RESEARCH ARTICLE

The Complete Genome Sequence of *Clostridium aceticum*: a Missing Link between Rnf- and Cytochrome-Containing Autotrophic Acetogens

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ABSTRACT ***Clostridium aceticum*** **was the first isolated autotrophic acetogen, converting CO_2 plus H_2 or syngas to acetate. Its genome has now been completely sequenced and consists of a 4.2-Mbp chromosome and a small circular plasmid of 5.7 kbp. Sequence analysis revealed major differences from other autotrophic acetogens.** ***C. aceticum*** **contains an Rnf complex for energy conservation (via pumping protons or sodium ions). Such systems have also been found in** ***C. ljungdahlii*** **and** ***Acetobacterium woodii*****. However,** ***C. aceticum*** **also contains a cytochrome, as does** ***Moorella thermoacetica*****, which has been proposed to be involved in the generation of a proton gradient. Thus,** ***C. aceticum*** **seems to represent a link between Rnf- and cytochrome-containing autotrophic acetogens. In** ***C. aceticum*****, however, the cytochrome is probably not involved in an electron transport chain that leads to proton translocation, as no genes for quinone biosynthesis are present in the genome.**

IMPORTANCE Autotrophic acetogenic bacteria are receiving more and more industrial focus, as CO_2 plus H_2 as well as syngas are interesting new substrates for biotechnological processes. They are both cheap and abundant, and their use, if it results in sustainable products, also leads to reduction of greenhouse gases. ***Clostridium aceticum*** **can use both gas mixtures, is phylogenetically not closely related to the commonly used species, and may thus become an even more attractive workhorse. In addition, its energy metabolism, which is characterized here, and the ability to synthesize cytochromes might offer new targets for improving the ATP yield by metabolic engineering and thus allow use of** ***C. aceticum*** **for production of compounds by pathways that currently present challenges for energy-limited acetogens.**

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The anaerobic autotrophic formation of acetate from a CO_2-H_2 gas mixture was first described in some detail in 1932 (1). Anaerobic sludge was used for those experiments, so that no single organism could be identified that was responsible for this metabolic activity. The first pure culture of such a bacterium was isolated by Wieringa (2), who later described it as *Clostridium aceticum* (3, 4). This is an anaerobic, endospore-forming bacterium, able to grow autotrophically on a CO_2 plus H_2 gas mixture as well as heterotrophically on sugars, organic acids, and alcohols (3, 5). Unfortunately, the strain was thought to be lost in the late 1940s. Many attempts of reisolation failed until 1980 (6), and so the pathway of autotrophic acetogenesis (now called the Wood-Ljungdahl pathway) was elucidated by using *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) (for a review, see reference 7). Except for Wieringa, only the group of Barker and colleagues worked with *C. aceticum* in the 1940s, publishing a paper on the nutritional requirements of this organism (8). And it was in the culture collection of Horace A. Barker (University of California, Berkeley) that one of us (G. Gottschalk), during a sabbatical in 1979, rediscovered a spore preparation of the original *C. aceticum* (5), which thus became available for research again, after being apparently lost for 4 decades.

Shortly before, in 1977, another autotrophic acetogen was isolated, *Acetobacterium woodii* (9). Detailed investigations on its bioenergetics revealed a different mode of energy conservation. *A. woodii* is sodium dependent, uses a so-called Rnf system for generation of a sodium ion gradient, and possesses an Na^+-dependent ATPase for ATP generation (10–12). *M. thermoacetica* instead contains cytochromes and menaquinone, and it obviously generates a proton gradient, which is used by a H^+-dependent ATPase for energy conservation. A respective scheme has been proposed by Das and Ljungdahl (13). However, this organism grows poorly under autotrophic conditions (14). In his analysis of the rediscovered *C. aceticum*, Manfred Braun provided biochemical evidence for the presence of cytochromes in this bacterium, which was only published in his Ph.D. thesis (15). As *C. aceticum* shows good growth with a CO_2+H_2 mixture, we performed genome sequencing in order to elucidate possible differences with respect to energy conservation in *M. thermoacetica*.

TABLE 1 Features of closed genomes of acetogenic bacteria

Species and strain/culture collection ID	Accession no(s).	Genome size (bp)	Gene count	CRISPR count	% GC	16S rRNA gene count
A. woodii WB1/DSM 1030	CP002987	4,044,777	3561	2	39	5
Ah. arabaticum Z-7288/DSM 5501	CP002105	2,469,596	2438	1	37	5
Ca. hydrogenoformans Z-2901/DSM 6008	CP000141	2,401,520	2738	3	42	4
C. aceticum/DSM 1496[a]	CP009687, CP009688	4,207,069	3984	2	35	6
C. autoethanogenum/DSM 10061	CP006763	4,352,205	4131	4	31	9
C. difficile 630/DSM 27543[a]	AM180355, AM180356	4,298,133	3983	10	29	11
C. ljungdahlii PETC/DSM 13528	CP001666	4,630,065	4283	1	31	9
E. limosum KIST612/ATCC 8486	CP002273	4,276,902	4583	2	48	5
M. thermoacetica/ATCC 39073	CP000232	2,628,784	2634	2	56	1
T. kivui/DSM2030	CP009170	2,397,824	2378	3	35	4
Ta. phaeum PB/DSM 12270	CP003732	2,939,057	2897	11	54	3
Tr. primitia ZAS-2/DSM 12427	CP001843	4,059,867	3579	3	51	2

[a] The strain harbors a plasmid. *Ah.*, *Acetohalobium*; *Ca.*, *Carboxydothermus*; *Ta.*, *Thermacetogenium*; *Tr.*, *Treponema*.

