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Formate-Dependent Growth and Homoacetogenic Fermentation by a Bacterium from Human Feces: Description of *Bryantella formatexigens* gen. nov., sp. nov.

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Formate stimulates growth of a new bacterium from human feces. With high formate, it ferments glucose to acetate via the Wood-Ljungdahl pathway. The original isolate fermented vegetable cellulose and carboxymethylcellulose, but it lost this ability after storage at −76°C. 16S rRNA gene sequencing identifies it as a distinct line within the *Clostridium coccoides* supra-generic rRNA grouping. We propose naming it *Bryantella formatexigens* gen. nov., sp. nov.

Plant cell wall polysaccharides in human diets are not digested by host enzymes (3). The cellulose and hemicellulose in vegetables and fruits are digested in the colon (2, 11, 27) and are fermented by the colonic microbial community to molar ratios of ca. 56 acetate: 22 propionate: 22 butyrate (4, 32) and H_2, CO_2, and CH_4 (32). Bacteria that digest filter paper (FP) or Avicel are relatively unimportant in the human colon. Betian et al. (1) and Wedekind et al. (31) showed that the frequency of individuals that harbor them is low and, when present, their concentrations are ca. 0.001 times the concentration of all anaerobic bacteria. We hypothesized that bacteria that use amorphous cellulose found in vegetables, but not the crystalline cellulose in FP, are present in the colon. A goal of this study was to enumerate and isolate human colonic bacteria that use vegetable cellulose.

MPN study. We used a purified cellulose preparation from cabbage for most-probable-number (MPN) comparisons of the concentrations of bacteria that use FP cellulose, vegetable cellulose, and starch in human fecal suspensions. A modification of the method described by Ehle et al. (8) was used to prepare a cellulose-enriched fiber preparation (VCP) from fresh white cabbage (29). The hydrolysis of 1 g of VCP with 2 N HCl solubilized 287 mg of reducing sugar (glucose equivalent) (22). The insoluble residue contained 138 mg of glucose equivalents when measured by the anthrone procedure (26). Distilled water suspensions of the powder were ball milled for 18 h at 25°C prior to addition to media.

MPN analyses were run concurrently with 0.8% VCP, 0.5% corn starch (CS), or 1-cm by 5-cm strips of Whatman number 1 FP with 10 subjects and concurrently with VCP and FP cellulose with 15 subjects. The medium (B1C) contained (per liter): $NaHCO_3$, 5.0 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.3 g; $(NH_4)_2SO_4$, 0.3 g; NH_4Cl, 1 g; NaCl, 0.61 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $CaCl_2 \cdot 2H_2O$, 80 mg; $MnSO_4 \cdot H_2O$, 4.5 mg; $FeSO_4 \cdot 7H_2O$, 3.0 mg; $CoSO_4 \cdot 7H_2O$, 1.8 mg; $ZnSO_4 \cdot 7H_2O$, 1.8 mg; $CuSO_4 \cdot 5H_2O$, 100 μg; $AlK(SO_4)_2 \cdot 12H_2O$, 180 μg; $Na_2MoO_4 \cdot 2H_2O$, 100 μg; H_3BO_3, 100 μg; Na_2SeO_4, 1.9 mg; $NiCl_2 \cdot 6H_2O$, 92 μg; nitrilotriacetic acid, 15 mg; thiamine · HCl, 2.0 mg; D-pantothenic acid, 2.0 mg; nicotinamide, 2.0 mg; riboflavin, 2.0 mg; pyridoxine · HCl, 2.0 mg; biotin, 10.0 mg; cyanocobalamin, 20 μg; *p*-aminobenzoic acid, 100 μg; folic acid, 50 μg; cysteine · HCl · H_2O, 0.5 g; rumen fluid, 100 ml; sodium acetate, 2.5 g; sodium formate, 2.5 g; trypticase, 2.0 g. Resazurin (1 mg/liter) was added as an oxidation reduction potential indicator. The pH was adjusted to 7 with NaOH prior to gassing with 100% CO_2 and the addition of $NaHCO_3$. After dispensing into serum bottles and autoclaving under CO_2, a sterile solution containing 0.125 g each of cysteine and sodium sulfide/ml (30 μl per ml of medium) was added prior to inoculation. Incubation was at 37°C.

