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Sporulation of *Clostridium perfringens* in a Modified Medium and Selected Foods[1]

CHUNG HEE KIM,[2] ROBERTA CHENEY,[3] AND MARGY WOODBURN

Department of Foods and Nutrition, School of Home Economics, Purdue University, Lafayette, Indiana 47907

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A modified sporulation medium for *Clostridium perfringens* was formulated in which a larger number of spores were produced than in SEC broth and in which spores of greater heat resistance were produced than in Ellner's medium when it was also used as the suspending medium. This modified medium consisted of 1.5% peptone; 3.0% Trypticase; 0.4% starch; 0.5% NaCl; and 0.02% $MgSO_4$. The addition of 0.1% sodium thioglycolate and 0.0001% thiamine hydrochloride was optional. The optimal temperature for sporulation of five strains was 37 C in comparison with 5, 22, and 46 C. Sporulation had occurred by 6 hr and was essentially complete after 20 hr at 37 C. Noyes veal broth without glucose also supported the formation of heat-resistant spores but in smaller numbers than did the modified medium. Very low numbers of spores, or none, were produced under the same conditions in pea or tuna slurries.

The spore-forming characteristics of *Clostridium perfringens* are important not only in biological studies but also for understanding food-borne illness caused by the ingestion of large numbers of these bacteria. However, factors which influence the expression of this genetically determined trait are not well understood. Microbiologists, including Despaul (4), Gibbs and Hirsch (6), Perkins (10), and Smith (12), have reviewed the need for laboratory media which could be used for consistent production of spore crops. Commonly used for sporulation of *C. perfringens* is Ellner's medium. Ellner (5) formulated a medium in which frequently greater than 90% of the inoculum of *C. perfringens* had sporulated within 24 hr at 37 C. Although agreeing that this medium encouraged sporulation, Collee et al. (3) found yields to be variable. Angelotti et al. (1) modified Lund's sporulation medium (9) and referred to it as SEC broth. Hall et al. (8) compared five media for sporulation of *C. perfringens* and found that only SEC broth consistently yielded spore crops capable of resisting exposure to 100 C for prolonged intervals. In their study, Ellner's medium was found unsatisfactory for heat-resistance studies, although, in most instances, it was superior to SEC broth in numbers of spores produced. Groom and Strong (7) concluded, after comparisons among seven strains, that larger numbers of spores were produced in Ellner's medium than in SEC broth or the other two media tested. However, the recovery after additional heat treatment at 100 C for 10 min was generally less than 0.01% for all the media.

Riemann (11) promoted the production of *C. perfringens* spores by means of the addition of Trypticase to spent medium from PA3679. Perkins (10) has suggested that a combination of SEC broth, Ellner's, and Riemann's medium might be profitable in formulating a medium which would support the formation of large numbers of heat-resistant spores of *C. perfringens*. Other media which have been accepted for the sporulation of *C. perfringens* include Reinforced Clostridial Medium and Robertson cooked meat medium (4, 6). Noyes veal broth has been used by Angelotti et al. (1) and others for maintaining cultures; without glucose, it favored the production of heat-resistant spores in comparison with others tested by Groom and Strong (7).

The present investigation was undertaken to modify Ellner's medium and SEC broth to obtain consistent numbers of heat-resistant spores of *C. perfringens*. In addition, sporulation in food items was included as a possible comparison. Spores are defined in this paper as those cells which reproduce in the plating medium after a heat treatment of 80 C for 15 min.

[1] Journal Paper 3012, Purdue University Agriculture Experiment Station.

[2] Present address: Research and Technical Division, Wilson and Co., Inc., Chicago, Ill.

[3] Present address: School of Household Economics, University of Alberta, Edmonton, Alberta, Canada.

MATERIALS AND METHODS

Organisms. Two strains of heat-resistant *C. perfringens*, NCTC 8239 and 8797, were used for developing a modified sporulation medium. Three additional heat-resistant strains of *C. perfringens*, NCTC 8238, 9851, and 8799, and a transfer of NCTC 8238 designated E 2 plus less heat-resistant strains including FH 111 from H. E. Hall (Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio) were obtained and were used for checking optimal levels of medium components and also for comparison of the three media. Stock cultures were maintained in Noyes veal broth without glucose (1).

