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Isolation and Characterization of Two New Homoacetogenic Hydrogen-Utilizing Bacteria from the Human Intestinal Tract That Are Closely Related to *Clostridium coccoides*

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Two gram-positive, strictly anoxic, coccoid- to rod-shaped strains of bacteria, *Clostridium coccoides* 1410 and *C. coccoides* 3110, were isolated from human feces on the typical homoacetogenic substrates formate plus H_2 plus CO_2 (strain 1410) and vanillate plus H_2 plus CO_2 (strain 3110) in the presence of 2-bromoethanesulfonate to inhibit methanogenesis. On the basis of 16S rRNA sequencing, DNA-DNA hybridization, and physiological and morphological parameters, both isolates are closely related to *C. coccoides* DSM 935^T. The G+C contents of the DNA were 46.1 and 46.2 mol% for *C. coccoides* 1410 and *C. coccoides* 3110, respectively. Cytochromes could not be detected. Formate was degraded exclusively to acetate, whereas vanillate was *O*-demethylated, resulting in acetate and 3,4-dihydroxybenzoate, the latter being further decarboxylated to catechol. In the presence of organic substrates, H_2 was cometabolized to acetate, but both strains failed to grow autotrophically. Lactose, lactulose, sorbitol, glucose, and various other carbohydrates supported growth as well. Untypical of homoacetogens, glucose and sorbitol were fermented not exclusively to acetate; instead, considerable amounts of succinate and D-lactate were produced. H_2 was evolved from carbohydrates only in negligible traces. Acetogenesis from formate plus H_2 plus CO_2 or vanillate plus H_2 plus CO_2 was constitutive, whereas utilization of carbohydrates was inducible. Hydrogenase, CO dehydrogenase, formate dehydrogenase, and all of the tetrahydrofolic acid-dependent, C_1 compound-converting enzymes of the acetyl-coenzyme A pathway of homoacetogenesis were present in cell extracts.

Dietary fiber such as pectin, cellulose, hemicellulose, resistant starch, and polyfructosanes, as well as monomeric dietary carbohydrates that escape digestion and absorption in the human ileum, enter the colon and are fermented by the colonic microflora. The short-chain fatty acids butyrate, propionate, and acetate are the main anionic fermentation products in the human colon and have been suspected of exerting beneficial effects on human health (39). It has been assumed that about 95% of the short-chain fatty acids produced in the colon are absorbed and utilized by the host. About 60 to 70% of the energy requirement of the colonic mucosa epithelial cells can be provided by the oxidation of butyrate produced by the colonic microflora (37).

The intestinal production of about 10 to 30 g of acetate daily (36) may be partly attributed to the activity of homoacetogenic bacteria (44), a group of strictly anoxic microorganisms capable of reducing CO_2 to acetate. Detailed information on the presence and activity of these organisms in the human intestinal tract and their physiological significance for their host is scarce. The biochemical pathway used by most homoacetogens is the acetyl-coenzyme A (CoA) pathway (Wood-Ljungdahl pathway) (28). Although strains differ in their ability to use various substrates, homoacetogenic bacteria as a group utilize sugars, one-carbon compounds such as formate, methanol, CO, and CO_2 plus H_2, as well as the methyl groups of methoxylated aromatic compounds, all of which are converted to acetate as the main fermentation product.

Homoacetogenic bacteria may be considered useful inside the human intestinal tract because of their proposed capability to decrease the intestinal H_2 concentration and to increase the acetate concentration of the colonic content. The synthesis of [^{13}C]acetate from $^{13}CO_2$ as a major human colonic microbiological process was demonstrated for the first time by Lajoie et al. (26). Wolin and Miller (43) isolated from human feces three bacterial strains that produced acetate from CO_2. However, these isolates were not further classified. Very recently, Bernalier et al. (3) isolated several H_2-consuming acetogenic strains from human feces and Miller and Wolin (30) demonstrated the formation of [1,2-^{14}C]acetate from [3,4-^{14}C]glucose by fecal incubations, indicating that acetogenesis occurred by way of the Wood-Ljungdahl pathway.

A negative correlation exists between the activity and cell counts of methanogenic archaea on the one hand and those of autotrophic acetogenic bacteria on the other hand in humans (1, 13) and pigs (11). A competition for H_2 between these groups was shown recently (2).

