

## Evaluation and Modifications of Media for Enumeration of *Clostridium perfringens*

A. H. W. HAUSCHILD AND R. HILSHEIMER

Food Research Laboratories, Health Protection Branch, Health and Welfare, Ottawa, K1A 0L2, Canada

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The suitability of the Shahidi-Ferguson perfringens, TSC (tryptose-sulfite-cycloserine), and oleandomycin-polymyxin-sulfadiazine perfringens agars for presumptive enumeration of *Clostridium perfringens* was tested. Of these, the TSC agar was the most satisfactory. The TSC agar method was improved by eliminating the egg yolk and using pour plates. The modified method allowed quantitative recoveries of each of 71 *C. perfringens* strains tested and is recommended. For confirmation of *C. perfringens*, the nitrite test in nitrate motility agar was unreliable, particularly after storage of the medium for a few days. In contrast, positive nitrite reactions were obtained consistently when nitrate motility agar was supplemented with glycerol and galactose.

The most common solid media for presumptive enumeration of *Clostridium perfringens* contain iron and sulfite ions which allow sulfite-reducing clostridia to produce black colonies. Of these, the following are currently being used: SPS (sulfite-polymyxin-sulfadiazine) (1), TSN (tryptose-sulfite-neomycin) (9), SFP (Shahidi-Ferguson perfringens) (12), and TSC (tryptose-sulfite-cycloserine) agars (6).

SPS agar selectively inhibits growth or interferes with the formation of black colonies by the sulfite-reducing *Enterobacteriaceae* and *Achromobacteriaceae*; it also inhibits growth of most other facultative anaerobes and of the genera *Pseudomonas*, *Bacillus*, and *Lactobacillus* (1, 5). However, low recoveries of *C. perfringens* in commercial SPS agar have been reported (12; L. F. Harris and J. V. Lawrence, *Bacteriol. Proc.* **70**:6, 1970). Hauschild et al. (7) recovered 12 strains of *C. perfringens* quantitatively in SPS agar prepared in the laboratory from its ingredients, but in only one out of four commercial lots of SPS. Handford and Cavett (4) and Harmon et al. (5) also obtained low recoveries in laboratory-prepared SPS agar. In this laboratory, we have usually obtained complete recoveries of *C. perfringens* in SPS agar prepared from its ingredients, but in two preparations the recoveries of some *C. perfringens* strains were below 1% (D. Dobosch and A. H. W. Hauschild, unpublished data). In one preparation, the cause could be traced to a particular lot of yeast extract. It appears that the selective ingredients of this agar are at a level where a slight adverse change in the medium may result in inhibition of *C. perfringens*.

TSN agar has been used less extensively than SPS agar, but the few reports on the suitability of this medium indicate that it is inhibitory to a number of *C. perfringens* strains (4, 5; Harris and Lawrence, *Bacteriol. Proc.* **70**:6, 1970).

SFP agar appears to allow quantitative recovery of *C. perfringens* (4-6, 12). Unfortunately, it does not prevent growth of a large number of facultative anaerobes, some of which are sulfite reducing (6, 12). Its applicability, therefore, seems to be limited to specimens in which *C. perfringens* is the predominant microorganism, i.e., foods responsible for *C. perfringens* enteritis or fecal samples from patients recovering from the disease. The use of neomycin-blood agar commonly used in the United Kingdom (8, 14) is similarly limited to investigations of food-poisoning incidents. Another disadvantage of the SFP agar is its relatively elaborate preparation: it requires addition of fresh egg yolk, surface plating, and pouring of cover agar.

Harmon et al. (6) modified SFP agar by replacing polymyxin B and kanamycin with 0.04% D-cycloserine. This antibiotic had been shown to selectively inhibit growth of essentially all of the common facultative anaerobes (2). In this modified medium (TSC agar), each of 10 *C. perfringens* strains tested was enumerated quantitatively by Harmon et al. (6).