Table 1 shows the results of MPN analyses of enema samples of patients presenting for flexible sigmoidoscopy. Five tubes of B1C plus the indicated substrate were inoculated with each dilution of the enema samples. Disappearance of substrate in inoculated tubes indicated the presence of hydrolytic bacteria. FP hydrolysis was found in only 3 out of 15 subjects. The concentrations of FP-digesting bacteria were appreciably lower than those for bacteria that used CS or VCP. Our results on the frequency and concentrations of FP-digesting bacteria confirm those of Betian et al. (1) and Wedekind et al. (31). VCP-digesting bacteria were found in 11 of 15 subjects at ca. 16 times higher concentrations than those of FP-digesting bacteria. CS-digesting bacteria were present in all subjects examined, and their concentrations, similar to those previously found by colony enumeration (30), were the highest of the polysaccharide-digesting populations (ca. 45 times higher than those for VCP-digesting bacteria).

Isolation. We isolated a novel gram-positive bacterium after enrichment of 0.5 ml of 10^{-6} to 10^{-9} dilutions (five tubes per dilution) of one human fecal suspension (28) in B1C plus 0.8% VCP. Hydrolysis was monitored by observation of the disappearance of insoluble VCP. The sample yielded an MPN of 1.1×10^{10} hydrolytic bacteria per g of dry feces. Transfers were made from a tube with 0.5 ml of the 10^{-9} dilution to the same medium with VCP. Growth was also obtained when 2.0% carboxymethylcellulose type 4M6F (CMC) (Hercules Inc., Wil-

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TABLE 1. MPN concentrations of bacteria in human fecal suspensions that use vegetable cellulose, FP cellulose, or starch[a]

Substrate	Total no. of subjects	No. positive	Bacteria concn (per g of dry feces) ± SD
Vegetable cellulose	15	11	Log_{10} 9.66 ± 0.70
FP	15	3	Log_{10} 8.45 ± 0.40
Starch	10	10	Log_{10} 11.31 ± 0.41

[a] Samples with more than 4×10^6 bacteria per ml of fecal specimen. The dilutions necessary to detect samples with less than 4×10^6 bacteria per ml were not inoculated into MPN tubes.

mington, Del.) replaced 0.8% VCP. Transfers from the VCP medium were diluted and plated on WM medium with 2% agar and 0.6% CMC in anaerobic roll tubes (17). WM medium, with mineral concentrations based on those used by McInerney et al. (15), contained (per liter): $NaHCO_3$, 3.5 g; KH_2PO_4, 0.5 g; NH_4Cl, 1 g; NaCl, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.33 g; $CaCl_2 \cdot 2H_2O$, 50 mg; $FeCl_2 \cdot 4H_2O$, 1.5 mg; $CoCl_2 \cdot 6H_2O$, 0.2 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $MnCl_2 \cdot 4H_2O$, 0.03 mg; $CuCl_2 \cdot 2H_2O$, 0.01 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg; H_3BO_3, 0.3 mg; Na_2SeO_4, 1.9 mg; $NiCl_2 \cdot 6H_2O$, 0.02 mg; thiamine · HCl, 2.0 mg; D-pantothenic acid, 2.0 mg; nicotinamide, 2.0 mg; riboflavin, 2.0 mg; pyridoxine · HCl, 2.0 mg; biotin, 10.0 mg; cyanocobalamin, 20 μg; p-aminobenzoic acid, 100 μg; folic acid, 50 μg; cysteine · HCl · H_2O, 0.5 g; sodium acetate, 1.0 g; isobutyric acid, 0.54 ml; 2-methylbutyric, valeric, and isovaleric acids (0.6 ml each); casein hydrolysate, 2.0 g (Type I, No. C-9386; Sigma Chemical Co., St. Louis, Mo.); and resazurin, 1 mg. Adjustment of pH, the gas phase, $NaHCO_3$ addition, autoclaving, addition of cysteine and sodium sulfide, and incubation were the same as for B1C.