MATERIALS AND METHODS

Isolation procedure. For enrichment and isolation of the two strains of homoacetogenic bacteria, a medium containing the following compounds per liter was used: 0.348 g of K_2HPO_4, 0.227 g of KH_2PO_4, 0.5 g of NH_4Cl, 1.16 g of NaCl, 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2$, 4.0 g of $NaHCO_3$, 3 ml of trace element solution, 1 mg of resazurin, 0.2 g of Casamino Acids, 0.2 g of tryptic peptone from casein, 0.4 g of yeast extract, 0.5 ml of 0.5% vitamin K_1 solution (in ethanol), and 0.6 g of L-cysteine · HCl. The pH was 6.8 to 7.1. The trace element solution described by Widdel et al. (42) was used except that both the nickel and cobalt concentrations were increased 10-fold. The gas atmosphere was 80% H_2 plus 20% CO_2 (subsequently referred to as H_2 plus CO_2) or 80% N_2 plus 20% CO_2 (subsequently referred to as N_2 plus CO_2). After being autoclaved, the medium was supplemented with 4 ml of filter-sterilized vitamin solution per liter of medium. The vitamin solution contained the following compounds per liter: 10 mg of biotin, 10 mg of folic acid, 50 mg of pyridoxine-HCl, 25 mg of thiamine-HCl, 25 mg of riboflavin, 25 mg of nicotinic acid, 25 mg of pantothenic acid-Ca^{2+} salt, 0.5 mg of vitamin B_{12}, and 25 mg of *p*-aminobenzoic acid.

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The following compounds served singly or in combination as growth substrates at a final concentration of 20 mM each: sodium formate, sodium vanillate, betaine, and methanol. During the enrichment period, sodium 2-bromoethanesulfonate was added from a filter-sterilized stock solution to a final concentration of 3 mM to inhibit growth of methanogens. Incubations were done under strictly anoxic conditions in rubber-stoppered tubes or bottles at 37°C. The anoxic techniques for medium preparation were those of Bryant (8). With H_2 plus CO_2 as the gas phase, the bottles were filled to only one-fifth to one-third of the total volume, and the cultures were shaken at 161 rpm on a rotary shaker. During the enrichment phase, the H_2 plus CO_2 atmosphere was pressurized to 140 kPa after inoculation. The gas was repressurized daily. Since gas pressurization did not improve the growth of the pure cultures, it was subsequently omitted.

Solid media were prepared by adding 1.5% agar to the media and pouring the media inside an anoxic chamber (Coy Laboratory Products, Grass Lake, Mich.) into petri dishes. The gas atmosphere inside the chamber consisted of 95 to 97% N_2 and 3 to 5% H_2. Enrichment cultures were diluted 10^6- to 10^{10}-fold, and aliquots of 0.5 to 1.0 ml were plated onto the agar plates. After inoculation, the plates were placed into anoxic jars and the gas atmosphere was replaced by H_2 plus CO_2 by evacuating the jars and flushing with the gas mixture to a final pressure of 140 kPa. The evacuation and flushing cycle was repeated three times.

For the enrichment procedure, fresh feces from a volunteer who had not undergone antibiotic therapy for the previous 4 months and whose breath methane levels did not exceed the trace levels of ambient air were anoxically diluted 10^6-fold in media and the bacteria present were grown on the substrates mentioned above under a gas atmosphere of both H_2 plus CO_2 and N_2 plus CO_2. Growth was monitored by measuring the change in turbidity at 600 nm with a spectrophotometer (Beckman, Munich, Germany). The optical path length of the cuvettes was 10 mm. The resazurin in the samples was kept colorless by adding a few grains of sodium dithionite. The optical density of uninoculated medium was subtracted from those of the samples, and samples whose optical densities exceeded 0.3 were diluted with medium. As a control, media without energy sources were inoculated in the same way under N_2 plus CO_2. Cultures exhibiting a greater increase in turbidity than the controls were used for further enrichment.

Determination of substrates and fermentation products. Enzymatic methods were used to determine concentrations of succinate (4), L-lactate (18), D-lactate (16), ethanol (5), formate (38), acetate (15), and glucose (25). Sorbitol was quantified with sorbitol dehydrogenase as follows. One assay mixture consisted of 0.667 ml of 0.1 M glycine (pH 9.5), 0.033 ml of 27 mM NAD solution, 0.333 ml of H_2O, and 0.03 ml of sample. The reaction was started with 7 μl of sorbitol dehydrogenase (240 U/ml) from sheep liver (Sigma Chemicals, Deisenhofen, Germany). The absorption at 365 nm was determined before the start of the reaction and after its completion. The difference in absorptions was used to calculate the sorbitol content of the sample. Since sugar alcohols other than sorbitol were absent in experiments with resting cells, the possible unspecific dehydrogenation of other sugar alcohols by sorbitol dehydrogenase was not taken into consideration.

Vanillate, 3,4-dihydroxybenzoate, and catechol levels were determined by high-performance liquid chromatography (HPLC; Gynkotek HPLC, Munich, Germany) at 40°C on a nucleosil reversed-phase C_{18} column (250 by 4.6 mm) with a particle size of 5 μm (Bischoff Chromatography, Leonberg, Germany). Methanol (20% [vol/vol]) in 0.1 M acetic acid was used as a chromatographic solvent. The compounds eluted were identified and quantified at 220 nm by comparison with appropriate standards.

