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Improved Growth Medium for *Campylobacter* Species

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Campylobacter species were grown in a base containing proteose peptone no. 3, yeast extract, K_2HPO_4, $(NH_4)_2SO_4$, Na_2SO_3, soluble starch, and agar. Concentrations and sources of organic nitrogen and growth factors were critical, and the optimal pH range was 7.0 to 7.5. Cultures tolerated 0.7% NaCl in addition to the salt present in the organic constituents and were sensitive to surface-active agents at concentrations recommended for enrichment of other gram-negative bacteria. Cultures were maintained on the proposed medium for 1 year with transfer every 2 weeks.

Campylobacter has frequently been implicated in gastroenteritis, specifically after consumption of contaminated water, poultry, and raw milk (6, 7, 10). Recovery methods for these organisms frequently fail to recover the organism and often are not reproducible in different laboratories. The difficulty is partially explained by the organism's complex nutritional requirements and its requirement for a microaerophilic environment.

Strains of *Campylobacter fetus* vary considerably in their minimal nutritional requirements. Smibert (9) has recognized five groups of *C. fetus* based on their nutritional requirements. The vitamin niacin is required by all; other vitamins are merely stimulatory. One subgroup grows with four amino acids: glutamic and aspartic acids, proline, and leucine; a second group requires methionine in addition to those listed; a third group also requires alanine and arginine; and the fourth and fifth groups require 17 or 18 amino acids, respectively. These findings, however, have not been applied to the formulation of media used for maintenance or for the preparation of inocula for physiological, serological, or pathogenicity studies. Instead, *Brucella*, Mueller-Hinton, and fluid thioglycolate broths (1, 2, 4) have been recommended for these purposes. The formulas for these media are not uniform. For growth on a solidified medium, one commercial preparation (5) specifies 10 g of pancreatic digest of casein, 10 g of peptic digest of animal tissue, 1 g of dextrose, 2 g of yeast autolysate, 5 g of NaCl, 0.1 g of $NaHSO_3$, and 50 ml of sheep blood per liter. Antibiotics are included for selectivity. In liquid medium many cultures grow poorly and are frequently lost. This report elucidates the major requirements and the tolerances of several strains of *Campylobacter* sp. to selected environmental pressures.

MATERIALS AND METHODS

To obtain a microaerophilic environment, we used anaerobic jars (BBL model 60465) containing three plates of veal infusion agar streaked confluently with a strain of *Escherichia coli*. The jar was then filled with petri dishes, tubes, or bottles and closed as specified. The jars were incubated at 35 or 42°C in an environment of 8% O_2 and 11% CO_2 for 2 to 4 days. The screw-capped tubes containing cultures for maintenance were stored at room temperature with the caps slightly loosened.

Our proposed growth medium for *Campylobacter* sp. included 15 g of proteose peptone no. 3, 7.5 g of yeast extract, 5 g of Casamino Acids (Difco Laboratories), 5 g of K_2HPO_4, 1.5 g of $(NH_4)_2SO_4$, 3 g of agar, 1 g of soluble starch, and 0.5 g of Na_2SO_3 per liter. With the exception of Na_2SO_3, all ingredients were dissolved with gentle heating. The pH was adjusted to 7.3 with KOH. Portions of 8 ml were placed in 16- by 125-mm screw-capped tubes; 100-ml portions were placed in 6- or 8-oz (ca. 170- to 230-ml) screw-capped bottles. All tubes were autoclaved for 15 min at 121°C. After the medium was cooled to 48°C and the 2.5% Na_2SO_3 solution was filter sterilized, 0.2 ml of the solution was added to 8 ml of medium, and 2 ml was added to 100 ml of medium. *Campylobacter* cultures tolerated 7.9 mmol of Na_2SO_3. Contents were mixed gently. For subculture, 0.1 ml of a gently agitated culture was transferred to 8 ml of medium or 1 ml of culture to 100 ml of medium.