Pure-culture features. A culture derived from the transfer of an isolated colony to B1C plus 0.6% CMC was replated on WM plus 2% agar with 0.6% CMC. An isolated colony was transferred and grown on B1C with 2.0% CMC. The isolate, strain I-52^T, consisted of gram-positive, nonmotile short rods (ca. 1.2 by 0.7 μm) that grew mainly in pairs and short chains (Fig. 1). It grew in carbohydrate-containing medium. Poor growth occurred in the absence of formate. Strain I-52^T grew for 48 h in B1C medium with 18.5 mM glucose to an optical density at 660 nm (OD_{660}) (1-cm light path) greater than 2.0 with 2.5 or 25.0 mM formate. With 0.0 and 0.25 mM formate, the OD_{660} was 0.32 and 0.46, respectively. The inoculum was from an unwashed culture grown with 30 mM formate. Vanillate (24 mM), but not methanol (78 mM) or 80% H_2–20% CO_2, substituted for formate in the glucose-containing medium. In the presence of 54 mM formate, the isolate grew with added glucose, CMC, vegetable cellulose, stachyose, sucrose, lactose, maltose, galactose, mannose, or xylose. It did not grow with formate and Avicel, FP, lactate, starch, pectin, vanillate, syringate, methanol, 80% H_2–20% CO_2, or ethanol. No growth occurred with 37 mM formate in the absence of carbohydrates.

Strain I-52^T was further characterized by using the commercially available API Rapid ID32A (bioMérieux, Inc., Durham, N.C.) system according to the manufacturer's directions. Activity was detected for α-arabinosidase, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase, β-glucosidase, β-glucuronidase, and *N*-acetyl-β-glucosaminidase. No activity was detected for alkaline phosphatase, arginine arylamidase, arginine dihydrolase, alanine arylamidase, α-fucosidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucyl glycine arylamidase, phosphoamidase, phenyl alanine arylamidase, proline arylamidase, pyroglutamic acid arylamidase, serine arylamidase, tyrosine arylamidase, or urease. The organism was indole negative and did not reduce nitrate to nitrite.

The dependence of strain I-52^T growth on the addition of CMC to B1C broth was apparent from cell density measurements. It grew to a maximal OD_{660} of 1.0 (1.8-cm light path) in 24 h in a medium with 2% CMC added as the sole carbohydrate in B1C with added formate. With VCP containing 2 mg of cellulose/ml (26) instead of CMC, 70% (1.4 mg) of the cellulose disappeared after growth for 96 h (26). No cellulose utilization occurred when FP or Avicel was used as a growth substrate. Unfortunately, recent transfers from stock cultures maintained at −76°C for several years did not grow with CMC or the VCP. Other properties of the transfers were identical to those of the original isolate.

Fermentation. A brief report of the fermentation of the isolate was presented in previous publications (33, 34). Although 2.5 mM formate was sufficient for good growth of strain I-52^T, increasing the formate concentration ca. 20-fold dramatically altered the nature of the products formed from glucose (Table 2). Either without added formate or with 4.8 mM formate, succinate, lactate, and acetate are major products (Table 2). With 48 mM formate, acetate production increased considerably at the expense of succinate and lactate formation (Table 2). No H_2 was detected. Fermentations of glucose were conducted with added $NaH^{14}CO_3$ or $H^{14}COONa$ (18). Schmidt degradation of the acetate produced showed that fermentations with $NaH^{14}CO_3$ produced acetate with ^{14}C almost entirely in the carboxyl group of acetate (Table 3). The methyl group contained almost all of the ^{14}C in acetate when $H^{14}COONa$ replaced $NaH^{14}CO_3$ (Table 3). If CO_2 was the precursor of both carbons of the third acetate, then one third of all the carbon in the acetate formed by a homoacetogenic fermentation of glucose would be labeled by $NaH^{14}CO_3$. If the methyl of the acetate is formed from added formate and not from CO_2, then 1/6 of the acetate carbons (16.7%) would be labeled by $H^{14}COONa$ and 1/6 (16.7%) would be labeled by $NaH^{14}CO_3$. The percentage of the total acetate C that was labeled when $H^{14}COONa$ was used was 18% of the acetate carbon and 21% when the labeled substrate was $NaH^{14}CO_3$. This is consistent with the results of Table 3 that show that the methyl group of labeled acetate is produced from $H^{14}COONa$ and the carboxyl group is from $NaH^{14}CO_3$. Incorporation of $H^{14}COONa$ into the methyl group and $NaH^{14}CO_3$ into the carboxyl group of acetate establishes the operation of the Wood-Ljungdahl pathway in strain I-52^T.