Preparation of inoculum. Inoculum for sporulation was prepared by transferring 0.5 ml of stock culture into 10 ml of fresh Fluid Thioglycollate Medium (Difco) contained in 15- by 150-mm screw-capped test tubes; two more consecutive transfers into 10 ml of fresh Fluid Thioglycollate Medium were made in the same manner with incubation times of 14, 4, and 4 hr, respectively. The inoculum prepared in this way contained no spores. For inoculation into 10 ml of fresh sporulation medium, 0.5 ml of the vegetative inoculum was used. This gave approximately 3×10^6 cells/ml.

Determination of vegetative cells, spores, and heat-resistant spores of C. perfringens. Vegetative cell counts were made by plating samples of the medium before heat treatment. In the sporulation medium, this count also included spores which were able to germinate without heat shock. For spore counts, 3 ml of culture in sporulation medium was placed in 5-ml soft glass ampoules. The ampoules were sealed, submerged in a constant-temperature water bath adjusted to 80 ± 1.0 C, and heated for 15 min. Time to reach water temperature was 4 min. The heat-treated ampoules were immediately placed in cool tap water. After 1 to 5 min, the ampoules were opened and samples were plated. For determination of the heat resistance of the spores, 5-ml samples of the sporulation cultures were placed in screw-capped test tubes. These tubes were placed in racks in a covered boiling water bath. After 30 min, the tubes were cooled in tap water and samples were plated. SPS agar without antibiotics (1) was used as the plating medium, plates were placed in a Case anaerobic jar, and anaerobiosis was produced as described by Angelotti et al. (1). Colony counts were made between 24 and 48 hr of incubation at 37 C.

Sporulation media. Spore numbers and heat resistance of the spores were checked for the two strains with two replications for each as the general pattern for evaluating modifications of media. In the first series, each component of Ellner's medium (5) was added singly, as was NaCl to SEC broth. Combinations of four components which increased spore production in SEC broth were then tested. The preliminary modified medium was SEC broth plus 0.5% peptone (Difco), 0.15% starch, 0.01% $MgSO_4$, and 0.25% NaCl. This medium was compared with SEC broth and Ellner's medium by use of other strains of *C. perfringens*. The need for both Trypticase (BBL) and vitamin-free Casamino Acids (Difco) as in the SEC broth was determined by eliminating each singly from this medium. Subsequently, the optimal level of the four components and 2% Trypticase was determined, one at a time, with the levels of the remaining components unchanged and with the addition of 0.0001% thiamine hydrochloride, and 0.1% sodium thioglycollate. The modified sporulation medium which was formulated on the basis of these experiments was compared with SEC broth and Ellner's medium for additional strains.

In addition, the three sporulation media were compared for protection of spores during heat treatment when used as the suspending media. Spores of three strains produced in each media were collected by an adaptation of Zoha and Sadoff (14) and were subjected to a heat-resistance test after the spores grown in one medium had been suspended in each of the three sporulation media. Spores grown in Ellner's medium, SEC, and modified medium were each collected by centrifuging at 800 × *g* for 1 hr. The supernatant liquid was discarded, and the spores were washed with sterile distilled water in the same manner as before. The packed spores were resuspended in 10 ml of sterile distilled water. A 1-ml sample was transferred into 6 ml of each of the fresh media, SEC broth, Ellner's medium, and the modified medium, and the tubes were placed in a boiling water bath. After 10 and 30 min of boiling, surviving spores were counted after incubation in SPS agar without antibiotics. One replication was done for each of three strains.

Incubation temperatures of 5, 22, 37, and 46 C were compared in the last series; five strains were utilized in a comparison of sporulation in the modified medium, SEC broth, Noyes veal broth without glucose, blended canned tuna, and pea puree. The tuna was diluted with two parts of sterile distilled water before being blended. The canned pea puree was diluted 2:1. All five media in 10-ml samples in capped test tubes were steamed for 10 min and were cooled 5 min before being equilibrated to incubation temperature for approximately 1 hr. A split-split plot experimental design with three replications was followed. Sampling times

TABLE 1. *Sporulation of Clostridium perfringens in SEC broth, Ellner's, and a modified medium*[a]

Strains	Sporulation medium	No. of spores[b]/ml at		No. of heat-resistant spores[c]/ml at 24 hr
		6 hr	24 hr	
NCTC 8238	SEC[d]	13,000	27,000	520
	Ellner's[e]	0	45,000	0
	Modified	5,200	250,000	71,000
NCTC 8239	SEC	46	1,800	90
	Ellner's	4	3,100	0
	Modified	1,100	490,000	71,000
NCTC 9851	SEC	1	2	0
	Ellner's	17	11	0
	Modified	17	170	3