RESULTS

General features of the *C. aceticum* genome. The major features of the *C. aceticum* genome in comparison to other completely sequenced acetogens are listed in Table 1. The circular genome has a size of 4.201 Mbp. In addition, the bacterium carries a plasmid of 5.72 kbp, whose existence had been shown previously by use of molecular biology tools (16). Two putative prophage regions (CACET_c15210-CACET_c15960 and CACET_c31610-CACET_c32220) were detected. The G+C content is 35.28%, there are two gene clusters encoding two CRISPR-Cas systems (CACET_c10480-CACET_c10550 and CACET_c22220-CACET_c22290), and 74 tRNAs and 6 rRNA clusters are present. The *C. aceticum* genome contains 3,853 predicted protein-encoding genes, of which 24.45% have not been assigned a tentative function.

As expected for an endospore-forming bacterium, the genes for the master regulator of sporulation, Spo0A, as well as those for sporulation-specific sigma factors (σ^H, σ^F, σ^E, σ^G, and σ^K) were identified (CACET_c18630, CACET_c36390, CACET_c00100/CACET_c15100, CACET_C21700, CACET_c21690, and CACET_c18180). As with all other clostridia, *C. aceticum* does not carry genes encoding a phosphorelay (Spo0F and Spo0B), in contrast to the genus *Bacillus*.

The bacterium is motile by means of flagella. The respective genes are organized in four small clusters and one large cluster (CACET_c02310-CACET_c02330, CACET_c02510-CACET_c02770, CACET_c03000-CACET_c03010, CACET_c03140-CACET_c03160, and CACET_c20140-CACET_c20400). Chemotaxis genes (CACET_C20070-CACET_c20110) are present as well and are located adjacent to the large flagellum cluster, while *cheY* (CACET_c20210) is located within this cluster.

In addition to CO_2, H_2, and CO (17), *C. aceticum* is also able to use a fourth gas, i.e., N_2. Nitrogenase genes are located in a cluster (CACET_c13970-CACET_c14040), followed by genes encoding ferredoxin (CACET_c14050) and an ABC transporter for molybdate (*modABC*; CACET_c14060-CACET_c14080). A tungstate ABC transporter (*tupCBA*) is also present, but in a different location (CACET_c08690-CACET_c08710).

In the following sections, only coding sequences (CDS) involved in special metabolic features (the Wood-Ljungdahl pathway, energy conservation, and intermediary metabolism) and the small plasmid will be discussed.

Wood-Ljungdahl pathway genes. The cluster of genes encoding enzymes of the methyl and carbonyl branches of the Wood-Ljungdahl pathway is remarkably conserved within acetogenic bacteria. In *C. aceticum*, genes coding for both branches are organized in one cluster, showing an arrangement identical to that of *C. ljungdahlii* and all other acetogenic bacteria of the genus *Clostridium* (Fig. 1, CACET_c10200-CACET_c10340). This is also true for *Thermoanaerobacter kivui*, but in addition, genes coding for a thymidylate synthase, a formate transporter, different subunits of a hydrogen-dependent carbon dioxide reductase, and a molybdate ABC transport system, as well as genes for a lipoyl synthase and an octanoyltransferase, are also localized in this cluster (Fig. 1, in gray). In *A. woodii* and *Eubacterium limosum*, genes coding for the methyl and carbonyl branches are located in two different regions. In all other bacteria depicted in Fig. 1, only the genes of the carbonyl branch of the Wood-Ljungdahl pathway are organized in one cluster. In addition, some genes of the methyl branch of the pathway are also part of this cluster (in different numbers for the different organisms). The remaining genes of the methyl branch are located elsewhere in the various genomes. It is also evident from Fig. 1 that phylogenetically closely related organisms show an identical or very similar arrangement of the Wood-Ljungdahl genes. *acsA* and *cooC* encode the CO dehydrogenase, *fhs* encodes the formyltetrahydrofolate (THF) synthetase, *fchA* encodes the methenyl-THF cyclohydrolase, *folD* encodes the methylene-THF dehydrogenase, *metV* and *metF* encode the two subunits of methylene-THF reductase, *lpdA* encodes a dihydrolipoyl dehydrogenase, *cooC1* encodes an accessory protein for Ni^{2+} insertion, *acsD* and *acsC* encode the two subunits of the corrinoid/Fe-S protein, *acsE* encodes the methyltransferase, *acsB* encodes the acetyl coenzyme A (CoA) synthase, *gcvH* encodes the lipoate-containing protein H of the glycine cleavage system, and *acsV* encodes a ferredoxin. As in *C. ljungdahlii*, *C. autoethanogenum*, and *C. carboxidivorans* (20), a binding site for the redox-responsive repressor Rex (TTGTTATATAATTAACAA) is located upstream of the cluster, which contains just one mismatch to the consensus (TTGTTAANNNNTTAACAA).

For converting CO_2 to formate, a reversibly acting formate dehydrogenase is required. *C. aceticum* contains a non-selenocysteine-containing paralog (CACET_c07250) and a selenocysteine-containing paralog (CACET_c32700); the latter one is directly associated with a gene cluster coding for an iron-only hydrogenase (CACET_c32700-CACET_c32740). The genome of *C. aceticum* contains three additional iron-only hydrogenase gene clusters comparable to those identified in *A. woodii* (21) (CACET_c03920-CACET_c03940, CACET_c07230-CACET_c07250, and CACET_c35700-CACET_c35750) but does