Presumptive enumeration of *C. perfringens* is followed by confirmatory tests. The simplest of these involves stab culturing of an adequate number of black colonies into nitrate motility (NM) agar (1). However, the nitrite test as described by Angelotti et al. (1) is unreliable (3, 12). Shahidi and Ferguson (12), therefore, intro-

duced egg yolk into their medium and proposed to enumerate only black colonies with an opaque halo around them and to confirm these in lactose motility (LM) agar. Of the clostridial species that produce sulfide as well as lecithinase, only *C. perfringens* is nonmotile and lactose positive. In our experience, this method has the following main shortcomings: (i) several *C. perfringens* species do not produce a discernible halo after 20 to 24 h of growth in SFP and TSC agars; (ii) due to excess gas formation in LM agar, nonmotility of these isolates is difficult to ascertain.

This work was initiated to evaluate the suitability of the SFP and TSC agars for enumeration of *C. perfringens* and to determine the conditions required to obtain consistent results in the nitrite motility test. While this work was in progress, Handford and Cavett (4) published a note on the enumeration of *C. perfringens* in OPSP (oleandomycin-polymyxin-sulfadiazine perfringens) agar. An evaluation of this medium is included in the present paper.

#### MATERIALS AND METHODS

**Cultures.** Seventy-one strains of *C. perfringens* were examined; 51 of these were isolated from food-poisoning incidents, 11 from pathological specimens, 7 from soil and normal feces, and 2 were of unknown origin. Strains were supplied by C. R. Amies, Willowdale, Ontario (six strains); R. J. Avery, Hull, Quebec (two strains); C. L. Duncan, Madison, Wis. (one strain); the late H. E. Hall, Cincinnati, Ohio (18 strains); S. M. Harmon, Washington, D.C. (two strains); A. G. Helstad, Madison, Wis. (one strain); B. C. Hobbs, London, England (23 strains); R. Malo, St. Hyacinthe, Quebec (two strains); M. A. Mason, Ottawa, Ontario (six strains); and L. D. S. Smith, Blacksburg, Va. (one strain); seven strains were isolated by us.

The working cultures were preserved in 15% glycerol (10) at  $-18^{\circ}\text{C}$ ; they were thawed, inoculated into screw-cap test tubes containing 15 ml of cooked meat medium (Difco), and incubated at  $37^{\circ}\text{C}$  for 20 h.

**Enumeration procedures.** The cultures were diluted in 0.1% peptone (13). When egg yolk-containing media were used, 0.1-ml volumes of diluted culture were spread on the agar surface in standard petri plates. Two plates were used per dilution. When completely dry, the surface was covered with about 10 ml of cover agar. Egg yolk-free media were used in pour plates with 1.0-ml volumes of diluted culture per plate. All plates were incubated anaerobically at  $37^{\circ}\text{C}$  for 20 h.

All plating media were prepared from the same agar base consisting of 1.5% tryptose (Difco), 0.5% Soytone (Difco), 0.5% yeast extract, 0.1% ferric ammonium citrate (British Drug Houses), 0.1% sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ ; British Drug Houses), and 2% agar. The ingredients were dissolved in distilled

water to either 92% of the final volume to allow for subsequent addition of egg yolk suspension, or to the final volume. The pH was adjusted to 7.6 before addition of the agar. The agar base was also obtained commercially (SFP agar base, Difco). Antibiotics and egg yolk suspension were added to the autoclaved medium at  $50^{\circ}\text{C}$ .

**SFP agar.** Complete SFP agar was prepared by adding to 920 ml of agar base: the contents of one antimicrobial vial P (30,000 U of polymyxin B [Difco] in 10 ml of distilled water); 4.8 ml of the contents of an antimicrobial vial K (25 mg kanamycin [Difco] in 10 ml of distilled water); and 80 ml of egg yolk suspension containing one egg yolk per 20 ml of 0.85% NaCl. The SFP cover agar had the same composition as the complete SFP agar, except that it contained no egg yolk.

**Media with D-cycloserine.** The second group of plating agars contained varying amounts of D-cycloserine (D-CS; Nutritional Biochemical Corp., Cleveland, Ohio) instead of polymyxin B and kanamycin (6). The medium containing 400  $\mu\text{g}$  of D-CS per ml (0.04%) is identical with the TSC agar of Harmon et al. (6). The antibiotic was added as a 4% filter-sterilized solution in water. The plating procedure was as described for the SFP agar.