[a] Inoculum was 2.8×10^6 to 5.0×10^6 vegetative cells per ml.
[b] After heating for 15 min at 80 C.
[c] After heating for 30 min in boiling water bath.
[d] Angelotti et al. (1).
[e] Ellner (5).

were 6 and 20 hr and 6 days. Heat-resistant spores were also determined after 3 weeks. Diluent was 0.1% peptone water, and plating was done with Sulfite-polymyxin-sulfadiazene Agar (BBL). Other procedures were those described above except that two rather than three transfers were used in growing the inoculum.

RESULTS

Development of a modified sporulation medium for C. perfringens. Components of Ellner's medium and sodium chloride were added to SEC broth. With peptone or starch, larger numbers of spores which were also more resistant to boiling were found. Peptone had the greater effect. Three lots which were tried gave equivalent results. The addition of $MgSO_4$ or NaCl seemed to slightly enhance the heat resistance of the spores. When Na_2HPO_4 was added, spores of strain NCTC 8239 increased. However, spores produced in this medium were not as heat-resistant. Yeast extract tended to increase heat resistance of spores of strain NCTC 8239 without increasing numbers.

TABLE 2. *Survival after boiling of spores of Clostridium perfringens produced in one of three media and resuspended in fresh sporulation media*[a]

Sporulation medium	Suspending medium for heating	Survival (%) after boiling for	
		10 min	30 min
SEC	SEC	6.0	0.0
	Modified	8.3	1.4
	Ellner's	0.0	0.0
Modified	SEC	18.0	6.8
	Modified	28.0	12.0
	Ellner's	0.0	0.0
Ellner's	SEC	18.0	4.1
	Modified	22.0	6.5
	Ellner's	0.0	0.0

[a] Average survival based on one replication for each of two strains: NCTC 8797 and NCTC 8239. Total was number recovered after heating to 80 C for 15 min. Heat treatment was in capped test tubes in a covered boiling water bath.

TABLE 3. *Spores of Clostridium perfringens produced in a modified medium and in Noyes veal broth after three incubation periods at various temperatures*

Strain	Temp (C)	No. of spores per ml[a]					
		Modified medium			Noyes veal broth[b]		
		6 hr	20 hr	6 days	6 hr	20 hr	6 days
E 2	5	18	16	13	31	37	28
	22	20	45	43	31	40	49
	37	11,000	5,000,000	2,300,000	2,400	22,000	13,000
	46	57	58	11	40	25	2
NCTC 8239	5	6	10	10	7	6	5
	22	8	9	6	7	6	6
	37	690,000	12,000,000	10,000,000	56	12,000	20,000
	46	2,000	1,100	79	12	35	6
A 48	5	0	0	0	0	0	0
	22	0	0	27	0	0	63
	37	18,000	2,700,000	2,000,000	190,000	1,700,000	660,000
	46	21	17	5	10	21	10
FH 111	5	0	0	0	0	0	0
	22	0	1	0	0	0	0
	37	1,100,000	2,700,000	2,100,000	83	120	51
	46	2	2	2	3	0	0
NCTC 8797	5	2	2	2	2	2	2
	22	2	2	2	2	2	2
	37	170,000	1,900,000	600,000	2,200	3,600	1,600
	46	1,700	890	130	2	8	3

[a] After heating for 15 min at 80 C. Average of three replications, by use of logarithms and then the antilog.

[b] Angelotti et al. (1), made without glucose.

In other series, the addition of yeast extract, KH_2PO_4, or Na_2HPO_4 was not effective.

When Trypticase was eliminated from the medium, very poor sporulation was observed. In addition to peptone, Trypticase was essential for the production of heat-resistant spores. No differences in spore numbers or heat resistance were noted in comparisons of three lots of Trypticase. Elimination of vitamin-free Casamino Acids had no detrimental effect on the medium. The effect of thiamine on spore yields was discussed by Lund (9). He suggested that the differences between lots of Trypticase could be eliminated by addition of thiamine. With the lots used, no differences were noted with levels of thiamine. The effect of sodium thioglycolate has already been discussed (C. H. Kim, Ph.D. Thesis, Purdue Univ., Lafayette, Ind., 1965). This reducing agent improved spore numbers when the surface of the medium was large, as in a flask culture, and had little effect in test tube culture.