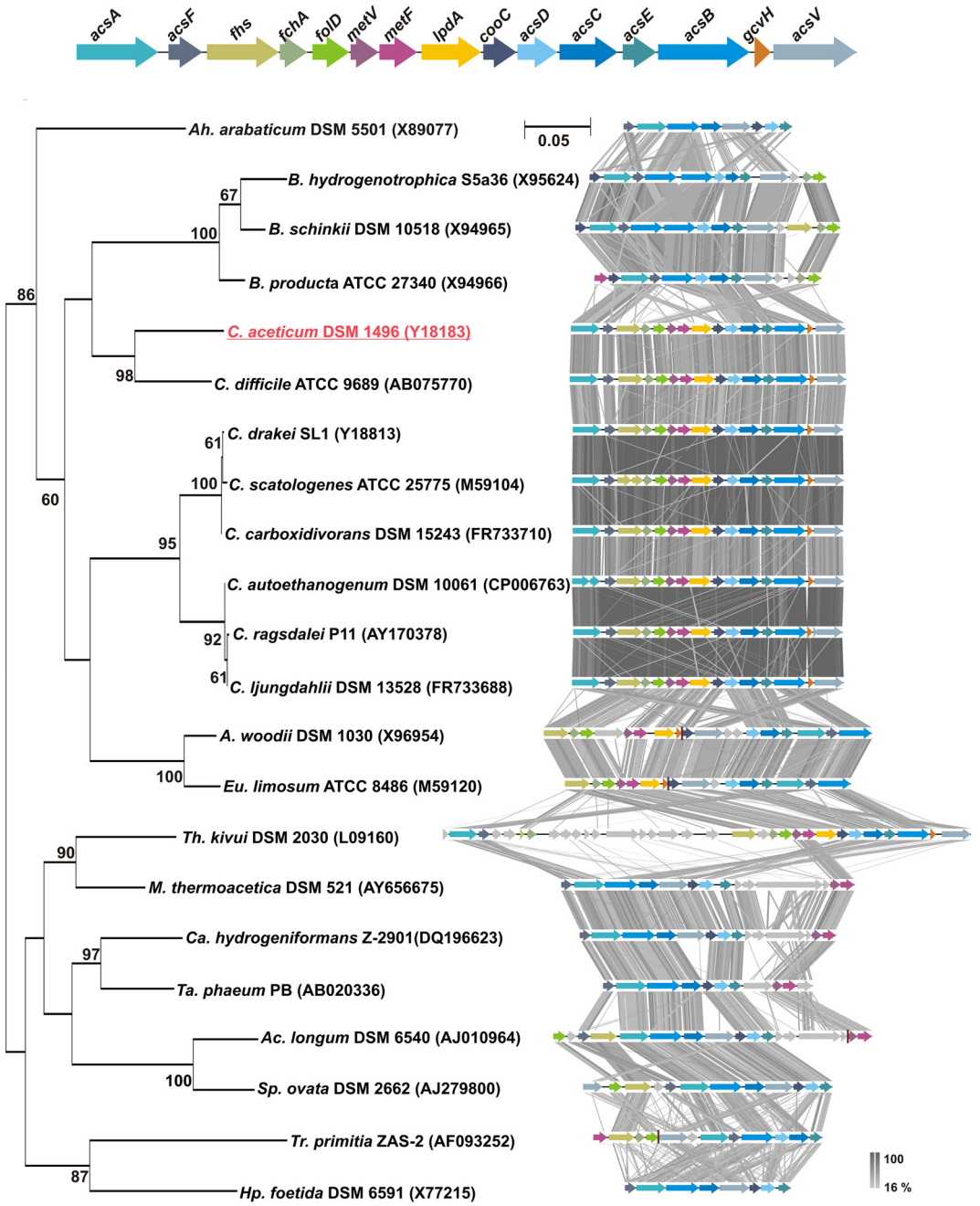

FIG 1 TBLASTx comparison of the Wood-Ljungdahl and CO-dehydrogenase/acetyl-CoA synthetase gene clusters of *C. aceticum* with all other autotrophic acetogens sequenced to date, in comparison with the phylogeny of these organisms based on 16S rRNA gene sequences. For the comparison, an *E value* cutoff of 1e−10 was used, and visualization of the gene clusters was done with the program Easyfig (18). Only genes associated with at least one other gene involved in the Wood-Ljungdahl pathway are depicted here. In some genomes, the Wood-Ljungdahl pathway genes are organized in separate clusters localized in different regions. In these cases, borders between the single clusters are marked with red vertical lines. The dendrogram was prepared using MEGA6 (19) and the maximum likelihood method. The clustering of the sequences was tested by a bootstrap approach with 1,000 repeats. The lengths of the tree branches were scaled according to the number of substitutions per site (see size bar). *C. aceticum* is underlined and marked in red. The following abbreviations for genus names were used: A, *Acetobacterium*; Ah, *Acetohalobium*; Ac, *Acetonema*; B, *Blautia*; Ca, *Carboxydothermus*; C, *Clostridium*; Eu, *Eubacterium*; Hp, *Holophaga*; M, *Moorella*; Sp, *Sporomusa*; Th, *Thermoanaerobacter*; Ta, *Thermacetogenium*; Tr, *Treponema*.

not harbor an iron-nickel hydrogenase. Sequence comparisons indicated that *C. aceticum* does not contain an Ech-type hydrogenase (energy-converting hydrogenase, which is able to generate a proton gradient across the cytoplasmic membrane), but it contains two gene clusters coding for a multisubunit sodium/proton antiporter (CACET_c29680-CACET_c29750 and CACET_c33270-CACET_c33340). Two genes encoding a formate hydrogen lyase are directly localized upstream of the latter gene cluster (CACET_c33250-CACET_c33260).

Growth on CO (17) is possible by employing an additional CO

dehydrogenase (CO-DH). The respective genes encode the catalytic subunit of a nickel-dependent, anaerobic-type CO-DH as well as a FAD/NAD^+-dependent oxidoreductase (CACET_c16200-CACET_c16210).