Interspecies H_2 transfer between strain I-52^T and *Methanobrevibacter smithii* strain PS (DSM861^T) did not occur. I-52^T grew by itself with glucose and formate or in a coculture with *M. smithii* without formate but not with glucose alone. Using a washed inoculum, the OD_{660} (1-cm light path) of cultures grown for 48 h were 0.06, 1.78, and 1.67 with glucose alone, glucose plus formate, and glucose plus methanogen, respectively. Although the addition of the methanogen allowed

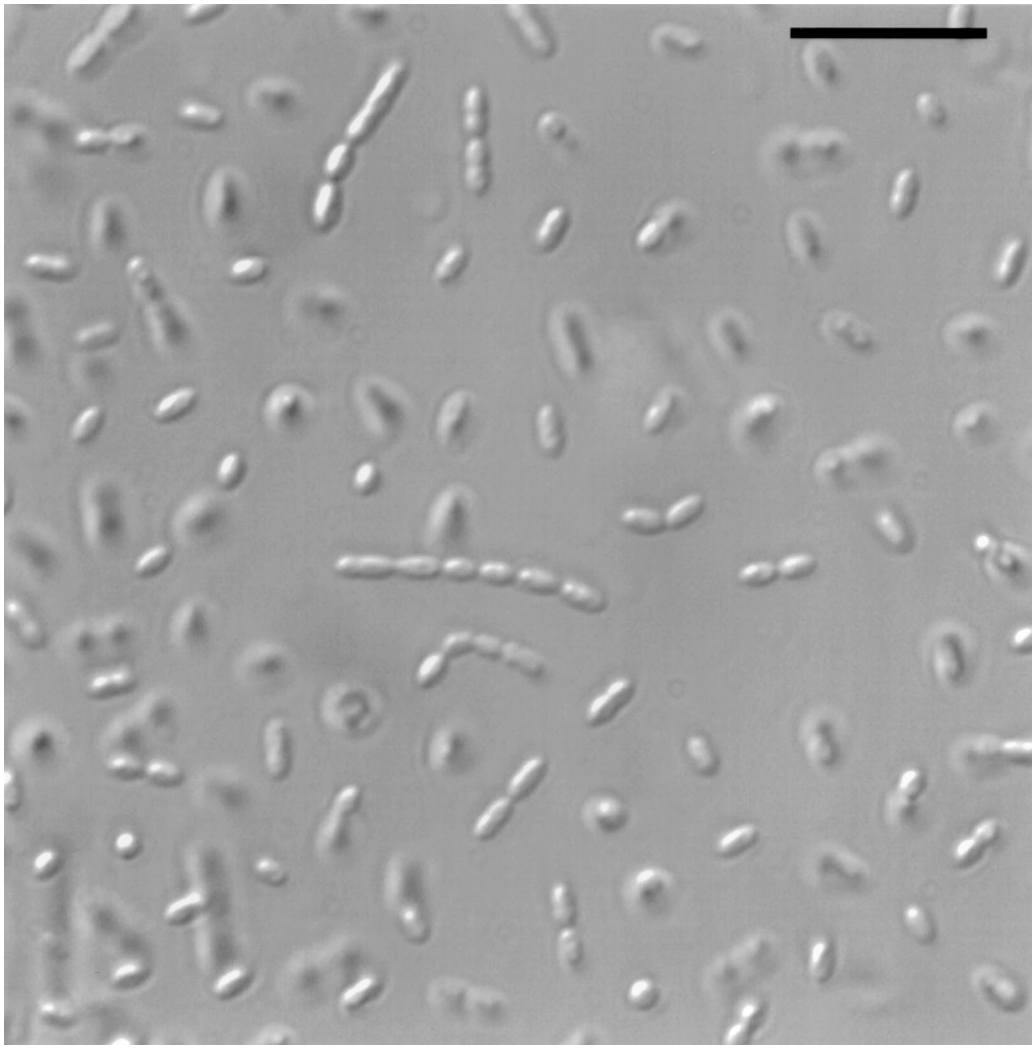

FIG. 1. Morphology of strain I-52^T. The strain was grown 18 h in B1C. A wet mount was examined with Nomarski optics. The bar represents 10 μm.

growth in the absence of formate, analysis of the fermentation products indicate incomplete interspecies H_2 transfer (32), i.e., only a small amount of methane was formed and succinate was a major product. No lactate was formed when the methanogen was present. Some interspecies transfer may have occurred at the expense of lactate but not succinate production. However, the major influence of the methanogen appeared to be the production of a nutrient that substituted for the low formate requirement for growth, and methanogenesis did not use electrons used for succinate formation by strain I-52^T.

The homoacetogenic fermentation of glucose by strain I-52^T

TABLE 2. Fermentation of glucose with different concentrations of formate[a]

Amt of added formate (mM)	Fermentation with the following substrates and products (mM):							C recovery (%)	O/R[b]
	Used			Formed					
	Glucose	Formate	CO_2	Succinate	Acetate	Lactate	CO_2		
0	14.3	0.0	5.8	11.6	10.0	7.0	0.0	95	1.00
4.8	23.2	4.8	9.3	18.6	23.1	13.0	2.8	106	1.03
48	25.1	27.2	2.2	4.4	70.3	1.6	13.6	98	1.00