Improvement of the medium was also sought through changing the level of certain ingredients. Starting with 0.25% peptone, an increase in the amount of peptone to 1.5% produced better results. The upper limit beyond which the numbers of spores decreased was not the same when different food poisoning strains of *C. perfringens* were checked. Five strains had maximal numbers of spores at 2.5%, whereas one strain sporulated best at a level of 1.5% peptone. Three levels of Trypticase were compared with the peptone level held at 0.5%. Five strains showed best sporulation at 6%, whereas one strain sporulated best at 4%. On the basis of results from experiments when levels of both were increased, 3% Trypticase and 1.5% peptone were used in the modified medium. Four levels of starch ranging from 0.15 to 0.75% were compared, and 0.4% was found to be the optimal level. Four levels of NaCl from 0.25 to 1.25% were compared, and 0.5% was chosen. Different strains reacted differently to the level of $MgSO_4$. On an overall basis, 0.02% was selected. When the modified medium was compared with SEC broth after 6 and 24 hr of incubation at 37 C, the modified medium consistently gave the larger number of spores after 24 hr of incubation. Numbers of spores produced in Ellner's medium were occasionally as large as in the modified.

Data from experiments with the modified medium are presented in Table 1 for several strains. Of the 13 strains tested, one did not sporulate satisfactorily in any of the three media. The greatest number of heat-resistant spores was produced in the modified medium; the second, in SEC broth. No spores resistant to 30 min of boiling were recovered from Ellner's medium when this was also the heating substrate.

The composition of the modified sporulation medium for *C. perfringens* is: 1.5% peptone, 3% Trypticase, 0.4% starch, 0.5% NaCl, and 0.02% $MgSO_4$. Sodium thioglycolate at a concentration of 0.1% may be included. After sterilization by autoclaving for 15 min at 121 C, a filter-sterilized solution of thiamine hydrochloride to give a final concentration of 0.0001% may be added.

Influence of medium on heat resistance. The results in Table 2 indicate different degrees of protection against heating when the three media were used as suspending media for testing the heat resistance of spores produced in other media. Although the numbers of spores per milliliter from Ellner's or SEC broth were low after resuspension (290 and 100, respectively), recovery after boiling was greater for those resuspended in SEC broth or in the modified medium than in Ellner's. The same trend was apparent with spores resuspended from the modified medium. The third strain produced very few spores, but results were in agreement.

Comparisons of sporulation in three media and in two food slurries. The modified medium and, to a significantly less extent, Noyes veal broth without glucose consistently favored sporulation of the five strains of *C. perfringens* utilized in this experiment (Table 3). The optimal temperature was 37 C. Analysis of variance was done for the

TABLE 4. *Spores of Clostridium perfringens produced in SEC broth, pea puree, and tuna after three incubation periods at 37* C

Medium	Strain	6 hr	20 hr	6 days
SEC[a]	E 2	72[b]	1,700	22
	NCTC 8239	81	2,700	1,200
	A 48	580	47,000	1,800
	FH 111	0	32	2
	NCTC 8797	910	400	13
Pea puree	E 2	17	19	16
	NCTC 8239	6	7	5
	A 48	0	0	0
	FH 111	0	0	2
	NCTC 8797	2	1	0
Tuna	E 2	27	59	180
	NCTC 8239	10	6	37
	A 48	0	0	15
	FH 111	0	0	0
	NCTC 8797	2	2	2

[a] Angelotti et al. (1).

[b] Number of spores per milliliter after heating for 15 min at 80 C. Average of three replications by use of logarithms and the antilog.