Energy conservation during autotrophic growth. The presence of a cytochrome has been demonstrated via redox difference spectroscopy at room temperature and −196°C in cell-free extracts of *C. aceticum* (15). The bands obtained indicated a *c*-type cytochrome. Indeed, all genes required for synthesis of a cytochrome *c* could be identified in the genome of *C. aceticum* (*hemACDBL* [CACET_c00470-CACET_c00520], *hemE* [CACET_c22420], *hemG* [CACET_c38340], *hemH* [CACET_c21910], *hemN* [CACET_c17390, CACET_c23610, and CACET_c24640], *ccdA* [CACET_c29170], *resA* [CACET_27250], *resB* [CACET_c22000 and CACET_c22170], and *resC* [CACET_c22010 and CACET_c22160]). However, it came as a surprise that no genes required for the biosynthesis of quinones could be found. Neither *ubiA/B* nor *tmz* genes are present. Also, no genes encoding quinone-dependent enzymes (e.g., encoding succinate dehydrogenase) could be detected in the genome. Thus, generation of a proton gradient by means of an electron transport chain that includes cytochrome and quinone, as suggested for *M. thermoacetica* (22), is unlikely in *C. aceticum*. However, the genes encoding an Rnf complex, a membrane-located proton or Na^+ pump, driven by coupled ferredoxin/NAD^+ oxidation/reduction (the original designation stems from *Rhodobacter* nitrogen fixation, by which Rnf was first discovered), are present (CACET_c16320-CACET_c16370). As *C. aceticum* does not show a sodium dependence and its ATPase (encoded by CACET_c02130-CACET_c02220) does not contain an Na^+-liganding amino acid motif (23, 24), the Rnf complex probably generates a proton gradient, as found for *C. ljungdahlii* (25, 26). Thus, *C. aceticum* is the first acetogenic *Clostridium* species that contains both Rnf and a cytochrome, but apparently it does not use the latter for energy conservation. The Rnf complex and the ATPase seem to be the only energy-conserving system, in addition to a phosphotransacetylase/acetate kinase system, as no Ech-type hydrogenase could be identified. However, the ATP generated by acetate kinase is consumed during the formation of formyl-THF, so that Rnf and ATPase are responsible for net ATP formation.

Acetate formation in *C. aceticum* also differs from that in all other acetogens sequenced to date. An acetate kinase gene was found (CACET_c20710), but no *pta* gene encoding a phosphotransacetylase was revealed. However, such an enzyme activity could be clearly measured in crude extracts of *C. aceticum* grown under both autotrophic and heterotrophic conditions (0.65 and 0.59 U/mg of protein, respectively, in cells from the exponential growth phase). There are two genes that possibly encode a phosphotransbutyrylase (CACET_c10660 and CACET_c34950). In addition, *C. aceticum* contains three *pduL* genes. PduL has been described as an evolutionarily distinct phosphotransacylase with known transacetylase activity and is responsible for 1,2-propanediol as well as propionyl-CoA degradation in *Salmonella enterica* (27). The first *pduL* gene lies in a cluster of genes required for ethanolamine utilization/microcompartment/shell proteins (CACET_c03950-CACET_c04100). The second *pduL* gene (CACET_c30070) is also clustered with genes required for ethanolamine utilization/microcompartment/shell proteins. However, the third *pduL* gene (CACET_c21930) is an orphan and might be responsible *in vivo* for phosphotransacetylase activity.

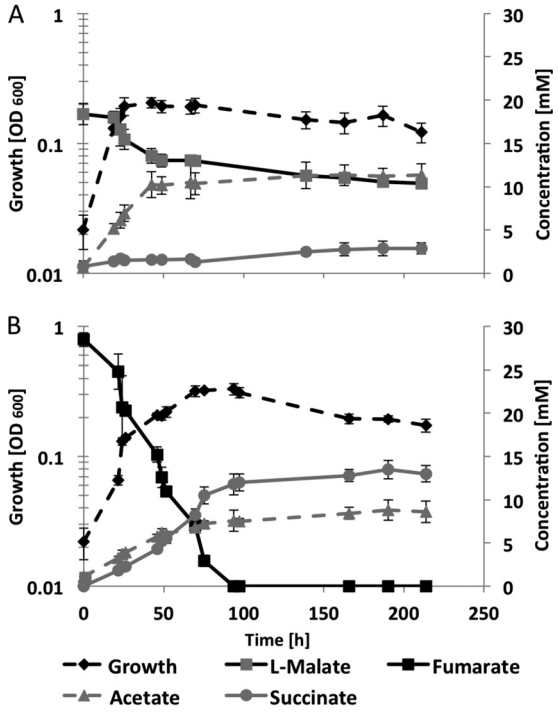

FIG 2 Growth, substrate consumption, and product formation by *C. aceticum* in batch culture. (A) Growth with L-malate; (B) growth with fumarate. Symbols: ◆, growth; ■, L-malate formation; ■, fumarate formation; ●, succinate formation; ▲, acetate formation.

Intermediary metabolism. CO_2 or CO are converted via the Wood-Ljungdahl pathway to acetyl-CoA. Pyruvate can be generated from reduced ferredoxin and acetyl-CoA by a pyruvate:ferredoxin oxidoreductase (encoded by CACET_c05500-CACET_c05530 and CACET_c25340-CACET_c25370). Formation of C_4 compounds is possible via a pyruvate carboxylase (encoded by CACET_c05600 and CACET_c19570); a phosphoenolpyruvate carboxykinase could not be found. As with most anaerobes, *C. aceticum* operates a branched tricarboxylic acid pathway, with one side leading to the formation of 2-oxoglutarate and the other to the formation of succinate. However, a citrate synthase gene could not be annotated.

