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Characterization of an Unclassified Microaerophilic Bacterium Associated with Gastroenteritis

JOHN R. ARCHER,[1]* SANDRA ROMERO,[1,2] AL E. RITCHIE,[3] MARJORIE E. HAMACHER,[1] BRET M. STEINER,[4] JOHN H. BRYNER,[5] AND RONALD F. SCHELL[1,2]

State Laboratory of Hygiene[1] and Departments of Medical Microbiology[2] and Medical Technology,[4] University of Wisconsin, Madison, Wisconsin 53706; National Animal Disease Center, Ames, Iowa 50010[5]; and Small World Services, Boone, Iowa 50036[3]

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Four isolates of an unclassified microaerophilic bacterium resembling *Campylobacter* species were characterized by growth requirements, microscopic examination, biochemical characteristics, antimicrobial susceptibility tests, and protein profile analysis. The unclassified isolates were differentiated from *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter fetus* subsp. *fetus*, *Campylobacter laridis*, *Campylobacter pylori*, and an ovine isolate. The bacterium was fusiform shaped with a corrugated surface due to the presence of periplasmic fibers and had multiple bipolar flagella. Biochemically, the bacterium was separated from the *Campylobacter* controls by its negative catalase reaction, negative nitrate reduction, and no growth in 1% glycine. It was also resistant to ampicillin. Protein profile analysis demonstrated nine major protein bands present in the unclassified isolates that were absent in the *Campylobacter* controls. The bacterium also differed from the ovine isolate by its negative catalase reaction, rapid urea hydrolysis, and susceptibility to clindamycin, erythromycin, and tetracycline. Our results showed that the unclassified bacterium was distinct from the recognized *Campylobacter* species.

We have previously isolated (16) an unusual microaerophilic gram-negative bacterium from the stools of two individuals presenting with symptoms of chronic gastroenteritis. The bacterium shared some isolation requirements, colonial morphology, and biochemical reactions with *Campylobacter* species. Transmission electron microscopy, however, demonstrated a fusiform rod with a corrugated surface, rather than a spiral rod. The number and arrangement of flagella differed from those of *Campylobacter* species, since the unclassified bacterium (UCB) had bipolar tufts of sheathed flagella, instead of single bipolar flagella.

Continued studies have now characterized this bacterium by comparing biochemical reactions, growth requirements, antimicrobial susceptibilities, and protein profiles with *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter fetus* subsp. *fetus*, *Campylobacter laridis*, *Campylobacter pylori*, and a similar UCB isolated from ovine abortions (12).

MATERIALS AND METHODS

Sources of the UCB isolates. Four isolates of a UCB were isolated from the stool specimens of three humans and one dog received for examination of enteric pathogens as previously described (16).

Other microorganisms. *C. jejuni*, *C. coli*, and *C. fetus* subsp. *fetus* were obtained from the State Laboratory of Hygiene, Madison, Wis. *C. laridis* and *C. pylori* (D-1872) were kindly provided by M. J. Blaser, Veterans Administration Medical Center, Denver, Colo., and by C. M. Patton, Centers for Disease Control, Atlanta, Ga., respectively. These classified *Campylobacter* species demonstrated consistent biochemical reactions. An ovine isolate (SD-86-1755) was obtained from C. A. Kirkbride, Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings. This isolate was recovered from ovine abortions and has been shown to induce abortions in guinea pigs (3, 11, 12).

Growth requirements and microscopic examination. Stool specimens were cultured on Skirrow medium (17) for isolation of *Campylobacter* species under microaerophilic conditions (5 to 10% O_2) at 42°C for 72 h. Once isolated, the bacteria were cultured on blood agar plates (BAP) (7% sheep blood; Difco Laboratories) with a 10-λ inoculum of a 0.5 McFarland standard suspension. Cultures were incubated at three temperatures (25 to 27, 35 to 37, and 42 to 43°C) under different atmospheric conditions (strict anaerobic, microaerophilic, and aerobic). Attempts at growth under CO_2 atmosphere were done, but results were negative. Presence or absence of bacterial growth was determined 72 h after incubation. The bacterial isolates were maintained in liquid nitrogen or subcultured to the transport medium of Wang et al. (20) or BAP. The isolates were microscopically examined by phase-contrast microscopy and transmission electron microscopy.