For measurement of H_2 levels a Hewlett-Packard gas chromatograph (series 6890) equipped with a molecular sieve 5A capillary column (30 m by 0.32 mm by 12 μm film thickness) and having N_2 (1 ml/min) carrier gas was used. The temperatures of the oven and the thermal-conductivity detector were 40°C and 205°C, respectively. The injection volume was 0.5 ml. Methane in breath was quantified with the same gas chromatograph equipped with an HP-5 capillary column (5% phenyl methyl siloxane) and having N_2 (7.2 ml/min) carrier gas. The temperatures of the oven and the flame ionization detector were 40°C and 250°C, respectively. The injection volume was 0.5 ml. Calibration was done in the range of 20 to 100 ppm methane.

Determination of G+C content of the DNA. The base composition of the DNA was determined by HPLC after digestion of isolated DNA with P1 nuclease and alkaline phosphatase as reported previously (29) except that the methanol content of the chromatographic buffer was decreased to 8% and the temperature was increased to 37°C. Lambda DNA (base composition, 49.85 mol% G+C) served as a standard. DNA isolation from glucose-grown cells of the two isolates and of *C. coccoides* DSM 935^T, obtained from the DSMZ (formerly DSM; German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany, was performed as described previously for gram-positive cells (17).

Determination of phylogenetic parameters. Sequencing of the 16S rRNA gene of strain 1410 was done at the DSMZ after extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA, and subsequent sequencing of the purified PCR product as described previously (33, 35). Evolutionary distances were computed by using the correction of Jukes and Cantor (21).

DNA-DNA hybridization studies were also performed at the DSMZ by the thermal denaturation method described by DeLey et al. (12) with a Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer. DNA was isolated by chromatography on hydroxylapatite as described by Cashion et al. (9).

Preparation of crude extracts and determination of enzyme activities. Crude extracts were prepared from 1- to 2-liter cultures of cells grown on formate or vanillate under an atmosphere of H_2 plus CO_2 or on glucose or sorbitol under an atmosphere of N_2 plus CO_2. The cells were harvested anoxically by centrifugation (10,000 × *g*, 20 min, 4°C), and the resulting pellet was washed in 50 ml of anoxic buffer containing 50 mM potassium phosphate (pH 6.9), 5 mM $MgSO_4$, 5 mM dithioerythritol, and 1 mg of resazurin per liter as the redox indicator. The sedimented cells were resuspended in a final volume of 2.5 to 5 ml, homogenized, and passed once through a French pressure cell at 130 MPa. The cell lysate was centrifuged (3,000 × *g*, 30 min, 4°C) to remove unbroken cells and cell debris. The supernatant, referred to as crude extract, was analyzed for the presence of various enzyme activities as described previously (22, 23), with the exception that a Cary spectrophotometer (Varian, Darmstadt, Germany) was used. Protein contents of crude extracts were determined following the method of Bradford (6), with human serum albumin as a standard.

Anoxic conditions during centrifugation steps and cell lysis were maintained by performing all critical steps inside the anoxic chamber. During cell rupture, the lysate was collected via gas-tight tubing into rubber-stoppered anoxic tubes. For enzymatic assays, rubber-stoppered cuvettes flushed with N_2 were used. All additions were made by syringe.

Biochemical and physiological analyses. The biochemical features of the two isolated strains were partly determined with an automatic identification system for bacteria (Vitek system; bioMérieux, Nürtingen, Germany) according to the instructions of the manufacturer.

Catalase was tested for by placing a drop of H_2O_2 (30%) on a colony of bacteria. The production of gas bubbles indicated a positive reaction. Nitrate reduction to nitrite was measured by supplementing growing cultures with 10 mM sodium nitrate. After growth, 0.5 ml of the culture fluid was mixed with 0.5 ml of 1% sulfanilamide in 3 M HCl and 0.5 ml of 0.02% *N*-(1-naphthyl)-ethylenediamine hydrochloride. In the presence of nitrite, the solution turns pink to red. Uninoculated media treated in the same way gave negative reactions.

Nitrate reduction to N_2 was monitored in tubes containing medium supplemented with 0.5% agar and 10 mM pyruvate. Inoculation of the tubes was done while the agar was still liquid. The reduction of nitrate to N_2 was detected by monitoring the production of gas bubbles during growth. Uninoculated tubes served as controls.

Reduction of sulfate to hydrogen sulfide was determined after the cells had been grown on vanillate or formate under H_2 plus CO_2 or on 10 mM pyruvate under N_2 plus CO_2 in the presence of 10 mM sodium sulfate. Formation of hydrogen sulfide was detected by mixing equal volumes of culture supernatant and lead acetate (10%). The formation of a black precipitate indicated the presence of sulfide. Since the presence of cysteine in the standard media led to the formation of a black precipitate even when the media were not inoculated, these experiments were done in media in which cysteine was replaced by titanium(III) citrate as a reducing agent (45). Titanium(III) citrate was added to the media from an 88 mM stock solution until the resazurin turned colorless.