Expanding the data obtained by Lux and Drake (17), growth and metabolic profiles of *C. aceticum* were compared using 20 mM of either fructose, fumarate, lactate, D-malate, D/L-malate, or L-malate as a substrate under a H_2 gas atmosphere. These batch experiments were repeated at least three times in 125-ml anaerobic bottles. *C. aceticum* could not use D-malate or D/L-malate as a carbon source. However, based on the culture optical density at 600 nm (OD_{600}), slight growth was observed (OD_{600}, 0.21 ± 0.01 [mean ± standard deviation]) with L-malate. The organism used 8 ± 0.4 mM of L-malate and produced acetate (11.4 ± 1.3 mM) as well as succinate (2.9 ± 0.6 mM) (Fig. 2A). Fumarate as a carbon source led to a higher OD_{600} (0.33 ± 0.03) and was used completely by *C. aceticum*. Fermentation products were acetate (8.6 ± 1.2 mM) as well as succinate (13 ± 0.9 mM) (Fig. 2B). As a control, growth of *C. aceticum* on fructose was monitored. A maximal OD_{600} of 1.23 was reached after 24 h, and only acetate (35 mM) was produced as a metabolic end product (data not shown). In none of the performed growth experiments was production of

TABLE 2 Occurrence in acetogens of systems involved in energy conservation

Species and strain/culture collection ID	Presence (+) or absence (−) of system				
	Rnf complex	Ech hydrogenase	Cytochromes	Quinones[a]	NfnAB
A. woodii WB1/DSM 1030	+	−	−	−	−
Ah. arabaticum Z-7288/DSM 5501	+	−	−	+	+
Ca. hydrogenoformans Z-2901/DSM 6008	−	+	+	+	+
C. aceticum/DSM 1496	+	−	+	−	−
C. autoethanogenum/DSM 10061	+	−	−	−	+
C. difficile 630/DSM 27543	+	−	−	−	+
C. ljungdahlii PETC/DSM 13528	+	−	−	−	+
E. limosum KIST612/ATCC 8486	+	−	−	−	−
M. thermoacetica/ATCC 39073	−	+	+	+	+
T. kivui/DSM 2030	−	+	−	−	+
Ta. phaeum PB/DSM 12270	−	+	−	+	−
Tr. primitia ZAS-2/DSM 12427	+	−	−	−	+

[a] Based on presence of key enzymes (UbiA, UbiD, UbiX, and UbiE). *Ah.*, *Acetohalobium*; *Ca.*, *Carboxydothermus*; *Ta.*, *Thermacetogenium*; *Tr.*, *Treponema*.

ethanol observed. In addition to previous reports, the utilization of acetoin could be shown, whereas arabinose was not metabolized. Neither fermentation nor production of lactate could be observed under any of the conditions tested.

The small plasmid. The small plasmid (5.72 kbp) of *C. aceticum* has a replication region consisting of *copG* (CACET_5p00070) and *repB* (CACET_5p00010). BLAST analysis of the RepB (rolling circle replication) protein sequence via NCBI's protein BLAST tool (BLASTp) revealed 47% identity to the RepB protein of *C. butyricum* (98% query coverage) and 46% identity to the replication protein RepB of *C. acidurici* (84% query coverage). Analysis of the CopG protein sequence revealed 58% identity to the CopG protein of *Streptococcus agalactiae* with a query cover of 60%. CopG (synonym, RepA) from *S. agalactiae* is known to play a role in regulating the plasmid copy number by binding to the *repAB* promoter (28). Thus, CopG presumably controls the synthesis of the plasmid replication initiator protein RepB (28). Five annotated genes of the small plasmid code for hypothetical proteins as well as transposases and show identity to other hypothetical proteins and transposases found in different *Clostridium* species. No annotated genes with essential functions could be found on this plasmid.

DISCUSSION

The most surprising feature arising from the *C. aceticum* genome sequence is the (so far) unique position with respect to energy conservation in autotrophic acetogens (Table 2). The closest autotrophic acetogenic relatives are *Clostridium difficile* AA1, *Clostridium glycolicum* DSM 1288, and *Clostridium mayombei* DSM 6539 (29). However, BLAST analysis of *C. aceticum* 16S rRNA gene sequences and a subsequent reconstruction of a phylogenetic tree indicated *Natronincola ferrireducens* was the closest relative (Fig. 3). This organism is described as an alkaliphilic, anaerobic, peptolytic, and iron-reducing bacterium (30). Inspection of the genome sequence revealed that *N. ferrireducens* also contains the Wood-Ljungdahl gene cluster, in an identical arrangement as found in *C. aceticum*. *N. ferrireducens* also contains gene clusters encoding sodium/proton antiporters highly similar to the ones from *C. aceticum*. It is noteworthy that such gene clusters can also be found in phylogenetically related bacteria whose genome sequences are publicly available. These include more distantly related bacteria, such as *N. peptidivorans*, *Alkaliphilus transvaalensis*,