[a] Cultures, inoculated with an unwashed inoculum, were incubated in B1C medium with 28 mM glucose without formate, except as indicated in the table, for 48 h. After gas analysis (23), cultures were acidified and analyzed by high-performance liquid chromatography (9, 18). The amounts of the acid products in the medium immediately after inoculation were similarly determined and were subtracted from the final values. The following equations were used to estimate CO_2 utilization and production: $3\ C_6H_{12}O_6 + 4\ HCO_2H \rightarrow 10\ CH_3COOH + 2\ CO_2 + 2\ H_2O$; $3\ C_6H_{12}O_6 + 2\ CO_2 \rightarrow 4\ HOOC(CH_2)_2COOH + 2\ CH_3COOH + 2\ H_2O$; and $C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$.

[b] O/R, oxidation equivalents/reduction equivalents.

TABLE 3. Labeling of acetate by $H^{14}COOH$ and $^{14}CO_2$[a]

Radiolabeled substrate	Sp act of:		
	Acetate	Acetate methyl	Acetate carboxyl
$H^{14}COOH$	762	593	32
$^{14}CO_2$	858	35	682

[a] Cultures were incubated for 48 h in 10 ml of B1C medium with a solution of 2 μCi of $NaH^{14}CO_3$ or 0.5 μCi of $Na^{14}COOH$, 30 mM glucose, and 25 mM formate.

with high concentrations of formate is reminiscent of that of *Syntrophococcus sucromutans* (13). *S. sucromutans* uses fructose when supplied with formate as an electron-accepting cosubstrate (13). Growth was also obtained with fructose without formate when *S. sucromutans* was cocultured with *M. smithii* strain PS. Apparently, *S. sucromutans* and strain I-52^T cannot reduce CO_2 to formate or produce formate from other nutrients. No formic dehydrogenase was detected in *S. sucromutans*, although an active CO dehydrogenase was found (5, 14). Formate is probably also used by both organisms for biosynthetic pathways, e.g., purine synthesis (10).