Medium productivity was evaluated by observing the formation of a continuous film of growth within 72 h of incubation at 35°C from an initial inoculum of 50 cells/ml. Cell populations were enumerated by most probable number and by colony counts. Serial dilutions of a gently agitated culture were prepared in 0.5% saline. Portions containing 1 ml of each dilution were added to three separate tubes of melted semisolid medium tempered at 48°C. The tubes were cooled immediately and incubated for 96 h at 35 or 42°C and examined for growth. Early growth appeared as a cottony ball in the medium; later the cells formed a film. Most-probable-number values were calculated by the standard procedure (3). For colony counts, media were prepared with agar concentrations of 0.8 and

TABLE 1. Elucidation of major requirements of *C. jejuni* culture CH[a] incubated for 96 h at 42°C

Nutrient[b]	Inoculum (cells/ml)	
	Large (5×10^6)	Small (7,000)
BE	—	—
CA	—	—
YE	—	—
BE + CA	+2	—
YE + CA	+4	—
BE + CA + YE	—	—
BE + CA + YE + Sol st	+4	—
BE + CA + YE + Ag	+4	+1
BE + CA + YE + Sol st + Ag	+3	+1
Mueller-Hinton broth	+2	—
Mueller-Hinton broth + YE + Ag	+3	+1
Brucella broth	+1	—

[a] CH, Children's Hospital culture isolate.
[b] BE, 0.3% beef extract; CA, 1.75% Casamino Acids; YE, 0.5% yeast extract; Sol st, 0.15% soluble starch; Ag, 0.15% purified agar.

1.5%. Medium containing 1.5% agar was poured into standard 15- by 100-mm plastic disposable petri dishes; 5-ml portions of medium containing 0.8% agar were prepared in 16- by 125-mm tubes and maintained in the melted state at 48°C after sterilization, and two 1-ml portions of each dilution were added to tubes of semisolid medium, mixed, and spread on the surface of solidified 1.5% agar. After solidification, the plates were inverted and incubated under microaerophilic conditions at 35°C for 72 h. Counts were determined in the standard manner. *Campylobacter* sp. cultures either do not grow in 1.5% agar or else produce pinpoint colonies. In the proposed semisolid medium, the titers of *Campylobacter* sp. cultures approached those obtained with *Enterobacteriaceae*, i.e., 5×10^8 to 1×10^9 cells/ml after dispersal of the film.

RESULTS AND DISCUSSION

Table 1 shows qualitative nutrient requirements of *C. jejuni* cultures. The cultures were unable to grow in beef extract, yeast extract, or Casamino Acids tested individually, but did grow in Casamino Acids plus yeast extract and to a lesser extent, beef extract when the inoculum was large. The minimal requirements for growth from a small inoculum were beef and yeast extracts, Casamino Acids, and agar. The requirements were clarified by use of many inocula (Table 2). Soluble starch and agar promoted growth in a Casamino Acids-yeast extract from a small inoculum. High concentrations of starch or agar, however, were inhibitory. Further supplementation with K_2HPO_4, KH_2PO_4, and $(NH_4)_2SO_4$ resulted in growth from an initial inoculum of 50 cells/ml. The initial studies with one batch of Casamino Acids gave good growth; another batch gave poor results. Proteose peptone no. 3, a satisfactory substitute, gave a minimal effective concentration of 0.1 to 1.0%, depending on the culture; a level of 2.5% was inhibitory. The minimal concentration of yeast extract varied from 0.1 to 1%, depending on the culture; a level of 1% was inhibitory. The inclusion of 0.07% $NaHCO_3$ was also inhibitory. The sodium salts of malic, aspartic, and glutamic acids at a concentration of 0.05% were not stimulatory in the proposed medium.

Because the cultures required a source of amino acids, a variety of commercially available hydrolysates were examined (Table 3). With the exception of Casamino broth, none of the sources yielded excellent growth from all strains. Casamino broth, which contains Casamino Acids, two amino acids, three vitamins, and three inorganic salts, was satisfactory when used individually or in conjunction with proteose peptone no. 3. Since it is no longer available, however, we recommend the use of Casamino Acids at a level of 5 g per liter of medium described in Materials and Methods in addition to proteose peptone no. 3.

TABLE 2. Establishment of nutritional requirements of *C. jejuni* culture CH[a] incubated for 96 h at 42°C, pH 7.0

Medium[b]	Inoculum (cells/ml)					
	5,000,000	500,000	50,000	5,000	500	50
CA + YE	+2	—	—	—	—	—
CA + YE + 0.1% agar	+2	+1	—	—	—	—
CA + YE + 0.3% agar	+3	+2	+1	—	—	—
CA + YE + 0.5% agar	+2	+1	—	—	—	—
CA + YE + 0.15% Sol st	+2	+1	—	—	—	—
CA + YE + 0.3% Sol st	+1	−2	—	—	—	—
CA + YE + 0.2% Sol st + 0.3% agar	+3	+2	+1	—	—	—
CA + YE + 0.2% Sol st + 0.3% agar + 0.2% K_2HPO_4 + 0.2% KH_2PO_4	+3	+2	+2	+2	—	—
CA + YE + 0.2% Sol st + 0.2% K_2HPO_4 + 0.2% KH_2PO_4 + 0.15% $(NH_4)_2SO_4$ + 0.3% agar	+4	+4	+3	+2	+2	+2

[a] CH, Children's Hospital culture isolate.
[b] CA, 1.75% Casamino Acids; YE, 0.5% yeast extract; Sol st, soluble starch.

