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Hydrogenase Activity in Catalase-Positive Strains of *Campylobacter* spp.

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A rapid hydrogenase assay has been developed which may be useful in separating the species *Campylobacter jejuni* and *C. coli* from the subspecies *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. This assay employs the impermeant redox dye benzyl viologen, and positive determinations can be made within 20 min. All strains of *C. jejuni* and *C. coli* were found to be strongly hydrogenase positive. All strains of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* were negative for hydrogenase when the assay was performed at a benzyl viologen concentration of 2 mM and an incubation temperature of 30°C. Some strains of *C. fetus* had low levels of hydrogenase as determined with cell extracts but were hydrogenase negative by the benzyl viologen assay. Since there are few rapid diagnostic tests available for screening *Campylobacter* isolates, we hope that the rapid hydrogenase assay will prove useful.

Catalase-positive *Campylobacter* species are associated with a variety of human and animal diseases (1, 8, 13–15). *C. jejuni* and *C. coli*, inhabitants of the intestinal tracts of many animals and fowl (14, 15), are now recognized as common causes of human enteritis. Since the campylobacters are nonfermentative and do not catabolize sugars (15), few diagnostic tests are available that distinguish between members of this genus. The thermophilic species (*C. jejuni* and *C. coli*) are presently distinguished from the nonthermophilic species (*C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*) based on growth at 42°C but not at 25°C, sensitivity to nalidixic acid, and resistance to cephalothin (8, 14, 15). However, nalidixic acid-resistant strains of *C. jejuni* have been reported (4, 12). Additional differential tests have recently been reported by Razi et al. (12), based on the inability of *C. jejuni* to grow anaerobically with nitrate. Payne et al. (11) have confirmed this observation and reported the absence of nitrite reductase in the *C. jejuni-C. coli* group. Although these methods are useful in distinguishing members of the genus *Campylobacter*, they all involve additional growth-oriented tests.

Presently, there is only one rapid diagnostic test (hippurate hydrolysis) which separates *C. jejuni* from *C. coli* (3, 14). There are currently no rapid tests for differentiating *C. jejuni* and *C. coli* from subspecies of *C. fetus* other than initial isolation at 42°C. However, there are reports that some strains of *C. fetus* can also grow at this temperature (7). Our studies into the energy-yielding metabolism of *C. jejuni* indicated that *C. jejuni* isolates contain high levels of hydrogenase (5). This study reports on the distribution of hydrogenase in the various *Campylobacter* species and on a new rapid test for distinguishing between the species *C. jejuni* and *C. coli* and the subspecies of *C. fetus*.

MATERIALS AND METHODS

Strains. Stock cultures of *C. jejuni*, *C. coli*, *C. fetus* subsp. *fetus*, and *C. fetus* subsp. *venerealis* were obtained from the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va., and from the Centers for Disease Control, Atlanta, Ga. The following type or neotype strains were also used in this study: *C. jejuni* ATCC 33560, *C. coli* CIP 7080, *C. fetus* subsp. *fetus* ATCC 27374, and *C. fetus* subsp. *venerealis* ATCC 19438. Recent clinical isolates were obtained from local hospitals and identified by growth at 42°C, hippurate hydrolysis, and resistance to cephalothin. All strains were maintained in brucella semisolid medium (Difco Laboratories, Detroit, Mich.), prepared by adding 0.16% agar. Cultures were maintained at 37°C and transferred weekly.

Growth and preparation of cell extracts. Starter cultures for batch cultures were grown in 250-ml flasks containing 100 ml of brucella broth, incubated statically at 37°C for 24 h and shaken for an additional 24 h. Three 2-liter flasks containing 1.5 liters of brucella broth were inoculated with a starter culture and similarly incubated for 48 h. Cells were harvested during the late log phase by centrifugation at 10,000 × *g* for 15 min, washed once in argon-sparged (anaerobic) 50 mM potassium phosphate buffer (pH 7.0), and suspended to 1 g/2.2 ml of buffer. Cell extracts were prepared by passages through a French pressure cell, followed by

centrifugation at 21,000 × *g* to remove cell debris. The cell extract was then subjected to ultracentrifugation at 100,000 × *g* for 90 min to obtain the membrane vesicles.

For the rapid hydrogenase and formate dehydrogenase assays, the pellicle from 48-h-old brucella semisolid medium was removed with a pipette, washed once in anaerobic 50 mM phosphate buffer, centrifuged, and suspended to 2 ml in anaerobic buffer. Colonies were also removed from 48-h-old brucella agar plates, incubated under microaerophilic conditions (6% O_2, 5% CO_2, 89% N_2) at either 37 or 42°C, and examined for hydrogenase activity. Several colonies (three to four) were scraped off the agar surface to provide sufficient cells to match the turbidities of suspended pellicles from the semisolid medium (absorbancy at 680 nm = 0.15 to 0.3).