TABLE 5. *Number and percentage of heat-resistant spores of Clostridium perfringens produced at 37 C in a modified medium*

Strain	Time	Replication					
		1		2		3	
		No./ml[a]	Per cent[b]	No./ml[a]	Per cent[b]	No./ml[a]	Per cent[b]
E 2	20 hr	50,000	0.7	210,000	62.0	12,000	0.2
	6 days	110,000	3.6	51,000	4.6	3,700	0.1
	3 weeks	—[c]	—	34,000	1.1	4,300	0.3
NCTC 8239	20 hr	4,100,000	23.0	520,000	14.0	2,500,000	10.0
	6 days	4,100,000	68.0	400,000	9.0	1,200,000	28.0
	3 weeks	—[c]	—	85,000	6.0	680,000	3.5
FH 111	20 hr	83,000	1.5	28,000	3.6	61,000	1.2
	6 days	70,000	1.5	31,000	8.0	67,000	1.4
	3 weeks	—[c]	—	9,700	0.3	1,700	0.2
NCTC 8797	20 hr	16,000	1.4	150,000	4.4	26,000	1.5
	6 days	2,100	1.6	79,000	1.9	1,200	0.3
	3 weeks	600	0.1	120,000	4.4	3,600	0.5

[a] After heating for 30 min at 100 C.
[b] Per cent = (heat-resistant spores)/(total spores formed) × 100.
[c] Heat resistance not tested.

data from this temperature. The *F* values for the interaction of the two media with strains and times with strains were significant ($P < .01$). The F value for replications was not significant, and spore production appeared to be consistent. Numbers of spores in SEC broth varied markedly with strain (Table 4). Very few spores were produced in pea puree or tuna (Table 4). Spore production at 5, 22, or 46 C was poor in all of the media. The average inoculum of vegetative cells was 2.1×10^8. Total numbers from direct plate counts after incubation, but prior to heat treatment, plus spore counts were similar to or less than two times the inoculum level in this series.

Spores resistant at the temperature of boiling water for 30 min were a small percentage of the total number of spores formed (Table 5), although the numbers of spores were large. After maturation for 3 weeks of incubation, spore numbers were generally slightly less, but they had comparable heat resistance.

Values for the *p*H of the media as determined before inoculation were 7.0 for modified medium, 6.6 for SEC broth, 6.5 to 7.5 for veal broth, 6.0 to 6.3 for pea puree, and 5.8 to 5.9 for tuna. Ellner's medium was adjusted to *p*H 7.8 before autoclaving.

DISCUSSION

When components of Ellner's medium and SEC broth plus NaCl were checked, each had some beneficial effect on either or both spore production and heat resistance, but the degree of benefit differed. Adding peptone to the two other varieties of hydrolyzed proteins in SEC broth, Trypticase and vitamin-free Casamino acids, enhanced spore production and heat resistance to a great extent. Under the same conditions, the addition of Proteose Peptone No. 3 (Difco) in place of peptone (Difco) did not produce a comparable result. Previously, four kinds of peptones were compared for spore production in Ellner's medium (C. H. Kim, Ph.D. Thesis). When each peptone was used singly, Proteose Peptone No. 3 yielded the largest spore crops among the four kinds tested. However, a combination of Trypticase and peptone gave better results than Proteose Peptone No. 3 alone. Use of more than one kind of peptone is apparently beneficial, perhaps one supplementing the deficiencies of the other. Lund (9) mentioned that with putrefactive anaerobe 3679 the difference between sporogenic and nonsporogenic lots of Trypticase depended upon the thiamine level. The great variation in sporulation of *C. perfringens* could not be corrected in previous studies in this laboratory by adding thiamine. Apparently, there are additional factors affecting the consistency of spore production. Other proteins may be desirable. Noyes veal broth without glucose supported sporulation. Despaul (4) observed greater sporulation in Robertson cooked meat medium than in Ellner's medium.

In addition, some spores may not have survived the heat treatment used to obtain spore counts. Yamagishi et al. (13) isolated from soil strains which produced spores that did not withstand 80 C for 10 min.

From this experiment, it was evident that heat-resistant spores were produced in Ellner's medium, but that this medium had a detrimental effect on the survival of heated spores. Groom and Strong (7) also observed higher recovery after heating of clean spores from Ellner's medium. The modified medium apparently gave large numbers of spores as well as protection during heating. The choice of a sporulation medium will thus be influenced by subsequent experimental steps.

Although the modified medium supported the consistent production of large numbers of heat-resistant spores of most strains of *C. perfringens* which were tested, less than 100% of the vegetative cells sporulated, and all were not resistant to boiling. Further development appears desirable, as has been recommended by Groom and Strong (7) and Perkins (10).

Under the conditions employed, sporulation of five strains occurred infrequently in pea puree or tuna. The lower *p*H may have prevented sporulation (10). Barnes et al. (2) concluded that sporulation did not occur on raw or cooked beef. However, the possibility that sporulation may occur in foods under natural conditions needs further study.

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