TABLE 3. Evaluation of organic nitrogen sources tested at 1.75% and incubated for 96 h at 42°C

C. jejuni culture[a]	Growth rate[b]								
	Base[c]	Casamino broth	Peptone	Tryptone	Proteose peptone no. 3	Bovine peptone	Casitone	Trypticase	Neopeptone
27374	+1	+2	+1	+1	+3	+1	+2	+1	+1
C7	+1	+2	+1	+1	+3	+1	+2	+1	+1
8260	+1	+3	+1	+1	+3	+1	+1	+1	+1
C43	+1	+3	+1	+1	+3	+2	+1	+1	+1
8259	+1	+3	+1	+1	+3	+1	+1	+1	+1

[a] Inoculum, 500 cells/ml.
[b] Rated on scale of 1 to 3 (poor to good).
[c] Base composed of 0.5% yeast extract, 0.5% K_2HPO_4, and 0.15% $(NH_4)_2SO_4$.

Examination of NaCl and pH tolerances (Tables 4 and 5), using formation of film as a criterion of growth, showed that all strains grew in the presence of 0.7% NaCl. Undoubtedly the level is higher because of the presence of NaCl in yeast extract and protein hydrolysates. The percentages of cultures which tolerated levels of 1.1, 1.5, 2.0, and 2.5% NaCl were 77, 31, 31, and 15, respectively. These data justify the existence of groups similar to those described by Skirrow and Benjamin (8). All cultures grew at pH 7.0 and 7.5. The percentages of cultures growing at pH 6.5 and 7.5 were 50 and 62, respectively. These data may explain the inconsistent recovery of *Campylobacter* sp. from enrichments or plates. The production of acid from carbohydrate or of amines from the decarboxylation of amino acids by other populations may inhibit growth of *Campylobacter* sp. These cultures are sensitive to 100 ppm of lauryl sulfate and 0.15% bile salts no. 3.

Considerable uncertainty about the physiological and biochemical activities of members of the genus *Campylobacter* exists in the literature.

TABLE 4. Tolerance of NaCl by *Campylobacter* sp. cultures incubated for 96 h at 42°C

C. jejuni culture[a]	Tolerance at given level						
	0%	0.3%	0.7%	1.1%	1.5%	2.0%	2.5%
8259	+4	+4	+2	+1	—	—	—
164	+4	+4	+2	+1	—	—	—
8260	+4	+4	+1	+1	—	—	—
46	+4	+2	+1	—	—	—	—
96	+4	+2	+1	—	—	—	—
72	+4	+4	+3	+1	—	—	—
8263	+4	+4	+4	+2	+2	+2	+2
66–76	+4	+3	+3	+3	+1	+1	—
201	+4	+4	+3	+2	+1	+1	—
9a	+4	+4	+4	+3	+2	+2	+1
54	+4	+3	+3	+1	—	—	—
27374	+4	+4	+2	+1	—	—	—
5b	+4	+3	+1	—	—	—	—

[a] Initial inoculum, 500,000 cells/ml.

TABLE 5. Tolerance for pH by *Campylobacter* sp. cultures incubated for 96 h at 42°C

C. jejuni culture[a]	Tolerance at given pH level[b]						
	5.5	6.0	6.5	7.0	7.5	8.0	8.5
CH	–	–	+	+	+	+	+
8259	–	+	+	+	+	+	+
2525	–	–	–	+	+	+	–
2440	–	–	–	+	+	–	–
66–53	–	–	–	+	+	–	–
27374	–	–	+	+	+	+	–
8260	–	+	+	+	+	+	+
66–76	–	–	–	+	+	–	–

[a] Initial inoculum, 600,000 cells/ml.
[b] pH adjusted with HCl and KOH, as needed.

Some of these discrepancies may be due to the failure to standardize the conditions of incubation, composition of medium, size of inoculum, and length of incubation. In addition, the cultures may not have been properly identified; e.g., *C. jejuni* and *C. coli* may have been misidentified. It is hoped that the proposed culture conditions or their modifications may provide a basis for more definitive studies of these microorganisms.

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