S. sucromutans cannot generate electron acceptors for metabolism of carbohydrates to acetate. It needs added formate or methoxy groups to produce intermediates of the Wood-Ljungdahl reactions that act as electron acceptors for carbohydrate metabolism. Acrylate side chains of benzenoid compounds or interspecies transfer of H_2 can substitute for formate or methoxy groups. In contrast, strain I-52^T produces its own electron sink reactions. It forms acetate, lactate, and succinate from glucose when supplied with low concentrations of formate. However, in the presence of high concentrations of formate, like *S. sucromutans*, it apparently produces intermediates of the Wood-Ljungdahl reactions and a homoacetogenic fermentation.

Significant steady-state concentrations of formate are not produced by the fermentation of the microbial community of the human colon (35). Added $H^{13}COOH$ is mainly converted to $^{13}CO_2$, and some ^{13}C is incorporated into the methyl group of acetate either by direct incorporation or after conversion of formate to CO_2 (35). The batch culture results in this study suggest that strain I-52^T would produce lactate, succinate, and acetate in the colonic environment. Drake (6) pointed out that acetogenesis by most acetogens is conditional and depends on the availability of a reductant and a terminal electron acceptor, including CO_2. The acetogen *Peptostreptococcus productus* U-1 produces lactate, succinate, and acetate under CO_2-limited conditions, and CO_2 enrichment increases acetate formation and decreases the formation of lactate and succinate (7, 19). Batch growth of strain I-52^T may be initiated with a homoacetogenic fermentation, with transition to other products when formate drops to much lower levels than the added 2.5 mM. Continuous cultures with varying levels of formate should reveal the transition point and aid in examining the mechanism of regulation of carbon flow to the alternative electron sink pathways.

Molecular characterization. The G+C content of the DNA was determined as described previously (16), except the methanol content of the chromatographic buffer was 8% and the temperature was 37°C. The G+C content was 50.3 mol%. An almost complete fragment of the 16S rRNA gene (ca. 1,450 bases) of strain I-52^T was amplified from DNA by PCR using universal primers pA (positions 8 to 28; *Escherichia coli* numbering) and pH* (positions 1542 to 1522) and was directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and an automatic DNA sequencer (model 373A; Applied Biosystems). The 16S rRNA gene fragments were generated by PCR and were sequenced as described by Hutson et al. (12). The closest known relatives of the new isolate were determined by database searches using the program FASTA (24). These sequences and those of other known related strains were retrieved from GenBank and were aligned with the newly determined sequences using the program DNATools (25). The resulting multiple-sequence alignment was corrected manually by using the program GeneDoc (20). A phylogenetic tree was constructed according to the neighbor-joining method with the programs DNATools and TREEVIEW (21), and the stability of the groupings was estimated by bootstrap analysis (1,000 replications) with the same programs. The 16S rRNA gene sequence of strain I-52^T has been deposited in GenBank under accession number AJ318527. Sequence database searches revealed that strain I-52^T was phylogenetically a member of the *Clostridium* subphylum of the gram-positive bacteria. Upon treeing analysis, the new isolate formed a hitherto unknown line of descent within the *Clostridium coccoides* rRNA group of organisms (Fig. 2). The sequence similarity to the nearest phylogenetic relative, *C. xylanolyticum*, was 92.5%. Sequence similarity comparisons revealed that strain I-52^T was only distantly related to other species within the *C. coccoides* group, with sequence divergence values of 7% or greater shown with all presently described members of this supra-generic grouping.

Morphologically, the short rod-shaped strain I-52^T somewhat resembles *S. sucromutans* but differs from the latter in staining gram positive and not requiring large amounts of rumen fluid for growth (13). Phylogenetically, strain I-52^T, like *S. sucromutans*, is a member of the *C. coccoides* rRNA supra-generic grouping. However, both sequence divergence (>10%) and treeing analysis show these organisms are phylogenetically only distantly related. Strain I-52^T forms a distinct line of descent and does not display a particularly close or a statistically significant phylogenetic affinity with any described species.