Experiments with resting cells. Experiments with resting cells were performed under strictly anoxic conditions. All manipulations for the preparation of the resting cells were done in an anoxic chamber containing a gas atmosphere of 95 to 97% N_2 plus 3 to 5% H_2. If not stated otherwise, cells were grown on the same organic substrate that was used for the resting-cell experiments. Growth on formate or vanillate was under H_2 plus CO_2, whereas growth on glucose was under N_2 plus CO_2.

Cells of the late logarithmic growth phase were harvested by centrifugation (10,000 × *g*, 20 min, 4°C) and washed twice in 50 mM anoxic potassium phosphate buffer (pH 6.8) containing 1 mg of resazurin per liter and 5 mM dithioerythritol as the reducing agent. The final cell sediment was resuspended in 5 to 10 ml of buffer and immediately used. The experiments were performed in 110-ml rubber-stoppered bottles by diluting the cells in the above-mentioned buffer supplemented with 4 g of $NaHCO_3$ per liter. The final volume of the cell suspension was 10 ml. Substrates were added from stock solutions to final concentrations of 10 to 20 mM. In experiments with vanillate as the substrate, the buffer was supplemented with 0.18 mM titanium(III) citrate as the reducing agent (45). The bottles were shaken in a water bath at 37°C. At defined time intervals, samples of 1 ml were taken by syringe and immediately cooled to 0°C followed by centrifugation at 4°C and 10,000 × *g* for 10 min. The supernatants were stored at −20°C until thawed for the determination of the concentrations of substrates and fermentation products.

Membrane preparation and cytochrome spectra. Cytoplasmic membrane preparations and low-temperature redox difference spectra to detect the presence of membrane-bound cytochromes after growth on formate or vanillate under H_2 plus CO_2 were done as described before (23), except that a culture volume of only 4 liters was harvested and DNase was omitted during cell rupture. For low-temperature redox-difference spectra a Cary spectrophotometer (Varian) was used. The temperature was decreased to −176°C by means of liquid nitrogen and an automated temperature controller.

Nucleotide sequences. The sequence of the 16S rRNA gene was deposited in the EMBL Nucleotide Sequence Database under accession number Y10584.

TABLE 1. Enrichment of homoacetogenic bacteria from human feces

Substrate	Gas atmosphere[a]	Growth[b]
Methanol	$N_2 + CO_2$	0.00
Methanol + formate	$N_2 + CO_2$	0.01
Methanol	$H_2 + CO_2$	0.00
Formate	$N_2 + CO_2$	0.00–0.03
Formate	$H_2 + CO_2$	0.14–0.39
Betaine	$N_2 + CO_2$	0.33–0.40
Betaine + formate	$N_2 + CO_2$	0.35–0.47
Betaine	$H_2 + CO_2$	0.28–0.44
Vanillate	$N_2 + CO_2$	0.01
Vanillate + formate	$N_2 + CO_2$	0.00–0.05
Vanillate	$H_2 + CO_2$	0.24–0.55
None	$H_2 + CO_2$	0.00

[a] The CO_2 content was 20% (vol/vol).
[b] The data given are final optical densities (for experimental details see Materials and Methods). The final optical density of cultures without added substrate (0.11) was subtracted from each measured value.

RESULTS

Isolation of two homoacetogenic bacterial strains from human feces. For enrichment cultures and isolation of homoacetogenic bacteria from the human gut, the growth substrates formate, methanol, betaine, H_2 plus CO_2 (autotrophic growth), and vanillate were used. All cultures with organic growth substrates were incubated under N_2 plus CO_2 as well as H_2 plus CO_2. In addition, the above-mentioned substrates were inoculated in combination with formate under N_2 plus CO_2. As controls, media without substrate and under N_2 plus CO_2 were inoculated. All cultures were supplemented with 3 mM 2-bromoethanesulfonate in order to inhibit growth of methanogenic organisms. The results of these experiments are given in Table 1. Methanol did not allow growth of the homoacetogenic enrichment cultures under any of these conditions. Formate and vanillate supported growth within 2 to 3 days, but only in the presence of H_2. Betaine was a growth substrate independent of the gas atmosphere. Growth on betaine occurred within 2 to 3 days. Autotrophic growth was not observed under these conditions, not even after incubation for several weeks. From the enrichment cultures containing formate under H_2 plus CO_2 or vanillate under H_2 plus CO_2, two strains were isolated by several cycles of plating on solid media followed by inoculation of liquid medium with a single colony. These two organisms were strain 1410 (isolated on formate plus H_2 plus CO_2) and strain 3110 (isolated on vanillate plus H_2 plus CO_2). Light beige colonies with diameters of 2 to 3 mm developed within two to three days with vanillate (under H_2 plus CO_2) or formate (under H_2 plus CO_2) as substrate and within one to two days with glucose as substrate. The organisms were coccoid to rod shaped and occasionally formed chains. The Gram staining was variable, i.e., actively growing cells displayed positive Gram staining, while with increasing age of the culture the cells tended to show negative Gram staining. The presence of cells exhibiting a gram-positive and a gram-negative reaction within the same cell chain indicated a weakening of the cell wall during growth. Therefore, the authentic Gram staining is reported as positive.