Media and biochemical tests. The following media were used to detect growth of the UCB: brucella broth and agar (BBL Microbiology Systems) with and without 5% defibrinated sheep blood (GIBCO Laboratories), Mueller-Hinton broth (Difco) with and without 50 mg of Ca^{2+} per liter and 25 mg of Mg^{2+} per liter, Mueller-Hinton agar (BBL) with or without 0.01% triphenyltetrazolium chloride (Difco), Hemopeptone broth with 2% Fildes enrichment (Difco), chocolate agar (GC and hemoglobin; Difco), brain heart infusion broth (Difco) with 2% horse serum, veal infusion broth (Difco) with 5% defibrinated sheep blood, and Trypticase soy broth (BBL) with and without 5% defibrinated sheep blood. Bacterial growth with conventional enteric media was also attempted (5). Cultures were incubated under microaerophilic conditions for 72 h at 42°C.

Biochemical tests were performed by the methods of Benjamin et al. (2). Briefly, these tests included: cytochrome oxidase activity, catalase reaction, tolerance to 3.5% sodium

* Corresponding author.

TABLE 1. Comparison of growth conditions and biochemical reactions of the UCB, an ovine isolate, and *Campylobacter* controls

UCB isolates and *Campylobacter* controls	Growth conditions				Biochemical reactions												
											Tolerance[a]						
	Atmosphere	Temp (°C) 25	35	42	Oxidase	Catalase	Nitrate reduction	Nitrite reduction	TSI	Pb-Ac	1% Glycine	3.5% NaCl	Nalidixic acid	Cephalothin	Urease[b]	Hippurate hydrolysis
Isolate 1	Anaerobic	−	−	−												
	5–10% O_2	−	+	+	+	−	−	−	−	$+^{tr}$	−	−	R	R	Rapid +	−
	Aerobic	−	−	−												
Isolate 2	Anaerobic	−	−	−												
	5–10% O_2	−	+	+	+	−	−	−	−	−	−	−	R	R	Rapid +	−
	Aerobic	−	−	−												
Isolate 3	Anaerobic	−	−	−												
	5–10% O_2	−	+	+	+	−	−	−	−	$+^{tr}$	−	−	R	R	Rapid +	−
	Aerobic	−	−	−												
Isolate 4	Anaerobic	−	−	−												
	5–10% O_2	−	+	+	+	−	−	−	−	$+^{tr}$	−	−	R	R	Rapid +	−
	Aerobic	−	−	−												
Ovine isolate SD-86-1755	Anaerobic	−	−	−												
	5–10%O_2	−	+	+	+	+	−	−	−	$+^{tr}$	−	−	R	R	+	−
	Aerobic	−	−	−												
C. laridis	Anaerobic	−	+	$+^{wc}$												
	5–10% O_2	−	+	+	+	+	+	−	−	+	+	−	R	R	−	−
	Aerobic	−	−	−												
C. pylori	Anaerobic	−	−	−												
	5–10% O_2	−	+	+	+	+	−	−	−	$+^{tr}$	−	−	R	S	Rapid +	−
	Aerobic	−	−	−												
C. jejuni	Anaerobic	−	+	+												
	5–10% O_2	−	+	+	+	+	+	−	−	+	+	−	S	R	−	+
	Aerobic	−	−	−												
C. coli	Anaerobic	−	+	−												
	5–10% O_2	−	+	+	+	+	+	−	−	+	+	−	S	R	−	−
	Aerobic	−	−	−												
C. fetus subsp. *fetus*	Anaerobic	+	$+^{w}$	$+^{w}$												
	5–10% O_2	+	+	$+^{w}$	+	+	+	−	−	+	+	−	R	S	−	−
	Aerobic	−	−	−												

[a] R, Resistant; S, sensitive.
[b] Rapid +, hydrolysis within 15 to 20 min; +, hydrolysis within 24 h; −, no hydrolysis.
[c] w, Weak growth.

chloride and to 1% glycine, and hydrogen sulfide (H_2S) production. The H_2S production was determined by inoculation of triple sugar iron (TSI) slants. Trace amounts of H_2S production were determined with lead acetate strips (Fisher Scientific) over TSI slants and brucella albimi broth (GIBCO) with 0.02% L-cysteine (J. T. Baker Diagnostics). Hippurate and urea hydrolysis were determined by the methods of Hwang and Ederer (9) and Christensen (4). Tolerance to nalidixic acid and cephalothin was performed with 30-μg disks (Difco) placed on BAP inoculated with a suspension of 10^8 bacteria per ml. Zones of inhibition were determined as described by Karmali et al. (10). Fermentation procedures were performed by the method of Hugh and Leifson (8).