Biochemical tests. Hippurate hydrolysis was determined by the method of Hwang and Ederer (6) as described by Harvey (3). Microaerophilic growth at 25°C was determined on Oxoid blood agar base no. 2 (Oxoid Ltd., London, England) supplemented with ferrous sulfate, sodium metabisulfite, and sodium pyruvate (0.025% each). Hydrogen sulfide production was determined by suspending lead acetate strips of filter paper over semisolid brucella broth supplemented with 0.2% of either cysteine or sodium thiosulfate.

A rapid assay was developed for determining hydrogenase and formate dehydrogenase activities, using the redox active dye benzyl viologen (BV) as an indicator. The assay was performed in stoppered 5-ml Vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) containing the following per 2.5 ml: 0.15 ml of BV (2 mM final concentration), 2.15 ml of 100 mM potassium phosphate (pH 7.0), 0.1 ml of 100 mM potassium formate, and 0.1 ml of 100 mM dithiothreitol (DTT). Control tubes and hydrogen-containing tubes lacked formate, and the differences in volume were adjusted by the addition of phosphate buffer. For the hydrogenase test, hydrogen gas was bubbled through the tubes whereas both formate and control tubes were rendered anaerobic by sparging with water-saturated argon passed through a heated copper column to remove oxygen. Suspensions of washed cells (0.5 ml) were injected into the tubes through the rubber stoppers, and the tubes were incubated at 30°C for 20 min. A positive test was visually judged as a reduction of the BV to a dark-blue to deep-purple color within 5 to 10 min. Negative tubes remained colorless or light blue. After 20 min, any increase in color was judged to be due to slight endogenous metabolism and was therefore a negative result.

To test the possibility that hydrogenase was inducible, some cultures were incubated in anaerobe jars (Oxoid); hydrogen (20% [vol/vol]) was added to the air in the jars. Cultures were examined for hydrogenase activity at 24, 48, and 72 h. Conditions for incubating and harvesting these cells were as described for the aerobically grown cells.

Respiratory activities were determined for selected strains with an oxygen electrode apparatus as described previously (3). The oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) was interfaced to a microcomputer, and collection of data and determinations of respiration rate were made over an interval of 2 to 3 min, using a time-based program and linear regression analysis of all traces of oxygen consumption. The concentration of various substrates, as well as the method for determining hydrogen oxidation was as described previously (5). Hydrogenase and formate dehydrogenase activities for selected strains were also determined spectrophotometrically, using an extinction coefficient for BV of 9.2 mM^{-1} cm^{-1} at 546 nm (9). Stoppered 1-ml cuvettes (1-cm light path) containing 5 mM BV and 100 mM potassium phosphate buffer (pH 7.0) were rendered anaerobic by sparging with argon or hydrogen, and to initiate the reaction, membrane vesicles or cell extracts were injected into the cuvette with a syringe. In addition to hydrogen, 0.02 to 0.05 ml of 100 mM sodium pyruvate, 100 mM potassium formate, 100 mM sodium lactate or 100 mM sodium succinate was used as substrates. Protein was estimated by the biuret method, using bovine serum albumin as the standard (2).

Anaerobic growth with nitrate and with nitrous oxide. Anaerobic growth with nitrate and with nitrous oxide was determined with cells grown in tryptose broth (Difco) containing thioglycolate, with formate (0.3%) as the energy source and nitrate (0.1%) as the electron acceptor (11). The oxygen-poor medium was prepared in screw-capped tubes (16 by 125 mm) and flushed with oxygen-free argon before incubation. Nitrous oxide was bubbled through some tubes lacking nitrate to determine growth with nitrous oxide as the electron acceptor. Control tubes lacking nitrate were flushed with argon. Batch cultures of selected strains of *C. fetus* were grown anaerobically in stoppered 2-liter flasks containing 1.5 liters of tryptose medium. Formation of nitrite from nitrate was determined colorimetrically, and conversion of nitrous oxide to nitrogen gas was determined by observing the gas evolution (11). Ammonia production was determined qualitatively with Nessler reagent.

RESULTS

All strains of *C. jejuni* and *C. coli* oxidized hydrogen in our rapid hydrogenase assay (Table 1). Considerable hydrogenase activity was observed with all clinical isolates, which included both *C. coli* and *C. jejuni* strains. In general, the recent clinical isolates oxidized hydrogen more rapidly than did most of our stock strains, as evidenced by the time required for BV reduction (5 to 10 versus 10 to 15 min).