Substrate spectra and biochemical parameters. The substrates utilized by the two isolates are shown in Table 2. In addition to formate and vanillate, various mono-, di-, and trisaccharide sugars, including lactose and lactulose, and the sugar alcohol sorbitol were good growth substrates for both organisms. Growth on formate as well as on vanillate depended on the presence of H_2 plus CO_2 and was finished within 2 days, whereas the overnight growth on sugars or on sugar alcohols was independent of the gas atmosphere. For cultures growing under H_2 plus CO_2 a partial vacuum developed in the growth vials. This did not occur under N_2 plus CO_2. Growth on H_2 plus CO_2 alone and growth on betaine or methanol were never observed, even after incubation for several weeks. The doubling time on vanillate plus H_2 plus CO_2 was 6.5 h. Growth depended strictly on the presence of yeast extract.

Biochemical parameters were determined to obtain further information about the strains' physiological capabilities. Both strains behaved the same with respect to every parameter tested. They were able to hydrolyze the respective *p*-nitrophenyl derivatives of β,D-galactopyranoside, α,D-galactopyranoside, β,D-glucopyranoside, α,D-glucopyranoside, β,D-glucuronide, β,D-lactoside, α,D-mannopyranoside, α,L-fucopyranoside, β,D-fucopyranoside, and β,D-xylopyranoside. In contrast, the *p*-nitrophenyl derivates of both α,L-arabinofuranoside and *N*-acetylglucosamine were not hydrolyzed. Furthermore, the *p*-nitroanilide derivates of *N*-benzoyl-D,L-arginine, L-leucine, L-proline, L-alanine, L-lysine, and L-glutamate (γ-position) were not degraded. Catalase, phosphatase, phosphatidylcholinesterase, arginase, urease, and the synthesis of indole from tryptophan could not be detected. These results show that both strains were preferentially saccharolytic and not peptolytic.

The isolated organisms were unable to utilize sulfate or nitrate as electron acceptors. Both strains exhibited heat resis-

TABLE 2. Growth substrates for strain 1410 and strain 3110

Substrate (gas phase)[a]	Optical density[b] for strain:	
	1410	3110
Formate ($H_2 + CO_2$)	0.49	0.47
Vanillate ($H_2 + CO_2$)	0.48	0.44
Fructose ($N_2 + CO_2$)	0.82	1.10
Galactose ($N_2 + CO_2$)	0.83	0.93
Sorbose ($N_2 + CO_2$)	0.11	0.10
Mannose ($N_2 + CO_2$)	0.56	0.72
Glucose ($N_2 + CO_2$)	2.03	1.91
myo-Inositol ($N_2 + CO_2$)	0.36	0.50
Arabinose ($N_2 + CO_2$)	0.24	0.49
Ribose ($N_2 + CO_2$)	0.29	0.38
Xylose ($N_2 + CO_2$)	0.27	0.34
Lactose ($N_2 + CO_2$)	0.88	0.73
Lactulose ($N_2 + CO_2$)	0.95	1.15
Maltose ($N_2 + CO_2$)	0.76	0.96
Sucrose ($N_2 + CO_2$)	0.86	1.06
Cellobiose ($N_2 + CO_2$)	0.93	1.00
Trehalose ($N_2 + CO_2$)	1.05	1.21
Melezitose ($N_2 + CO_2$)	0.96	0.87
Raffinose ($N_2 + CO_2$)	0.83	0.85
Mannitol ($N_2 + CO_2$)	1.13	1.35
Sorbitol ($N_2 + CO_2$)	1.15	0.74

[a] CO_2 content was 20%. Substrate concentrations were 0.1 M (formate), 25 mM (vanillate), 20 mM (monosaccharides and sugar alcohols), 10 mM (disaccharides), and 7 mM (trisaccharides).
[b] The data given are final optical densities (for experimental details see Materials and Methods). The final optical density of cultures without added substrate (0.08) was subtracted from each measurement. Formate (0.1 M; N_2 plus CO_2), vanillate (25 mM; N_2 plus CO_2), methanol (20 mM; N_2 plus CO_2 as well as H_2 plus CO_2), betaine (20 mM; N_2 plus CO_2 as well as H_2 plus CO_2), erythrose (20 mM; N_2 plus CO_2), starch (0.1%; N_2 plus CO_2), inulin (0.1%; N_2 plus CO_2), and $H_2 + CO_2$ did not support growth at all.