Antimicrobial susceptibility tests. Susceptibility to antimicrobial agents was determined by a modification of the Kirby-Bauer method (1). Mueller-Hinton agar plus 5% sheep blood was inoculated with a suspension of 10^8 bacteria per ml and overlaid with antimicrobial disks (Difco). Zones of inhibition were measured after incubation for 48 to 72 h at 37°C under microaerophilic conditions. Cultures were incubated at 37°C to grow the *Campylobacter* controls. The following concentrations and antimicrobial agents were tested: ampicillin (10 μg), cefamandole (30 μg), cefoxitin (30 μg), cephalothin (30 μg), clindamycin (2 μg), erythromycin (15 μg), nitrofurantoin (300 μg), gentamicin (10 μg), nalidixic acid (30 μg), oxacillin (1 μg), penicillin G (10 μg), and tetracycline (30 μg).

Protein profiles. Protein separations were performed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (13) with running and stacking gels containing 10 and 3.8% acrylamide, respectively. Suspensions of each microorganism (10^9 bacteria per ml) were pretreated with solubilization buffer containing bromophenol blue according to the method of Laemmli (13) and boiled for 10 min; 20 λ of these suspensions was used to load each lane. Gels were run at 20 mA for 3 to 4 h until the tracking dye had reached the bottom of the gel.

RESULTS

Isolation and growth characteristics. The four isolates of the UCB were recovered on Skirrow plating medium after 72 h of incubation under microaerophilic conditions at 42°C

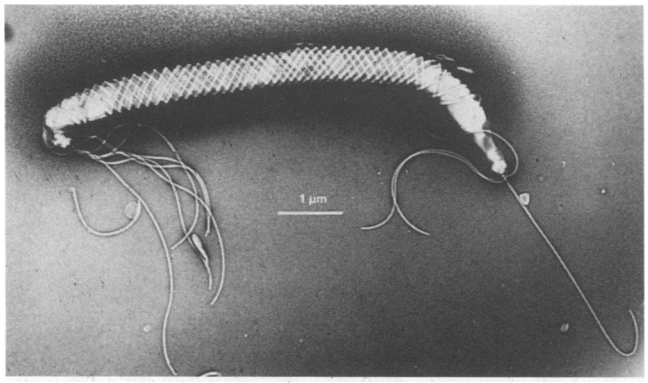

FIG. 1. Transmission electron micrograph of the UCB after a 24-h incubation. Magnification, ×14,000.

(17). Colonies were flat, runny, transparent, and spreading along the streak lines. When subcultured on BAP for 24 to 48 h, swarming growth was observed which subsequently developed a wavelike appearance. Initially, different atmospheres and incubation temperatures were used to determine optimal growth conditions (Table 1). The UCB grew only under microaerophilic conditions at temperatures ranging from 35 to 42°C. No growth was observed at 25°C under different atmospheric conditions. In general, these growth conditions were similar to those required by the *Campylobacter* controls and the ovine isolate (Table 1).

Although Skirrow plating medium and BAP supported growth of the UCB, additional media were evaluated. The following media supported growth: brucella agar with 5% defibrinated sheep blood, Mueller-Hinton broth with and without 50 mg of Ca^{2+} per liter and 25 mg of Mg^{2+} per liter, Mueller-Hinton agar with and without 0.01% triphenyltetrazolium chloride, and Mueller-Hinton agar with 5% defibrinated sheep blood. The UCB failed to grow on brucella broth with or without 5% defibrinated sheep blood, brucella agar without 5% defibrinated sheep blood, Hemopeptone broth, chocolate agar, brain heart infusion broth with 2% horse serum, veal infusion broth with 5% defibrinated sheep blood, Trypticase soy broth with and without 5% defibrinated sheep blood, and conventional enteric media. Overall, fresh solid media containing 5% defibrinated sheep blood enhanced the growth of the UCB.

Microscopic examination. Microscopically, the UCB was fusiform shaped (approximately 6.5 by 0.5 μm) after 24 to 48 h of incubation (Fig. 1). The fusiform-shaped rods began to develop coccoid forms (2.0 to 3.0 μm) after 48 h of culture (Fig. 2). The fusiform rods possessed multiple bipolar flagella (approximately seven flagella per tuft) that allowed for a random and, occasionally, polar oscillating movement. The bacteria also had a corrugated surface formed by periplasmic fibers.