The third group of plating agars differed from the second in two aspects: no egg yolk was added, and they were used in pour plates only.

**OPSP agar.** Details for the preparation of OPSP agar not contained in the note of Handford and Cavett (4) were obtained by personal communication. The basic ingredients, including ferric ammonium citrate and sodium metabisulfite, were the same as in the SFP and TSC agars. The final concentration of sodium sulfadiazine 272 molecular weight (American Cyanamid Co., Pearl River, N.Y.) was 109 mg/liter, which corresponds to 0.01% sulfadiazine (250 molecular weight) used by Handford and Cavett. Concentrations and origins of the antibiotics were the same as in the work of these authors; the final concentrations of oleandomycin phosphate (Pfizer Co., Montreal) and polymyxin phosphate (aerosporin; Burroughs Wellcome Co., Montreal) were 0.5 mg/liter and 10,000 IU (equivalent to 1.0 mg of polymyxin standard) per liter, respectively. The OPSP agar was used without egg yolk and in pour plates.

The results of all enumerations were expressed as percentages of the counts in the corresponding antibiotic-free control medium.

**Confirmatory tests.** Single colonies were stab-inoculated into LM agar (12), NM agar (1), and NM agar supplemented with 0.5% each of glycerol and galactose (11) and incubated at  $37^{\circ}\text{C}$  for 20 h. Nitrite formation was determined according to Angelotti et al. (1).

#### RESULTS AND DISCUSSION

**Evaluation of SFP and TSC agars.** In a preliminary experiment, 21 strains of *C. perfringens* were recovered quantitatively in SFP and TSC agars, both prepared from commercial

SFP agar base. The results were consistent with those of Shahidi and Ferguson (12) and of Harmon et al. (5, 6), but the experiment revealed two considerable shortcomings of both media. (i) Most *C. perfringens* strains produced large colonies in SFP and TSC agars; counts of over 50 per plate therefore became progressively inaccurate, and 10-fold dilutions were often inadequate. (ii) Of 21 strains, 8 had no discernible opaque halos around the black colonies after the first day of incubation; presumably, such colonies would not be counted in the procedure of Shahidi and Ferguson (12). These drawbacks as well as the lengthy plating procedure are all associated with the dependence of the method on the egg yolk reaction which had been introduced because the nitrite motility test was unreliable. The following experiments were designed to determine the conditions (i) for quantitative enumeration of *C. perfringens* in a selective medium without egg yolk and (ii) for consistent nitrite reactions in the confirmatory tests.

**Enumeration in egg yolk-free agar with D-CS.** Table 1 shows the recoveries of 71 strains in egg yolk-free agar with different concentrations of D-CS. The recoveries were essentially quantitative at D-CS concentrations of 200 and 400  $\mu\text{g/ml}$ ; the lowest count at 400  $\mu\text{g/ml}$  was 64% of the count in the control medium. Several strains were partially or totally inhibited at D-CS concentrations of 600 and 800  $\mu\text{g/ml}$ , with mean recoveries of 63 and 39%, respectively. About 40% of the strains listed in Table 1 were tested in medium with the commercial agar base; due to supply problems, the remaining strains were tested in medium prepared from the ingredients. This did not appear to affect the results. A detailed table showing the recoveries of each strain is available upon request.

At all concentrations of D-CS (Table 1), the strains produced only black colonies, even at the surface. However, a few strains occasionally produced colonies at the surface with a narrow, white halo around the black center.

The medium with the highest D-CS concentration (400  $\mu\text{g/ml}$ ) that does not significantly

inhibit *C. perfringens* appears to be the medium of choice for enumeration. Since its composition is similar to that of the TSC agar of Harmon et al. (6), we have designated it "EY" (egg yolk)-free TSC agar."