TABLE 3. 16S rRNA sequence similarity values between *C. coccoides* 1410 and related taxa

Organism	% Similarity between *C. coccoides* 1410 and organism no.:									
	1	2	3	4	5	6	7	8	9	10
1. *C. coccoides* 1410										
2. *C. coccoides* DSM 935^T	99.8									
3. *R. productus* DSM 2950	95.0	95.1								
4. *R. hansenii* DSM 20583	95.7	95.7	92.4							
5. *R. obeum* ATCC 29174	93.5	93.5	90.7	94.4						
6. *Clostridium xylanolyticum* DSM 6555	92.3	92.3	89.4	91.5	90.4					
7. *Clostridium celerecrescens* DSM 5628	92.1	92.1	89.2	91.3	89.9	98.0				
8. *Eubacterium cellulosolvens* ATCC 43171	90.9	90.9	88.6	90.3	89.9	91.0	90.3			
9. *Clostridium symbiosum* DSM 934	91.5	91.5	88.8	90.7	89.9	91.2	91.1	89.0		
10. *Clostridium clostridiiforme* DSM 933	93.3	93.3	90.7	92.2	90.8	92.4	92.0	89.9	93.6	

tance (10 min at 80°C), reduced triphenyl tetrazolium chloride, were highly oxygen sensitive, and required a medium with sufficiently low redox potential. Even small amounts of oxygen, as indicated by the weak pink color of the redox indicator resazurin, inhibited totally the growth of the cells. In contrast to other homoacetogens such as *Clostridium thermoaceticum* and *Sporomusa* species, both isolates were devoid of membrane-bound cytochromes, as was evident from the absence of the typical cytochrome bands in redox-difference spectra (at −175°C) taken of isolated cytoplasmic membranes after growth of the cells on formate plus H_2 plus CO_2 or on vanillate plus H_2 plus CO_2 (spectra not shown). The lack of catalase activity in whole cells supports the view that heme proteins are absent from the isolates.

The identical substrate spectra and identical biochemical parameters of the two organisms led to the hypothesis that the same organism had been independently isolated twice. The identity of the protein patterns of both strains after sodium dodecyl sulfate-polyacrylamide gel electrophoresis supported this assumption. In addition, pulsed-field gel electrophoresis of chromosomal DNA digested with restriction enzymes *Fse*I and *Not*I showed identical DNA fragment patterns (DNA fingerprinting; data not shown).

Phylogenetic classification and DNA base composition. In order to determine the phylogenetic position of the isolated organisms, 1,471 bases of the 16S rDNA of strain 1410 were sequenced. The sequence indicated a close relationship to *Clostridium coccoides* (Table 3). In DNA-DNA reassociation experiments, strain 1410 and strain 3110 exhibited DNA similarities of 103 and 99.6%, respectively, to *C. coccoides*. The DNA similarity of strain 1410 to strain 3110 was 92.8%. Therefore, both isolates are not new species but instead represent new strains of *C. coccoides*. *C. coccoides* was isolated in 1976 from the feces of mice fed a high-lactose diet (24). Other closely related species (33, 34) are *Ruminococcus hansenii*, *Ruminococcus productus*, and *Ruminococcus obeum.*

The G+C content of the DNA as detected by HPLC analysis was 46.1 and 46.2 mol% for strain 1410 and strain 3110, respectively. The G+C content of the DNA from the *C. coccoides* type strain (obtained from the DSMZ) determined by the same method was 44.8 mol%. This is close to the value of 44.3 mol% previously reported for the type strain of *C. coccoides* (24).

Enzyme activities of the acetyl-CoA pathway. In order to substantiate the ability of the new isolates and of *C. coccoides* to reduce CO_2 to acetate via the homoacetogenic pathway, we determined the activities of the enzymes of the acetyl-CoA pathway (Wood-Ljungdahl pathway) in anoxically prepared crude extracts of both strain 1410 and *C. coccoides* DSM 935^T after growth on formate plus H_2 plus CO_2. It is obvious from Table 4 that all enzymes of the pathway were present. Based on these results, the organisms can be considered true homoacetogens in spite of their inability to grow on H_2 plus CO_2 in the absence of formate or vanillate. At present, we have no explanation for the strains' dependency on formate or vanillate for H_2 oxidation.

Comparable enzymatic activities were also detected in anoxically prepared crude extracts (data not shown) when the cells had been grown on glucose, formate plus glucose, and sorbitol, indicating that the acetyl-CoA pathway is also operating during growth on carbohydrates or sugar alcohols. This allows the conclusion that the enzymes of the acetyl-CoA pathway are constitutively expressed. The capability of reducing CO_2 to acetate even during heterotrophic growth enables the cells to reoxidize NADPH without the necessity to evolve H_2. This is in agreement with the observation that the strains only evolved traces of hydrogen (about 0.04 to 0.08 mmol of H_2/mmol of C_6 unit) during growth on the substrates glucose, sorbitol, lactose, and lactulose.

Fermentation products. In order to get further information about the catabolic potentials of the two isolated strains, resting cells were incubated with various substrates and the degradation products were analyzed. CO_2 was present in excess and therefore CO_2 levels were not determined.