In addition to being distinct from *S. sucromutans*, strain I-52^T is phenotypically different from the plethora of other genera found within the *C. coccoides* rRNA complex. For example, in addition to its unusual homoacetogenic fermentation, strain I-52^T can be distinguished from *Clostridium* spp. and *Sporobacterium* in not producing endospores, from *Lachnospira* by the absence of curved cellular shapes, from *Roseburia* and *Butyrivibrio* in end products of glucose metabolism (i.e., not producing butyric acid), and in being nonmotile. It differs from *Coprococcus* and *Ruminococcus* in cellular morphology and in end products of glucose metabolism. Strain I-52^T is also metabolically distinct from the numerous putative *Eubacterium* species currently found within the *C. coccoides* rRNA complex. The putative *Eubacterium* species invariably displayed >10% sequence divergence with strain I-52^T and therefore cannot be considered members of the same genus. It is clear that the novel acetogen reported here is both pheno-

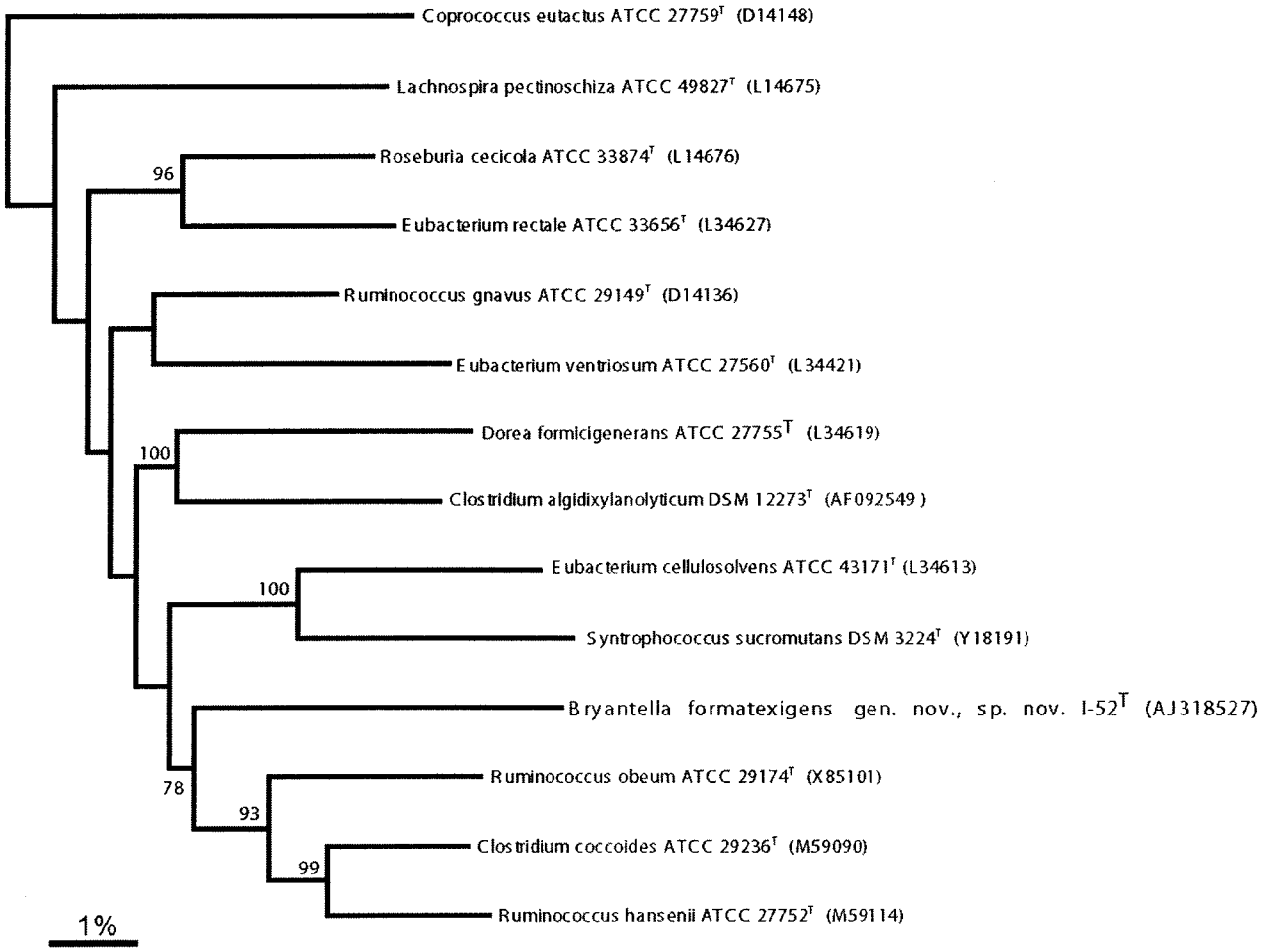

FIG. 2. Unrooted tree showing the phylogenetic relationships of strain I-52^T within the *C. coccoides* rRNA grouping. The tree, constructed by using the neighbor-joining method, was based on a comparison of ca. 1,300 nucleotides. Bootstrap values, expressed as a percentage of 1,000 replications, are given at the branching points.

typically and phylogenetically incompatible with all recognized genera within the *C. coccoides* rRNA cluster, and it merits classification as a new genus. Therefore, we propose the unknown rod-shaped bacterium be classified as a new genus and species, *Bryantella formatexigens.*

Description of *Bryantella*. *Bryantella* (Bry. an. tel'la. N.L. fem. n., named after the American microbiologist Marvin P. Bryant in recognition of his outstanding contributions to the microbial ecology of anaerobic ecosystems). It consists of short rod-shaped cells. Gram-positive, nonmotile, and non-spore forming. Anaerobic. Catalase and oxidase negative. Does not require rumen fluid for growth. Acetate is the sole product of glucose fermentation when grown in the presence of high concentrations of formate. Glucose fermentation with low concentrations of formate yield succinate, lactate, and acetate. Indole negative. Nitrate is not reduced. The G+C content of DNA is 50.3 mol%. The type species is *Bryantella formatexigens*.