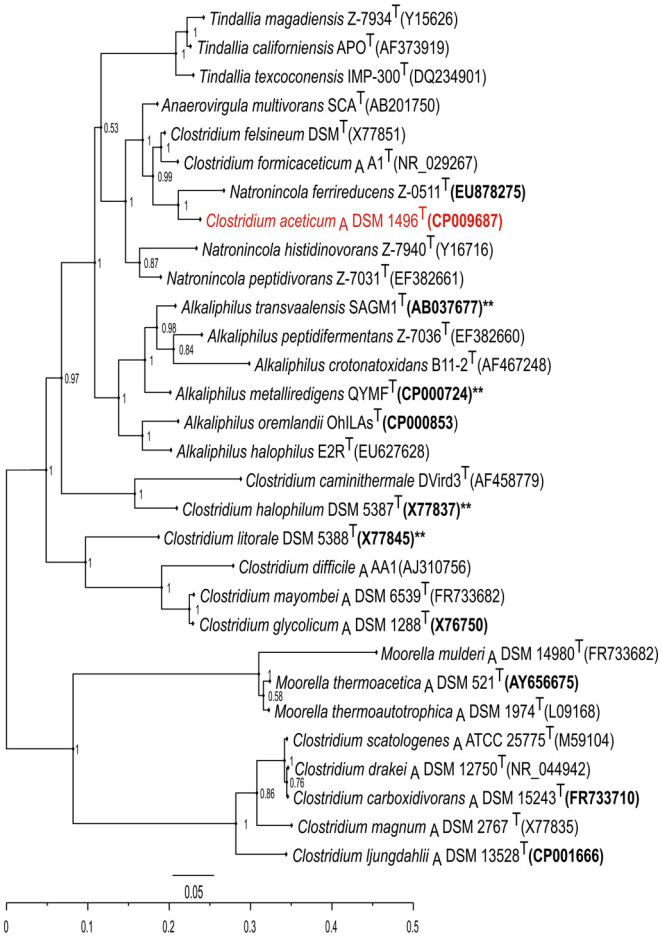

FIG 3 Phylogenetic tree based on bacterial 16S rRNA sequences from closely related bacteria of *C. aceticum* and selected autotrophic acetogenic bacteria. Accession numbers shown in bold indicate the availability of the genome sequence. Two asterisks after a strain name indicate the presence of a gene cluster coding for a multisubunit sodium/proton antiporter and formate:hydrogen lyase. An uppercase letter A following a name indicates that the strain has been described to be capable of autotrophic growth.

A. peptidifermentans, *A. metalliredigens*, *C. halophilum*, and *C. litorale* (Fig. 3). All of these bacteria are described as being alkaliphilic and may therefore be dependent on a sodium/proton antiporter.

In *C. aceticum*, the net ATP generation is obviously performed by an Rnf complex, which is probably proton dependent. However, as in *M. thermoacetica* one (or more) cytochrome(s) is present. In contrast to *M. thermoacetica*, the cytochrome(s) in *C. aceticum* cannot be used for generation of a proton gradient, as *C. aceticum* lacks the ability to synthesize quinones. This leaves the question as to the *C. aceticum* cytochrome function. In the archaeon *Methanosarcina acetivorans*, a cytochrome *c* is involved in the electron transfer from Rnf to the heterodisulfide reductase (31). Rnf might also be the electron donor for cytochrome *c* in *C. aceticum*, but the acceptor obviously might be different. Four nitrite reductase genes are present, but there is no gene encoding a nitrate reductase. Cytochrome-independent enzymes are encoded by *nirB* (CACET_c02780) and *nasD* (CACET_c16220). The other two nitrite reductase genes (CACET_c22020 and CACET_c22150) encode enzymes which are known to recruit cytochrome *c* for catalysis. So, this might be the physiological function of the cytochrome in *C. aceticum*. In *M. thermoacetica*, nitrate is a preferred electron sink over CO_2 when cells are grown on vanillin and vanillate, preventing acetate and favoring CO_2 formation (32). In this organism, the cytochrome was essentially absent in cells grown in the presence of nitrate (33). Also, the nitrite reductases of *M. thermoacetica* are, according to sequence homology, not cytochrome dependent; these findings therefore further strengthen the physiological function differences for cytochromes in *C. aceticum* and *M. thermoacetica*.

Another striking difference was the absence of a cluster of phosphotransacetylase and acetate kinase genes. In fact, a phosphotransacetylase could not be annotated. However, one or both of the annotated phosphotransbutyrylase genes or one of the *pduL* genes might take over this function. Phosphotransacetylase activity could clearly be determined in crude extracts, and acetate is the dominant fermentation product. As already mentioned, *pdu* genes in *S. enterica* are responsible for coenzyme B_{12}-dependent 1,2-propanediol (1,2-PD) degradation (27). PduCDE catalyze propionaldehyde formation (from which PduQ can generate propanol), and PduP catalyzes propionyl-CoA synthesis. PduL converts propionyl-CoA into propionyl phosphate, from which propionate is generated by PduW. PduL also exerts phosphotransacetylase activity (0.4 U/mg of protein), as shown by analysis of Pta^- and $PduL^-$ mutants (27). *pdu* genes have also been found in *A. woodii* and other acetogens (34).

Another important feature in some acetogens, including *M. thermoacetica*, *C. autoethanogenum*, and *C. ljungdahlii*, is the presence of an electron-bifurcating NAD^+-dependent reduced ferredoxin:$NADP^+$ oxidoreductase complex (NfnAB), which produces 2 molecules of NADPH from NADH and reduced ferredoxin (35, 36). In the two clostridia, *nfnA* and *nfnB* are fused into a single gene (35). No such gene(s) could be detected in *C. aceticum*. Also, *A. woodii* does not contain *nfnAB* genes (21). For *C. ljungdahlii* (25) and *M. thermoacetica* (37), the possibility has been discussed that the reduction of methylene-THF to methyl-THF could be a site of electron bifurcation. For *A. woodii*, such a possibility has been excluded (38). However, in *M. thermoacetica* the reaction is coupled via flavin-based electron bifurcation with the endergonic reduction of a still-unknown electron acceptor (37). Whether such a reaction also adds to energy conservation in *C. aceticum* cannot be answered yet. An alignment of methylene-THF reductases from *M. thermoacetica*, *A. woodii*, *C. aceticum*, *C. ljungdahlii*, and *C. autoethanogenum* shows almost complete identity of all important amino acid residues (based on data provided by Mock et al. [37], which show some inconsistencies in some amino acid positions). Thus, biochemical data are required to answer this question.

Finally, sodium/proton antiporters as found in *C. aceticum* are not present in *M. thermoacetica*, *C. ljungdahlii*, or *A. woodii*. Although we have not yet detected a sodium dependency of *C. aceticum*, there might be metabolic reactions which involve sodium ions or their transport across the cytoplasmic membrane. Thus, *C. aceticum* might not only represent the link between the cytochrome-containing, Rnf-lacking and the cytochrome-lacking, Rnf-containing autotrophic acetogens, but also it might close the gap between proton- and sodium-dependent species of this metabolic group. This might be further supported by the close relationship to *N. ferrioxidans*, which was isolated from a soda lake and is considered alkaliphilic.