Strains of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* were hydrogenase negative in the BV assay. Attempts to induce hydrogenase activity in selected strains were unsuccessful. Interestingly, some of our stock strains of *C. fetus* contained low levels of hydrogenase activity. Generally, these strains reduced BV in 20 to 30 min and constituted approximately 20% of our strains. In the rapid hydrogenase assay, these strains appeared pale blue by 15 min but could be easily distinguished from the hydrogenase-positive species which were dark purple to black by 15 min.

Low levels of BV were relatively impermeable to the cytoplasmic membrane, as evidenced

TABLE 1. Biochemical tests and hydrogenase activities

Strain	H_2S[a] cysteine	Growth		Nitrate growth[b]	Hippurate hydrolysis[c]	BV reduction[d]	
		26°C	42°C			H_2	Formate
C. jejuni							
H840 (ATCC 29428)	+3	−	+	−	+	+	+
11641-8-76	+3	−	+	−	+	+	+
Holy Cross	+3	−	+	−	+	+	+
12019	+3	−	+	−	+	+	+
ATCC 33560[e]	+2	−	+	−	+	+	+
CJ-1	+3	−	+	−	+	+	+
CJ-3	+3	−	+	−	+	+	+
CJ-4	+4	−	+	NT[f]	+	+	+
CJ-996	+2	−	+	NT	+	+	+
H477	+4	−	+	NT	+	+	+
CJ-755	+2	−	+	NT	+	+	+
CJ-909	+3	−	+	NT	+	+	+
CJ-248	+3	−	+	NT	+	+	+
C. coli							
76-3227	+4	−	+	−	−	+	+
H325	+2	−	+	−	−	+	+
H550	+4	−	+	−	−	+	+
7080	+	−	+	−	−	+	+
CJ-2	+2	−	+	NT	−	+	+
CJ-5	+3	−	+	NT	−	+	−
CJ-197	+4	−	+	NT	−	+	+
CJ-368	+3	−	+	NT	−	+	+
CJ-314	+4	−	+	NT	−	+	+
C. fetus subsp. *fetus*							
1510 MB	−	+	−	+	−	−	+
18151-A-76	−	+	−	+	−	−	+
HCB	−	+	−	+	−	−	+
10296	−	+	−	+	−	−	+
7953-B-77	−	+	−	+	−	−	+
V8	+	+	−	+	−	−	+
Suis I	−	+	−	+	−	−	+
Suis VI	−	+	−	+	−	−	+
ATCC 27324[e]	+2	+	−	+	−	−	+
319-A-77	+	+	−	+	−	−	+
C. fetus subsp. *venerealis*							
UM-HS	+	+	−	+	−	−	+
B-24-MK	−	+	−	+	−	−	+
ATCC 19438[e]	+	+	−	+	−	−	+
80-4577 (unidentified)	+2	−	+	−	−	+	+

[a] Detection of hydrogen sulfide was performed by the lead acetate method by suspending paper strips over brucella semisolid medium in screw-capped test tubes (cysteine added to medium). +4, Strip was dark brown (strong positive); +, bottom edge of strip was light brown (weak positive); −, no color change (negative).

[b] Anaerobic growth with nitrate in tryptose broth.

[c] Determined as described by Harvey (3).

[d] Assays for formate dehydrogenase and hydrogenase were performed with 2 mM BV as described in the text.

[e] Type or neotype strain.

[f] NT, Not tested.

by the inability of whole cells of the various *Campylobacter* strains to reduce BV in the presence of succinate or lactate. These dehydrogenases are located internally to the cytoplasmic membrane (5). When an equivalent volume of whole cells was disrupted by passage through a French pressure cell and then assayed for lactate and succinate dehydrogenase activities, BV was reduced (5 to 28 nmol/min per ml of equivalent volume of cell extract). Since the activities of succinate and lactate dehydrogenases were low, we added DTT to the reaction mixtures (whole cells and extracts) to hasten the reaction. In the absence of DTT, residual oxygen in the reaction

TABLE 2. Respiration rates of membrane vesicles of *Campylobacter* spp.