Description of *Bryantella formatexigens* sp. nov. *Bryantella formatexigens* (for.mat.ex'i.gens. N.L. neut. n. *formatum* formate, L. part. adj. *exigens* demanding, N.L. adj. *formatexigens* formate-demanding).

Cells consist of nonmotile, short rods (ca. 1.2 × 0.7 μm) which occur mainly in pairs and short chains. Gram positive. Strictly anaerobic chemoorganotroph. Catalase-negative. Does not require rumen fluid for growth. Cellulolytic but may lose this activity upon prolonged storage at −76°C. Acetate is the sole product of glucose fermentation when grown in the presence of high concentrations of formate. Glucose fermentation with low concentrations of formate yield succinate, lactate, and acetate. In the presence of formate (54 mM), strain I-52^T grows with added glucose, vegetable cellulose preparation, carboxymethylcellulose, stachyose, sucrose, lactose, maltose, galactose, mannose, or xylose. Does not grow with formate and FP cellulose, Avicel, lactate, starch, pectin, vanillate, syringate, methanol, ethanol, or H_2–CO_2. No growth with 0.25% formate in the absence of carbohydrates. By using the commercially available API Rapid ID32A system, activity is detected for α-arabinosidase, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase, β-glucosidase, β-glucuronidase, and *N*-acetyl-β-glucosaminidase. No activity is detected for alkaline phosphatase, arginine arylamidase, arginine dihydrolase, alanine arylamidase, α-fucosidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucyl

glycine arylamidase, phosphoamidase, phenyl alanine arylamidase, proline arylamidase, pyroglutamic acid arylamidase, serine arylamidase, tyrosine arylamidase, or urease. Indole negative. Nitrate is not reduced. The G+C content of DNA is 50.3 mol%. The type strain is I-52^T = DSM 14469^T = CCUG 46960^T. Isolated from human feces.

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