**Confirmatory tests.** The results of the confirmatory tests are summarized in Table 2. Of the 142 isolates (duplicate colonies of the 71 strains), only 112 showed a positive nitrite reaction in basic NM medium. Five tubes contained traces of nitrite as evidenced by a faint red color, and 25 tubes contained no detectable nitrite. Most of the negative tubes showed only little growth. All of the isolates grown in supplemented NM medium showed good growth and produced positive nitrite reactions; most of these were very intense, in contrast to the reactions in basic NM medium (Table 2). Two strains each of *C. sporogenes* and *C. bifermentans* were used as negative controls; none of these showed a color reaction.

During incubation, the pH of the *C. perfringens* stab cultures dropped from 7.1 to 6.7-6.9 in NM agar and to 5.6-6.1 in supplemented NM agar. Uninoculated tubes with supplemented NM agar, adjusted to pH 5.5, were therefore incubated as additional controls; they were all negative for nitrite.

All NM media were used within 2 weeks after preparation, and all were de-aerated before stabbing. In a separate experiment, we compared the suitability of freshly prepared, supplemented NM medium with the same medium stored for 5 weeks at 4 C. No difference was found. In contrast, basic NM medium deteriorated rapidly during storage: in the fresh medium, 24 out of 28 isolates showed positive nitrite reactions, and 4 gave trace reactions; in the same medium stored for 3 weeks at 4 C, only two isolates produced nitrite; the remaining 26 were negative for nitrite.

**Comparison of surface-plated egg yolk media with egg yolk-free pour media.** We compared the recoveries of 19 *C. perfringens*

TABLE 1. Enumeration of 71 *C. perfringens* strains in egg yolk-free medium with D-cycloserine in pour plates

D-Cycloserine concn ( $\mu\text{g/ml}$ )	Mean $\pm$ SD as % of counts in control agar	Range of counts (%)
200	100 $\pm$ 11	74-130
400	95 $\pm$ 17	64-131
600	63 $\pm$ 37	0-121
800	39 $\pm$ 34	0-104

TABLE 2. Nitrite reaction in basic and supplemented nitrate motility agar<sup>a</sup>

Reaction	No. of reactions	
	Basic NM agar	Supplemented NM agar <sup>b</sup>
Positive . . . . .	112 (57) <sup>c</sup>	142 (132) <sup>c</sup>
Doubtful (trace) . . . . .	5	0
Negative . . . . .	25	0

<sup>a</sup> Two isolates each of 71 strains were tested.

<sup>b</sup> With 0.5% galactose and 0.5% glycerol.

<sup>c</sup> In parentheses, very intense reactions.

strains in the presence of D-CS in pour plates without egg yolk (as in Table 1) and in surface-plated medium with egg yolk and cover agar (6). The SFP medium was included for comparison. In both media containing 400 µg of D-CS per ml and in SFP agar, the recoveries were essentially quantitative (Table 3). At the higher concentrations of D-CS, the recoveries in medium with egg yolk were considerably lower than in egg yolk-free medium. The differences were likely due to exposure of the *C. perfringens* cells to high oxygen tension in the surface plating procedure. The results demonstrate that the recoveries of *C. perfringens* in the proposed procedure are equal to or higher than recoveries in the existing procedures.

In this work, we have not tested the selective inhibition of single strains of facultative anaerobes by the EY-free TSC agar. However, applications of this medium for enumeration of *C. perfringens* in naturally contaminated foods and in fecal specimens (A. H. W. Hauschild and R. Hilsheimer, manuscript in preparation) have shown essentially the same degree of selectivity as that of the egg yolk medium of Harmon et al. (6).

Some shortcomings of the media containing egg yolk have been listed above: (i) the low selectivity of SFP agar; (ii) the relatively elaborate procedures; (iii) the frequent occurrence of *C. perfringens* colonies without discernible halos (false negatives); and (iv) the large and frequently spreading colonies which make 10-fold dilutions impractical. We have also found that SFP agar allows growth of egg yolk-positive facultative anaerobes from foods. In some cases, these organisms produced completely opaque plates and thus masked the egg yolk reaction of *C. perfringens*. The lack of selectivity of the

TABLE 3. Enumeration of 19 *C. perfringens* strains in egg yolk-free pour medium and on surface-plated egg yolk media<sup>a</sup>

Medium	D-cycloserine (µg/ml) <sup>b</sup>			
	0	400	600	800 <sup>c</sup>
Basic agar without egg yolk		95 ± 17	61 ± 35	28 <sup>d</sup>
Basic agar with egg yolk		84 ± 27	32 <sup>e</sup>	6 <sup>f</sup>
Complete SFP agar	86 ± 22			

<sup>a</sup> Strains relatively sensitive to D-cycloserine (Table 1) were selected.