Strain	Respiration rate[a]			
	H_2	Formate	Succinate	NADH
C. jejuni				
H840	219.6	226.8	15.7	5.3
11641-b-76	82.1	81.5	5.9	5.9
Holy Cross	32.8	10.2	8.8	1.6
12019	67.4	19.5	11.6	1.8
C. coli				
76-3227	42.6	32.9	6.8	1.4
H325	93.5	NA	7.9	2.3
H550	85.5	73.7	16.6	3.9
C. fetus subsp. *fetus*				
151OMB	NA	14.5	10.4	5.6
18151A	27.6	12.0	8.3	6.1
HCB	NA	NA	4.4	2.5
10296	NA	21.7	22.5	11.1
7953-B-77	NA	3.5	4.7	1.8
V8	NA	7.4	4.5	2.8

[a] Respiration rates are expressed in nanomoles of oxygen consumed per minute per milligram of protein. Substrate concentrations: hydrogen, 1 mM; NADH, 1 mM; succinate, 20 mM; and formate, 5 mM. NA, No activity.

mixture caused a delay in the onset of BV reduction. The presence or absence of DTT had no effect on BV reduction in the presence of hydrogen, and DTT did not spontaneously reduce BV.

Table 2 shows the respiratory activities of membrane vesicles of selected *Campylobacter* strains. Respiratory activities with hydrogen and formate (hydrogenase and formate dehydrogenase) were generally much greater in *C. jejuni* and *C. coli* strains than were activities observed with NADH and succinate. Similarly, the respiratory activities of the *C. fetus* strains with formate were also much greater than those observed with succinate and NADH. Only *C. fetus* 18151-A-76 exhibited oxygen consumption in the presence of hydrogen.

The respiratory activities and spectrophotometric activities were comparable (Table 3). The spectrophotometric assay for hydrogenase was much more sensitive than the respiratory method. Four strains of *C. fetus* (Suis I, 319-A-77, 18151-A-76, and B-24-MK) exhibited hydrogenase activities ranging from 5 to 35 nmol/min per mg of protein. In contrast to the *C. fetus* strains, selected strains of *C. jejuni* and *C. coli* (either membrane vesicles or crude cell extracts) had hydrogenase and formate dehydrogenase specific activities ranging from 80 to 320 nmol/min per mg of protein. Formate dehydrogenase activity was observed in all but one of the *Campylobacter* strains by the BV assay.

All of the *Campylobacter* strains examined in this study reduced nitrate to nitrite in the tryptose medium, as evidenced by the appearance by 24 h of a deep-red color upon the addition of nitrate reagents to broth cultures. Interestingly, only the *C. fetus* strains continued to reduce nitrite to ammonia as reported previously (11). The increase in ammonia in the medium was detected with Nessler reagent, but no effort was made to quantify the amount of ammonia produced. Similarly, the intensity of the nitrite color reaction also decreased over the 5-day test period. The *C. jejuni-C. coli* strains grew poorly in this medium (absorbancy at 680 nm = 0.2) and appeared to lack the ammonia-producing nitrite reductase. In contrast, all of our *C. fetus* strains grew well anaerobically with formate and nitrate (absorbancy at 680 nm = 0.5 to 0.8). Attempts to grow our *Campylobacter* strains anaerobically in brucella broth with nitrate and formate proved unsuccessful. The inhibitory effect of brucella broth was not explored further. Some of the *C. fetus* strains (40%) were also capable of growing in tryptose medium (no nitrate) with nitrous oxide as the terminal electron acceptor and evolved nitrogen gas. Those strains that did grow with nitrous oxide had turbidities similar to those reported for nitrate-grown cells.

DISCUSSION

A rapid hydrogenase assay has been developed which may be useful in the separation of subspecies of *C. fetus* from the species *C. jejuni* and *C. coli*. The assay can be done within 20 min. All recent clinical isolates were found to possess high levels of hydrogenase. However, if the BV concentration is increased over the 2 mM level recommended in this study, several *C. fetus* isolates (20%) will appear positive within the 20-min period. This is also true if the assay temperature is raised to 37°C. Since there are few rapid tests for distinguishing among the various *Campylobacter* species, the hydrogen-

TABLE 3. Specific activities of various enzymes for *C. jejuni* and *C. fetus*

Enzyme	Sp act[a]	
	C. jejuni H840	*C. fetus* Suis I
Succinate dehydrogenase	10.8	NT
Pyruvate dehydrogenase	26	54
Lactate dehydrogenase	11.2	15.3
Hydrogenase	236	18.2
Formate dehydrogenase	146	212

[a] Specific activities reported as nanomoles per minute per milligram of protein. The enzymatic activities were determined in cell extracts with 5 mM BV. NT, Not tested.

ase test may have diagnostic, as well as taxonomic value. Although *C. jejuni* is separated from *C. coli* by the hippurate hydrolysis test (3, 4), the only methods for separating these species from *C. fetus* subspecies require additional growth-related tests, which may take 3 to 5 days (8, 14, 15).