A recent hypothesis was proposed, however, to bioenergetically classify acetogens into those containing either an Rnf complex or an Ech complex (38). This is based on the fact that the generation of a proton gradient by cytochromes and quinones in *M. thermoacetica* has not been biochemically verified yet and that *M. thermoacetica* carries genes with homology to those of an Ech complex. However, the functions of these respective gene products have not been verified either. The genome sequence of *T. kivui* was recently published, and this organism carries neither *rnf* nor cytochrome/quinone genes, but instead *ech* genes (39). So, in this case the proposed classification might be suitable, but for general acceptance the biochemical functions of cytochromes and quinones in *M. thermoacetica* need to be verified.

Two recent publications reported a very incomplete and a 53-contig *C. aceticum* genome sequence (40, 41). Probably due to this incomplete sequence, those authors missed all the essential features described above.

The fermentation of L-malate and fumarate by *C. aceticum* very much resembles the reactions described for *Clostridium formicoaceticum* (42). Fumarate is dismutated to succinate, acetate, and CO_2. Succinate stems from direct reduction of fumarate, while acetate and CO_2 are produced from pyruvate, which is derived via malate and oxaloacetate. Fumarate clearly represented the better substrate, yielding succinate as the dominant product. With L-malate, mostly acetate was formed. A lactate dehydrogenase gene (CACET_c16820) was found in the genome sequence of *C. aceticum*. However, under all conditions tested, neither lactate formation nor lactate utilization could be detected. The genome organization does not show a combination with *etf* genes, which encode an electron transfer flavoprotein. Such a pattern has been found in *A. woodii*, where a lactate dehydrogenase/Etf complex uses flavin-based electron confurcation to drive endergonic lactate oxidation with NAD^+ (43). Thus, the function of the putative lactate dehydrogenase in *C. aceticum* still remains to be elucidated. The lack of a citrate synthase gene appears puzzling at first glance. However, the *Re*-citrate synthase from *C. kluyveri* was found to be phylogenetically related to homocitrate synthase and isopropylmalate synthase (44). A subunit of isopropylmalate synthase in fact exhibits *Re*-citrate synthase activity. In *C. aceticum*, CACET_c09870 and CACET_c09880 are annotated as isopropyl-

malate synthase subunit genes, but the gene products do not show high homology in a BLAST search to the *C. kluyveri* enzyme. However, the proteins are in the same COG and KOG categories (COG0119, KOG2367) and contain the same Pfam domains (00682 and 08502) as their *C. kluyveri* counterparts. Most likely, such an enzyme replaces the missing *Si*-citrate synthase in *C. aceticum*.

In general, acetogens with a low pH optimum (e.g., *C. ljungdahlii* and *C. autoethanogenum*) form acetate and ethanol, whereas those with a more neutral pH optimum (e.g., *A. woodii*) produce only acetate under standard growth conditions. Having an alkaliphilic pH optimum of 8.3 (5), *C. aceticum* belongs rather to the latter group and, in fact, produces only acetate under standard growth conditions. However, the situation is certainly not only pH dependent, but also more complex. In the genome of *C. aceticum*, genes encoding seven alcohol dehydrogenases and two acetaldehyde dehydrogenases were found. Also, *A. woodii* has been reported to produce ethanol at high (40 mM), but not low (10 mM), sugar concentrations (45). A significant effect of phosphate concentration on ethanol production by *A. woodii* could be demonstrated. Maximal ethanol production occurred at 3.2 mM phosphate; starting at a concentration of 8.4 mM phosphate, ethanol was no longer produced.

A striking feature is the often highly conserved clustering of Wood-Ljungdahl pathway genes in acetogens (Fig. 1). Surprisingly, if all genes involved in this pathway are organized in one or at most two gene clusters, two genes of the glycine decarboxylase or glycine cleavage complex are found in these clusters as well, namely, the genes for protein 3 (*lpdA*, dihydrolipoamide dehydrogenase) and protein 2 (*gcvH*, lipoate-containing protein H of the glycine cleavage system). On the other hand, genes for the remaining proteins of the complex (P1 and P4) are always missing. A recent hypothesis proposed formatotrophic growth via a reductive glycine pathway that contains elements of the Wood-Ljungdahl pathway as well as the glycine cleavage or synthesis system (46). In this pathway, formate is converted to methylene-THF, which is converted to glycine and further to serine and pyruvate. However, for this pathway to function in acetogens, all four proteins would be required (P1 to P4). So, why then would only two genes be located in the Wood-Ljungdahl gene cluster? However, there might be another possibility. About 30 years ago, Pezacka and Wood described a CO dehydrogenase-disulfide reductase in *M. thermoacetica* (47). The enzyme seems to be involved in acetyl-CoA formation. An amino acid composition was reported (47); however, it was impossible to identify the respective gene product from the genome sequence of *M. thermoacetica* based on these data. So, *lpdA* and *gcvH* gene products might catalyze such a reaction. Biochemical experiments are required to prove or disprove this hypothesis.

MATERIALS AND METHODS

Bacterial strain. *C. aceticum* DSM 1496 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Brunswick, Germany) was used in this study.

Media and cultivation. *C. aceticum* was grown under strictly anaerobic conditions in a slightly modified medium described earlier by Braun et al. (5) that contained, per liter of distilled water, resazurin (0.5 mg), NH_4Cl (1.0 g), K_2HPO_4 (0.54 g), KH_2PO_4 (0.27 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), vitamin solution (20.0 ml), mineral solution (20.0 ml), yeast extract (2.0 g), $NaHCO_3$ (10.0 g); cysteine-HCl $\cdot H_2O$ (0.5 g), $Na_2S \cdot 9H_2O$ (0.5 g). The vitamin (with a 200-fold B_{12} concentration) and mineral solutions (with additional $Na_2SeO_3 \cdot 5H_2O$ [0.003 g], $Na_2Wo_4 \cdot 2H_2O$ [0.004 g]) described by Wolin et al. (48) were used. The pH of the medium after autoclaving for 15 min at 120°C was about 7.8. Then, the gas atmosphere was changed from N_2 plus CO_2 (80:20, vol/vol) to H_2 (100%). This resulted in a pH increase to approximately 8.5. Heterotrophic growth was carried out in 50 ml of medium in 125-ml anaerobic bottles (Fisher Scientific, GmbH, Schwerte, Germany) with butyl rubber stoppers, at 30°C. Autotrophic growth was carried out in 100 ml of medium in 500-ml Müller and Krempel bottles (Müller and Krempel AG, Bülach, Switzerland) and a synthesis gas atmosphere (CO at 50% [vol/vol], H_2 at 45% [vol/vol], and CO_2 at 5% [vol/vol]).