Resting cells converted formate (under N_2 plus CO_2) as well as H_2 plus CO_2 exclusively to acetate. It is interesting to note,

TABLE 4. Enzyme activities in cell extracts of *C. coccoides* DSM 935^T and 1410[a]

Enzyme[b]	Sp act [μmol/(min × mg of protein)] of *C. coccoides* strain:	
	DSM 935^T	1410
CO dehydrogenase[c]	2.46	3.05
Formate dehydrogenase[c]	0.22	0.39
Hydrogenase[c]	2.14	2.17
10-Formyl-H_4F synthetase	2.57	2.12
5,10-Methenyl-H_4F cyclohydrolase[d]	0.03	0.08
5,10-Methylene-H_4F dehydrogenase[e]	3.93	4.41
5,10-Methylene-H_4F reductase[f]	0.26	0.14

[a] Cells were grown on formate plus H_2 plus CO_2, and crude extracts were prepared anoxically.
[b] H_4F, tetrahydrofolic acid.
[c] Benzyl viologen was the electron acceptor.
[d] Corrected for the spontaneous chemical hydrolysis of 5,10-methenyl-H_4F.
[e] Specific for $NADP^+$; does not react with NAD^+.
[f] Benzyl viologen was the electron donor; does not react with NADPH.

however, that growth on these substrates, when supplied individually, was not observed (Table 2). Fermentation balances were as follows (CO_2 neglected): 1 formate → 0.25 acetate and 1 H_2 → 0.30 acetate.

The theoretical values expected from the acetyl-CoA pathway are 0.25 mol of acetate per mol of formate and 0.25 mol of acetate per mol of H_2. Cells incubated with formate in the presence of 2 to 7% H_2 in the gas atmosphere cometabolized both substrates to acetate, i.e., neither formate nor H_2 inhibited the degradation of the other substrate.

Acetogenesis from vanillate (4-hydroxy-3-methoxybenzoate) by resting cells led to the accumulation of 3,4-dihydroxybenzoate and catechol (1,2-dihydroxybenzene), as is obvious from the following fermentation balance: 1 vanillate → 0.91 3,4-dihydroxybenzoate + 0.26 catechol + 0.73 acetate.

Supplementation of resting cells with 3,4-dihydroxybenzoate resulted in the production of catechol, but no acetate was formed. As is obvious from the fermentation balance, the aromatic system of vanillate remained intact. The theoretical value expected from the acetyl-CoA pathway is 0.75 mol of acetate per mol of vanillate since the methyl group of vanillate has to be disproportionated to acetate. Three-fourths of the methyl group is converted to the methyl group of acetate, whereas one-fourth is oxidized in order to gain reducing equivalents for the reduction of CO_2 to the carboxyl group of acetate. Similar to the results obtained with formate as substrate, acetogenesis from H_2 plus CO_2 by resting cells previously grown on vanillate was observed. In the presence of 1 to 4% H_2 in the gas phase, vanillate and H_2 were metabolized simultaneously to acetate. The decarboxylation of 3,4-dihydroxybenzoate to catechol has already been described for the homoacetogen *C. thermoaceticum* (20). Its significance was explained by the authors in terms of the gain of CO_2.

Cells grown on formate or vanillate were not capable of the degradation of glucose or sorbitol as resting cells. In contrast, cells grown on glucose or sorbitol were capable of fermenting formate or vanillate. This shows that the isolates have a preference for formate and vanillate over sugars and that the enzymes of the acetyl-CoA pathway are constitutively expressed.

The experiments in order to determine the fermentation balance for glucose led to the surprising finding of D-lactate and succinate in addition to acetate according to the following fermentation balance: 1 glucose → 1.84 acetate + 0.29 succinate + 0.44 D-lactate.

Whereas the formation of succinate had been reported for *C. coccoides*, the synthesis of D-lactate has not been described previously (24). L-Lactate, ethanol, and formate were not produced from glucose.

DISCUSSION

In this report the isolation from the human intestinal tract of two homoacetogenic bacterial strains closely related to *C. coccoides*, designated strain 1410 and strain 3110, is described. *C. coccoides* DSM 935^T was isolated in 1976 from the feces of mice fed a high-lactose diet (24) and was classified as a saccharolytic *Clostridium* species producing acetate and succinate. The homoacetogenic potential of *C. coccoides* DSM 935^T has not been investigated previously, but the results presented here demonstrate that *C. coccoides* DSM 935^T and the two closely related isolates are capable of acetate formation via the acetyl-CoA pathway with H_2 as the electron donor. These features are interesting since little is known about H_2-consuming and, especially, homoacetogenic bacteria in the human colon (2, 3, 26, 40).