The presence of hydrogenase in some *C. fetus* strains was confirmed with cell extracts, and preliminary studies suggest that this enzyme might be located internally to the cytoplasmic membrane. Additional studies are needed to resolve the location and function of the hydrogenase. We have previously reported that the hydrogenase of *C. jejuni* H840 is located externally to the cytoplasmic membrane and that hydrogen serves as a major energy source (5). Based on similar rates of reduction of BV by other strains of *C. jejuni* and *C. coli*, it is reasonable to assume a similar location for their hydrogenase. Formate dehydrogenase activity is also located on the periplasmic face of the cytoplasmic membrane in all of the *Campylobacter* species examined in this study. Since BV is generally impermeable to the cytoplasmic membrane (9, 10), enzymes located on the periplasmic side of the cytoplasmic membrane exhibit maximal activities, whereas enzymes located internally to the membrane exhibit activities lower than those observed in cell extracts. Enzymes, such as succinate dehydrogenase and lactate dehydrogenase, located internally to the cytoplasmic membrane exhibited lower activities with whole cells than with cell extracts. BV is preferred over methyl viologen because BV has a higher redox potential and reacts more rapidly with the dehydrogenases.

The presence of hydrogenase activity in the intestinal *Campylobacter* isolates may also suggest a role for hydrogenase in pathogenesis. This is supported by our finding that hydrogen is the most energy-efficient substrate oxidized by *C. jejuni* (5). Hydrogen produced by the anaerobic microflora of the intestine would be available to *C. jejuni* and *C. coli* strains during pathogenesis. Further, since *C. jejuni* and *C. coli* fail to grow anaerobically with alternative electron acceptors, their apparent requirement for oxygen may be met through invasion of the intestinal mucosal tissue where oxygen is present. Thus, it might be useful in future studies of the disease process of these intestinal campylobacters to consider their metabolic capabilities for survival, as well as for pathogenesis.

LITERATURE CITED

1. **Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravins, L. B. Reller, and W.-L. L. Wang.** 1979. *Campylobacter* enteritis: clinical and epidemiological features. Ann. Intern. Med. **91**:179–185.
2. **Gornall, A. G., G. J. Bardawill, and M. M. David.** 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **117**:751–766.
3. **Harvey, S. M.** 1980. Hippurate hydrolysis by *Campylobacter fetus*. J. Clin. Microbiol. **11**:435–437.
4. **Hébert, G. A., D. G. Hollis, R. E. Weaver, M. A. Lambert, M. J. Blaser, and C. W. Moss.** 1982. 30 years of campylobacters: biochemical characteristics and a biotyping proposal for *Campylobacter jejuni*. J. Clin. Microbiol. **15**:1065–1073.
5. **Hoffman, P. S., and T. G. Goodman.** 1982. Respiratory physiology and energy conservation efficiency of *Campylobacter jejuni*. J. Bacteriol. **150**:319–326.
6. **Hwang, M.-N., and G. M. Ederer.** 1975. Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. J. Clin. Microbiol. **1**:114–115.
7. **Janssen, D., and A. G. Helstad.** 1982. Isolation of *Campylobacter fetus* subsp. *jejuni* from human fecal specimens by incubation at 35 and 42°C. J. Clin. Microbiol. **16**:398–399.
8. **Kaplan, R. L.** 1980. Campylobacter, p. 235–241. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
9. **Kroger, A., E. Dorrer, and E. Winkler.** 1980. The orientation of the substrate sites of formate dehydrogenase and fumarate reductase in the membrane of *Vibrio succinogenes*. Biochim. Biophys. Acta **589**:118–136.
10. **Odom, J. M., and H. D. Peck, Jr.** 1981. Localization of dehydrogenases, reductases, and electron transfer components in the sulfate-reducing bacterium *Desulfovibrio gigas*. J. Bacteriol. **147**:161–169.
11. **Payne, W. J., M. A. Grant, J. Shapleigh, and P. Hoffman.** 1982. Nitrogen oxide reduction in *Wolinella succinogenes* and *Campylobacter* species. J. Bacteriol. **152**:915–918.
12. **Razi, M. H. H., R. W. A. Park, and M. B. Skirrow.** 1981. Two new tests for differentiating between strains of *Campylobacter*. J. Appl. Bacteriol. **50**:55–57.
13. **Skirrow, M. B.** 1977. *Campylobacter* enteritis: "a new" disease. Br. Med. J. **2**:9–11.
14. **Skirrow, M. B., and J. Benjamin.** 1981. '1001' Campylobacters: cultural characteristics of intestinal campylobacters from man and animals. J. Hyg. **85**:427–442.
15. **Smibert, R. M.** 1978. The genus *Campylobacter*. Annu. Rev. Microbiol. **32**:673–709.