Analytical methods. Cell growth was monitored by measuring the OD_{600} with a Genesys 20 system (Thermo Electron, Dreieich, Germany). Samples (2 ml) for subsequent analysis of product concentrations were withdrawn with a syringe and centrifuged at 18,000 × *g* for 15 min at 4°C, and the supernatant was stored at −20°C. Acetate, ethanol, fructose, fumarate, D-malate, L-malate, D/L-malate, lactate, and pyruvate concentrations were determined using an Agilent 1260 Infinity series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Böblingen, Germany) equipped with a refractive index detector operated at 35°C and a diode array detector. The organic acid resin column (300 mm by 8 mm; CS-Chromatographie Service GmbH, Langerwehe, Germany) was kept at 60°C. Twenty-five millimolar H_2SO_4 was used as the mobile phase, with a flow rate of 0.7 ml min^{-1}. The samples were centrifuged again at 18,000 × *g* for 10 min at 4°C. Twenty microliters of the supernatant was injected into the HPLC system for determination of the compound concentrations.

Nucleic acid isolation and manipulation. Genomic DNA of *C. aceticum* was isolated by using the Epicentre MasterPure Gram-positive DNA purification kit (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) according to the instructions of the manufacturer.

Preparation of cell extracts. Crude cell extract of *C. aceticum* was obtained from 40 ml of grown cells (OD_{600}, 1.2). Cells were harvested in the anaerobic chamber in 50-ml centrifugation tubes (Sarstedt AG and Co., Nümbrecht, Germany) by centrifugation (9,418 × *g*, 10 min, 4°C). Cell pellets were washed twice using anaerobic buffer (Tris-HCl, 0.1 M; $MgCl_2 \cdot 7H_2O$, 5 mM; pH 7.2). Cells were transferred into Cryo tubes (Sarstedt AG and Co.) filled with 250 mg of glass beads (0.1 mm; Carl Roth, Karlsruhe, Germany). Cell disruption was carried out using a Ribolyser (Hybaid Ltd., Middlesex, United Kingdom) adjusted to a speed of 4 m s^{-1} for 45 s. This step was repeated six times and in between, cells were cooled on ice (5 min). Finally, cell debris was removed by centrifugation (15,493 × *g*, 30 min, 4°C), and crude extracts were transferred from the anaerobic chamber into Hungate tubes.

Enzyme assays. Crude extract was diluted 1:10, 1:20, 1:50, 1:100, and 1:200 in test buffer (K_2HPO_4, 49.5 mM, KH_2PO_4, 50.5 mM). A phosphotransacetylase enzyme activity assay was carried out photometrically as described earlier (49). Briefly, the reaction took place in a 1-ml airtight cuvette (Hellma Analytics, Müllheim, Germany) filled with N_2 (anaerobic) at 37°C. Acetyl-CoA solution (acetyl-CoA, 0.4 mM; 5,5′-dithiobis-2-nitrobenzoic acid, 0.08 mM; dissolved in test buffer) was injected into the cuvette by using a Hamilton syringe (Hamilton Company, Europe, Bonaduz, Switzerland), and background activity was measured for 5 min at 405 nm. Addition of diluted crude extract started the reaction, and absorption of 5-thio-2-nitrobenzoate was recorded for a further 5 min. Specific enzyme activities refer to total protein concentrations, as determined in a Pierce bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL, USA).

Sequencing strategy. The extracted genomic DNA of *C. aceticum* was used to generate 454 shotgun, paired-end, and Illumina Nextera XT shotgun libraries according to the manufacturer's protocols. A combined approach using a 454 GS-FLX system (Titanium GS70 chemistry; Roche Life Science, Mannheim, Germany) and a Genome Analyzer II apparatus (Illumina, San Diego, CA) was used to sequence the created libraries. Sequencing resulted in 374,679 total 454 shotgun reads containing 182,232 paired reads, with an average pair distance of 5.7 kb and a pair distance

deviation of 1.4 kb, and 2,280,716 Illumina 112-bp paired-end reads. The hybrid *de novo* assembly was performed with the Mira 3.4 software (50) and Roche Newbler Assembly software 2.3 for scaffolding, resulting in 77 contigs within 24 scaffolds with an average coverage of 76.97%. The remaining gaps were closed by PCR-based techniques and primer walking with Sanger sequencing of the products using BigDye 3.0 chemistry and an ABI 3730XL capillary sequencer (Applied Biosystems, Life Technology GmbH, Darmstadt, Germany). For this purpose, the Gap4 software (version 4.11) of the Staden package (51) was used.

Gene prediction and annotation. For automatic gene predictions, the software tool Prodigal (prokaryotic dynamic programming gene-finding algorithm) (52) was used, while identifications of rRNA and tRNA genes were performed with RNAmmer (53) and tRNAscan (54), respectively. Automatic annotation was carried out with the IMG-ER (integrated microbial genomes-expert review) system (55), but annotation was afterward manually curated by employing BLASTP and the Swiss-Prot, TrEMBL, and InterPro databases (56).

Nucleotide sequence accession numbers. The genome sequence for *C. aceticum* has been deposited at GenBank/DDBJ/ENA under accession numbers CP009687 (chromosome) and CP009688 (plasmid).

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