume to loop length ratio of 0.25.

An attempt was made to elicit fluid accumulation by the administration of free spores of strain B-4ac. Spores were prepared as described in Materials and Methods and were resuspended in both physiological saline and BHI at levels ranging from 8.6×10^8 to 1.6×10^{10}/ml. Although both unheated and heat-shocked spores were administered, no fluid accumulation was elicited in any trials.

The loop activity of 12-hr BHI cultures of B-4ac was consistently high (i.e., fluid volume to loop length ratio of 0.8 to 1.1) when the cells were grown at temperatures between 25 C and 41 C and were demonstrable, though reduced, at 19 C and 43 C. Cultures incubated at 45 C were tested after 4, 6, 9, 12, 15, and 24 hr. All such tests were negative. Cultures tested at 18 C were also negative when tested at 12 hr, but when incubated for 24 hr a quantitative loop response (fluid volume to loop length ratio) of 0.64 could be demonstrated. Lower temperatures were not tested.

Loop fluid-inducing activity in BHI cultures of B-4ac could first be demonstrated about halfway through logarithmic growth phase

TABLE 4. *Total cell and spore counts in ileal loops challenged with cultures of various Bacillus spp.*[a]

Strain	Challenge[b]		Final		Fluid volume to length ratio
	Total ($\times 10^{-9}$)	Spores ($\times 10^{-4}$)	Total ($\times 10^{-9}$)	Spores ($\times 10^{-4}$)	
B. cereus					
B-2ac	1.9	17.0	5.0	19.0	0.53
B-4ac	1.4	0.24	3.4	0.99	0.91
B-5ac	0.97	1.9	3.0	4.2	0.62
B-6ac	1.0	29.0	2.4	34.0	0.94
B-7ac	1.0	1.7	4.4	5.2	1.00
F-77	0.88	2.3	1.3	6.0	0.75
F-66	0.83	25.0	1.2	28.0	0.68
F-42	0.65	3.2	1.9	2.8	1.04
A-1	2.0	5.2	1.8	5.6	0.99
A-13-1	0.71	0.97	0.83	37.0	0.47
A-16-4	1.3	39.0	0.98	15.0	0.60
F-19-2	1.2	4.0	1.6	4.4	0.62
H-4	1.5	2.2	2.3	14.0	0.32
T	1.0	6.7	1.5	34.0	0.44
USDA 201	1.9	0.24	6.5	0.53	0.88
331	1.0	0.0	2.3	0.0	1.22
1979	1.1	16.0	1.3	10.0	0.57
D_1	0.80	15.0	1.0	14.0	0.51
39 benz	0.83	3.7	3.3	7.5	1.39
B. thuringiensis					
ATCC 10792	1.4	0.80	4.4	0.1	0.79
2280	0.59	47.0	0.27	34.0	Neg.
D-1	1.2	1.4	1.0	5.0	Neg.
B-9ac	1.8	9.6	0.40	6.4	Neg.
B. licheniformis					
V-37	0.90	0.53	0.31	1.5	Neg.
B. subtilis					
WBS 1958	0.52	0.80	0.21	1.4	Neg.
B. megaterium					
USDA 234	0.40	3.7	0.01	0.10	Neg.
Other *Bacillus* spp.					
G-13s2	0.83	33.2	0.03	8.25	Neg.
D-12s1	1.2	0.34	0.33	0.14	Neg.
B-15s2	0.15	5.7	0.17	4.6	Neg.
A-8s	0.56	0.25	0.09	7.6	Neg.
G-13L	0.40	0.01	0.008	0.02	Neg.

[a] All values represent the average of tests in at least three different rabbits.
[b] Two milliliters of 12-hr brain heart infusion (BHI) cultures grown at 32 C injected per loop.

(Fig. 2). Maximum fluid-inducing activity was achieved at the same time growth ceased to be exponential and shortly before the *p*H reached a minimum. The temporal synthesis by *B. cereus* of fluid-inducing activity is thus in contrast to that of the *C. perfringens* enterotoxin, production of which is intimately associated with sporulating cells (10).