<sup>b</sup> Means ± SD expressed as percentage of counts in control agar.

<sup>c</sup> Only 13 strains were tested.

<sup>d</sup> Range, 0 to 75.

<sup>e</sup> Range, 0 to 100.

<sup>f</sup> Range, 0 to 62.

SFP agar has been overcome by replacing it with TSC agar (6). The remaining shortcomings of the egg yolk agars may be overcome by using EY-free TSC agar in pour plates and stab-culturing black colonies of supplemented NM agar for confirmation of *C. perfringens*.

**Comparison of D-CS from different sources.**

Since we did all of our work with D-CS from a single supplier (Nutritional Biochemicals Corp.), its effect on the enumeration of *C. perfringens* was compared with that of D-CS from another company (Sigma Chemical Co., St. Louis, Mo.). Five *C. perfringens* strains were tested. Essentially the same results were obtained with D-CS from both suppliers (Table 4).

**Comparison of EY-free TSC agar with OPSP agar.** Table 5 shows the recoveries of 22 *C. perfringens* strains in EY-free TSC and OPSP agars. As in preceding experiments (Tables 1 and 3), the recoveries of all strains were essentially quantitative in EY-free TSC agar. Twenty of these strains were also enumerated quantitatively in OPSP agar, but one of them (8247) produced only pin-point colonies that

TABLE 4. Enumeration of *C. perfringens* in egg yolk-free medium containing D-cycloserine from different suppliers

D-CS supplier	D-cycloserine (µg/ml) <sup>a</sup>			
	200	400	600	800
Sigma Chemical Co.	92 ± 6	88 ± 8	61 ± 30	29 ± 21
Nutritional Biochemical Corp.	103 ± 3	94 ± 12	75 ± 25	27 ± 17

<sup>a</sup> Means ± SD expressed as percentage of counts in control agar.

TABLE 5. Enumeration of 22 *C. perfringens* strains in EY-free TSC agar and in OPSP agar<sup>a</sup>

<i>C. perfringens</i> strain	Expt no.	Colony count (% of count in control agar)	
		EY-free TSC agar	OPSP agar
19 strains <sup>b</sup>		99 ± 11	97 ± 16
8247	1	96	97 <sup>c</sup>
	2	99	90 <sup>c</sup>
A-72	1	111	<2
	2	78	<1
	3	124	<1
S-88	1	68	27 <sup>c</sup>
	2	85	42 <sup>c</sup>
	3	98	<2

<sup>a</sup> Strains relatively sensitive to D-cycloserine (Table 1) were selected.

<sup>b</sup> Means ± SD of 19 out of 22 strains tested.

<sup>c</sup> Only pin-point colonies were produced.

were difficult to count even in pure culture. A few of the other 19 strains, namely 1194 and 11668, had relatively small colonies, but these could still be counted without difficulty. For the remaining two strains (A-72, S-88), the counts were low; no colonies of strain A-72 were found, and strain S-88 had pin-point colonies only. Handford and Cavett (4) obtained quantitative recoveries of *C. perfringens* in OPSP agar, but they tested only eight strains. Our work with naturally contaminated foods (A. H. W. Hauschild and R. Hilsheimer, manuscript in preparation) has shown that facultative anaerobes were considerably less inhibited in OPSP agar than in the TSC agars. The OPSP agar, therefore, is not satisfactory for enumeration of *C. perfringens*.

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#### ADDENDUM IN PROOF

D. A. A. Mossel and H. Pouw (Zentralbl. Parasitenk. Infektionskr. Hyg. Abt. 1. Orig. **223**:559-561, 1973) have recently modified the TSN agar (9) by replacing its antibiotics with D-CS. The modified medium has not as yet been thoroughly tested.

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