Biochemical characteristics. The four isolates of UCB had identical biochemical reactions, except for the failure of isolate 2 to produce trace amounts of H_2S (Table 1). This response was reproducible. The UCB shared the following characteristics with the *Campylobacter* controls and the ovine isolate (Table 1). The isolates were oxidase positive, nontolerant to 3.5% NaCl, and nonfermentative (Hugh and Leifson media and TSI). In general, the following biochemical characteristics differentiated the UCB from the *Campylobacter* controls: negative catalase reaction, negative nitrate reduction, and intolerance to 1% glycine. *C. pylori*, however, shared additional biochemical characteristics with the UCB. *C. pylori* was also nontolerant to 1% glycine and negative for nitrate reduction and hydrolyzed urea rapidly (15 to 20 min). The UCB and *C. pylori* differed in their catalase reaction and tolerance to cephalothin (Table 1). The UCB also shared many biochemical reactions with the ovine isolate. Major differences were the strong catalase reaction and the slow urea hydrolysis (24 h) of the ovine isolate.

Antimicrobial susceptibility tests. Twelve antimicrobial agents were tested against the UCB, ovine isolate, and *Campylobacter* controls. The UCB and ovine strain were resistant to penicillin G, oxacillin, ampicillin, cephalothin, cefoxitin, cefamandole, and nalidixic acid, whereas they were susceptible to nitrofurantoin (zone of inhibition, >60 mm) and gentamicin (17 to 18 mm). The UCB was also susceptible to tetracycline (30 to 33 mm), erythromycin (30 to 32 mm), and clindamycin (16 to 26 mm), whereas the ovine isolate was resistant.

The antimicrobial susceptibilities of the UCB and the *Campylobacter* controls were similar. A major difference between these groups was the resistance of the UCB to ampicillin. Minor differences were also detected. *C. jejuni* and *C. coli* were susceptible to nalidixic acid, *C. fetus* subsp. *fetus* was susceptible to cephalothin, and *C. laridis* was susceptible to penicillin G, oxacillin, and cephalothin. Similar antimicrobial patterns of susceptibility and resistance for the *Campylobacter* controls have been previously reported (10, 21).

Protein profile analysis. The results of the protein profile analysis are shown in Fig. 3. The four isolates of UCB had similar sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles, although only isolates 1, 2, and 4 are shown in Fig. 3. The UCB presented nine major bands of different molecular masses (<3.0, 4.5, 12.7, 30.0, 34.5, 40.0, 55.4, 59.1, and 74.6 kilodaltons [kDa]) which were not detected in the *Campylobacter* controls. A similar profile was observed with the ovine isolate, although the 30.0-kDa band was absent. The ovine strain also demonstrated four bands (3.6, 7.3, 51.8, and 65.4 kDa) which were absent in the protein profiles of the UCB.

DISCUSSION

The genus *Campylobacter*, as described in *Bergey's Manual of Systematic Bacteriology* (18), is composed of slender,

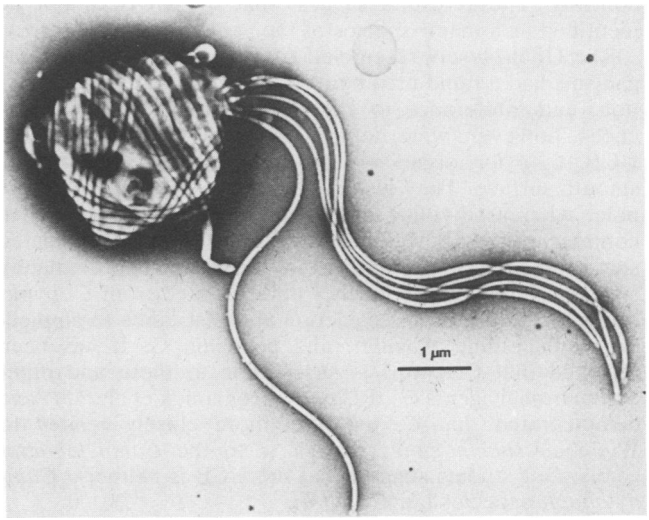

FIG. 2. Transmission electron micrograph of the UCB after a 72-h incubation. Magnification, ×28,000.