DISCUSSION

The ileal loop fluid-inducing activity of a given strain of *B. cereus* is markedly affected by the growth medium employed. The ability of a particular medium to support the production of this activity does not appear to be related simply to its effectiveness as a growth medium. Growth of *B. cereus* in fresh BI was consistently superior to growth in either BHI or TSB, yet the loop fluid-inducing activity of BI cultures was lower. The nutritional influences on loop fluid-inducing activity remain to be studied.

TABLE 5. *Rabbit ileal loop response to the injection of graded amounts of vegetative cells of Bacillus cereus B-4ac*[a]

Cells suspended in:	Total no. injected[b, c]	Fluid volume to length ratio[b]
Fresh BHI	0	Neg.
	2.2×10^2	Neg.
	1.6×10^4	Neg.
	1.6×10^6	0.14
	1.6×10^8	0.30
	1.6×10^{10}	0.81
Culture filtrate from 12-hr BHI culture	0	0.83
	4.2×10^2	0.72
	4.2×10^4	0.77
	4.2×10^6	0.83
	2.4×10^8	0.88
	3.0×10^{10}	1.02

[a] Vegetative cells obtained from 12-hr cultures in brain heart infusion (BHI) broth, incubated at 32 C on a reciprocal shaker. Cells washed twice with physiological saline solution before being resuspended in selected medium.

[b] Values represent average of tests in two different rabbits.

[c] Challenge inoculum volume was 2 ml.

The loop fluid-inducing activity among *Bacillus* spp. appears to be limited to *B. cereus* or *B. cereus*-like organisms. Of the cultures tested, 19 of 22 strains of *B. cereus* and 4 strains of *B. thuringiensis* were capable of eliciting a response. The ileal loop response did not differentiate *B. cereus* strains isolated from food poisoning outbreaks from those isolated from other sources. If the ileal loop response is related to *B. cereus* food poisoning in humans, most strains of *B. cereus* must be considered potentially enteropathogenic. Further, although no such relationship has as yet been established, our results offer at least presumptive evidence that such illness in humans is the result of an intoxication rather than an infection.

Further investigation into this technique is obviously required, yet the ligated rabbit ileal loop now appears to have significant potential as a model for the study of *B. cereus*-caused food poisoning in humans.

ACKNOWLEDGMENT

This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wis., by Food and Drug Administration grant FD 00447, and by contributions from the food industry to the Food Research Institute.

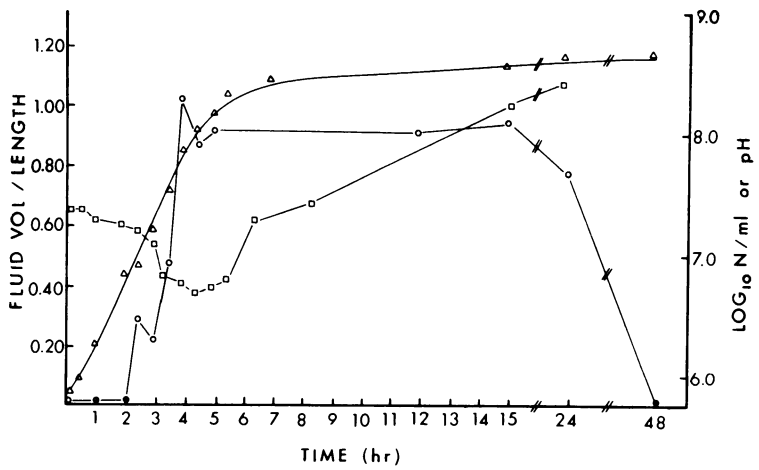

FIG. 2. *Loop fluid-inducing activity (O——O), cell numbers (Δ——Δ), and pH (□——□) of brain heart infusion (BHI) cultures of Bacillus cereus B-4ac (32 C shaken culture). Samples for loop activity determination were chilled to 4 C immediately upon removal from culture flask to prevent further growth. Each point is the average of 2 to 3 separate determinations.*

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