Although the fermentation balances obtained for formate, H_2 plus CO_2, and vanillate are typical of homoacetogenic bacteria and although the acetyl-CoA pathway has been demonstrated in the two newly isolated strains as well as in *C. coccoides* DSM 935^T, it is still a question of definition whether they can be considered true homoacetogens or not. As long as homoacetogenic organisms are defined as organisms capable of reducing CO_2 to acetic acid via the acetyl-CoA pathway, *C. coccoides* DSM 935^T and the isolated strains can be considered as homoacetogens. However, when homoacetogenic bacteria are defined as bacteria that ferment 1 mol of glucose exclusively to 3 mol of acetate, *C. coccoides* DSM 935^T and both isolates are not homoacetogens because of the formation of considerable amounts of succinate and D-lactate in addition to acetate. The ability to synthesize from carbohydrates other products besides acetate was recently demonstrated for the homoacetogen *Peptostreptococcus productus* U-1. Under CO_2-limiting conditions, this organism produced lactate from fructose (31).

From the fermentation balance of glucose it is hard to estimate the amount of CO_2 refixation via the acetyl-CoA pathway. Additional experiments with [^{14}C]glucose or $^{14}CO_2$ have to be done to elucidate this question.

The assignment of *C. coccoides* to the genus *Clostridium* (24) was based solely on gram-positive staining, spore formation, and strictly anoxic growth. Recently, strong arguments were presented against *C. coccoides*'s being a true *Clostridium* species. Rainey and Janssen (34) found that *C. coccoides* is closely related to *R. productus*, *R. hansenii*, and *R. obeum* and suggested the regrouping of *C. coccoides* together with the above *Ruminococcus* species and *Ruminococcus gnavus*, *Ruminococcus lactaris*, *Ruminococcus torques*, *Clostridium oroticum*, and *Clostridium nexile* in a new genus. According to the nomenclature of Collins et al. (10), this phylogenetic group is positioned in the cluster XIVa.

The fermentations of lactulose and sorbitol by the isolated homoacetogens were accompanied by very little H_2 formation. This is an unusual and important feature in light of the observation that the consumption of lactulose or sugar alcohols by human subjects for dietary or medical reasons is usually associated with an increased production of H_2, resulting in intestinal bloating and flatulence as an undesirable side effect (27).

The formation of D-lactate from glucose or sorbitol is another important feature of the isolates and *C. coccoides*. Kaneuchi et al. (24) did not find lactate at all when *C. coccoides* was grown on glucose. Since colonic production of D-lactate is thought to be important in serum D-lactate acidosis, a complication seen in patients with intestinal bypass or short bowels (19), it might be interesting in the future to investigate the contribution of homoacetogenic bacteria similar to those described here to the production of D-lactate in the colon and the possible dietary effects.

The isolates' failure to grow on methanol is surprising since methanol is usually a typical homoacetogenic substrate (28) and may be available to homoacetogens during colonic fermentation of pectin. Furthermore, growth on vanillate shows the general ability of the isolates to metabolize methyl groups. A possible explanation for this finding might be the absence of a methanol-specific methyltransferase that initiates the degradation of methanol. The presence of inducible, substrate-specific methyltransferases in the homoacetogen *Sporomusa ovata* has been demonstrated (41).

The simultaneous acetogenesis from organic compounds and H_2 is called mixotrophy and has already been described for the homoacetogen *Sporomusa termitida* isolated from the gut of termites (7) and for acetogenic bacterial strains from human

feces (43). This is an interesting metabolic capability since both H_2 and formate are fermentation products of the colonic microflora. Although resting cells synthesized acetate from H_2 plus CO_2 and although all enzymes of the acetyl-CoA pathway as well as hydrogenase were present in cell extracts, autotrophic growth did not occur. The ability of acetogenic bacteria to convert H_2 and CO_2 to the nongaseous product acetate may be of importance for the reutilization of microbiologically produced H_2 and the prevention of flatulence and intestinal discomfort. Additionally, the host is supplied with acetate. When discussing the significance of homoacetogenic bacteria for the host with respect to the amounts of H_2 consumed and of acetate produced, several arguments have to be taken into consideration. (i) Cell counts have to be determined, but one has to be aware that cell counts in fecal samples do not necessarily represent the real situation in the colon. (ii) The significance of a species in an ecosystem is also dependent on the metabolic activity of that species. Since acetogenesis from formate plus H_2 plus CO_2 is expected to result in the formation of less than 1 mol of ATP per mol of acetate, the amount of substrate consumed and acetate produced in the course of this fermentation is much higher than during the conversion of carbohydrates. Even relatively low numbers of homoacetogens may significantly contribute to H_2 removal and therefore be of importance for the host.

Experiments by Doré et al. (13) indicate that acetogens compete with methanogenic and sulfate-reducing organisms for H_2. Doré et al. (14) also demonstrated by analysis of human and rat fecal samples that the ratio of acetogenic bacteria to total bacteria is higher in non-methane-excreting subjects than in methanogenic individuals. The possible microbiological, dietary, or immunological reasons for this observation are completely unknown. Similarly, in the rumens of newborn lambs the growth of acetogenic organisms was suppressed as soon as methanogenic organisms became established (32).

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