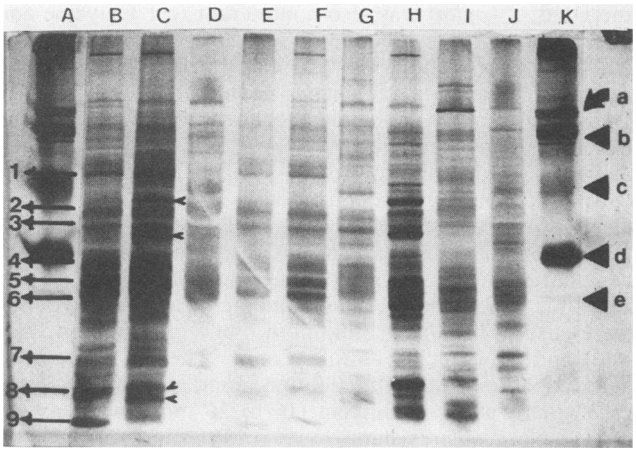

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles. Lanes: A, molecular weight standards; B, isolate 2; C, ovine isolate; d, *C. jejuni*; E, isolate 1; F, isolate 4; G, *C. fetus* subsp. *fetus*; H, *C. coli*; I, *C. laridis*; J, *C. pylori*; and K, molecular mass (in kilodaltons) standards (a, 116; b, 97.4; c, 66; d, 45; and e, 29). Protein bands (in kilodaltons): 1, 74.6; 2, 59.1; 3, 55.4; 4, 40.0; 5, 34.5; 6, 30.0; 7, 12.7; 8, 4.5; and 9, <3.0. The arrowheads pointing at bands in lane C indicate protein bands at 3.6, 7.3, 51.8, and 65.4 kDa.

spirally curved rods with a single polar flagellum at one or both ends of the bacterial cell. The UCB did not fulfill the recognized criteria for identification of a member of the genus *Campylobacter*. The UCB was fusiform, rather than a spiral rod, and had a corrugated surface due to the presence of periplasmic fibers. In addition, the UCB had multiple bipolar flagella that differed in number and arrangement. These features are absent in the *Campylobacter* genus.

The UCB was further separated from the *Campylobacter* controls (*C. jejuni*, *C. coli*, *C. fetus* subsp. *fetus*, and *C. laridis*) by biochemical reactions, antimicrobial susceptibilities, and protein profiles. The UCB was catalase negative and nitrate reduction negative and demonstrated no growth in 1% glycine. The UCB also was resistant to ampicillin, whereas the *Campylobacter* controls were susceptible. The protein profile analysis demonstrated nine major bands present in the UCB that were absent in the *Campylobacter* controls. These results suggest that the UCB cannot be identified as a major species of the genus *Campylobacter*.

The UCB closely resembled *C. pylori* (6). Both microorganisms had a rapid urea hydrolysis, negative nitrate reduction, and intolerance to 1% glycine. Fundamental differences, however, were detected between *C. pylori* and the UCB. *C. pylori* organisms are ox-bow shaped and have a smooth surface. They also have three to five flagella at one pole, whereas dividing cells have bipolar flagella (19). In contrast, the UCB was fusiform shaped with a corrugated surface and possessed bipolar tufts of flagella (approximately seven). The UCB was further differentiated from *C. pylori* by the negative catalase reaction and resistance to ampicillin, cephalothin, oxacillin, and penicillin G. It has been reported that *C. pylori* is susceptible to these and other antimicrobial agents (7). Recently, Romaniuk et al. (15) have demonstrated that *C. pylori* is more closely related to *Wolinella succinogenes* than it is to the *Campylobacter* genus. Our studies suggest that the UCB is neither a *Campylobacter* species nor *C. pylori*.

The UCB also closely resembled the ovine isolate. Both microorganisms were fusiform shaped with corrugated surfaces and had bipolar tufts of sheathed flagella (12). They also demonstrated similar growth characteristics and biochemical reactions (Table 1). The ovine isolate differed from the UCB by a strong catalase reaction, slower urea hydrolysis (24 h), and resistance to clindamycin, erythromycin, and tetracycline (Table 1). The ovine strain also had four major protein bands that were absent in the protein profiles of the UCB. In contrast, the UCB had an additional 30.0-kDa protein band that was not detected in the ovine isolate. These differences may be due to strain variability.

A variety of microaerophilic bacteria are recognized or tentatively included in the existing genus *Campylobacter*. Some of these bacteria share similar growth characteristics and biochemical reactions with our UCB. The UCB, however, did not fulfill the classical description of the *Campylobacter* genus (14, 18). Our results suggest that the UCB is distinct from the recognized *Campylobacter* species.

Additional studies involving DNA hybridization and cellular fatty acid composition are required to classify this bacterium.

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