RESEARCH ARTICLE

Converting Carbon Dioxide to Butyrate with an Engineered Strain of *Clostridium ljungdahlii*

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ABSTRACT **Microbial conversion of carbon dioxide to organic commodities via syngas metabolism or microbial electrosynthesis is an attractive option for production of renewable biocommodities. The recent development of an initial genetic toolbox for the acetogen *Clostridium ljungdahlii* has suggested that *C. ljungdahlii* may be an effective chassis for such conversions. This possibility was evaluated by engineering a strain to produce butyrate, a valuable commodity that is not a natural product of *C. ljungdahlii* metabolism. Heterologous genes required for butyrate production from acetyl-coenzyme A (CoA) were identified and introduced initially on plasmids and in subsequent strain designs integrated into the *C. ljungdahlii* chromosome. Iterative strain designs involved increasing translation of a key enzyme by modifying a ribosome binding site, inactivating the gene encoding the first step in the conversion of acetyl-CoA to acetate, disrupting the gene which encodes the primary bifunctional aldehyde/alcohol dehydrogenase for ethanol production, and interrupting the gene for a CoA transferase that potentially represented an alternative route for the production of acetate. These modifications yielded a strain in which ca. 50 or 70% of the carbon and electron flow was diverted to the production of butyrate with H_2 or CO as the electron donor, respectively. These results demonstrate the possibility of producing high-value commodities from carbon dioxide with *C. ljungdahlii* as the catalyst.**

IMPORTANCE **The development of a microbial chassis for efficient conversion of carbon dioxide directly to desired organic products would greatly advance the environmentally sustainable production of biofuels and other commodities. *Clostridium ljungdahlii* is an effective catalyst for microbial electrosynthesis, a technology in which electricity generated with renewable technologies, such as solar or wind, powers the conversion of carbon dioxide and water to organic products. Other electron donors for *C. ljungdahlii* include carbon monoxide, which can be derived from industrial waste gases or the conversion of recalcitrant biomass to syngas, as well as hydrogen, another syngas component. The finding that carbon and electron flow in *C. ljungdahlii* can be diverted from the production of acetate to butyrate synthesis is an important step toward the goal of renewable commodity production from carbon dioxide with this organism.**

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Acetogenic microorganisms are attractive catalysts for the conversion of syngas or carbon monoxide to organic commodities (1–7) and also have the ability to convert carbon dioxide to organic products with electrons derived from an electrode in a process known as microbial electrosynthesis (5, 8, 9). An attractive feature favoring the use of acetogens in these processes is that the Wood-Ljungdahl pathway, which is naturally present in acetogens, provides the most effective known pathway for converting carbon dioxide to organic compounds that are then excreted from the cell (5, 10).

Acetogens typically produce acetate as their primary end product, but other natural products include ethanol, 2,3-butanediol, butyrate, and butanol (1–3, 6, 7, 11–13). Furthermore, under the appropriate conditions, products other than acetate may predominate. For example, modifying growth conditions permitted *Clostridium ljungdahlii* and *Clostridium autoethanogenum* (C. D. Mihalcea, J. M. Y. Fung, B. Al-Sinawi, and L. P. Tran, U.S. patent application publication no. US/0230894 A1) to produce ethanol as the dominant product from syngas metabolism and allowed *Butyribacterium methylotrophicum* adapted for growth on carbon monoxide to produce butyrate at molar levels comparable with those of acetate (14).

Genetic engineering is another approach to enhance the production of commodities other than acetate (1). Transient production of butanol was achieved by expressing genes required for butanol production on a plasmid introduced into *C. ljungdahlii* (15). Expression of heterologous genes for acetone in *C. ljungdahlii* diverted carbon and electron flow to this product (16). *Moorella thermoacetica* was genetically manipulated to produce lactate (17). However, in none of these studies was production of the target commodity optimized with additional genetic modifications, such as deletion of genes for competing pathways. Much more impressive yields of multicarbon products from *Clostridium* strains were reported in studies by researchers at Syngas Biofuels Energy, Inc., but the validity of these results is doubtful (1).

An improved array of tools for genetic manipulation of *C. ljungdahlii* (16, 18) and a steadily improving understanding of its physiology (11, 15, 19–23) suggested that *C. ljungdahlii* might

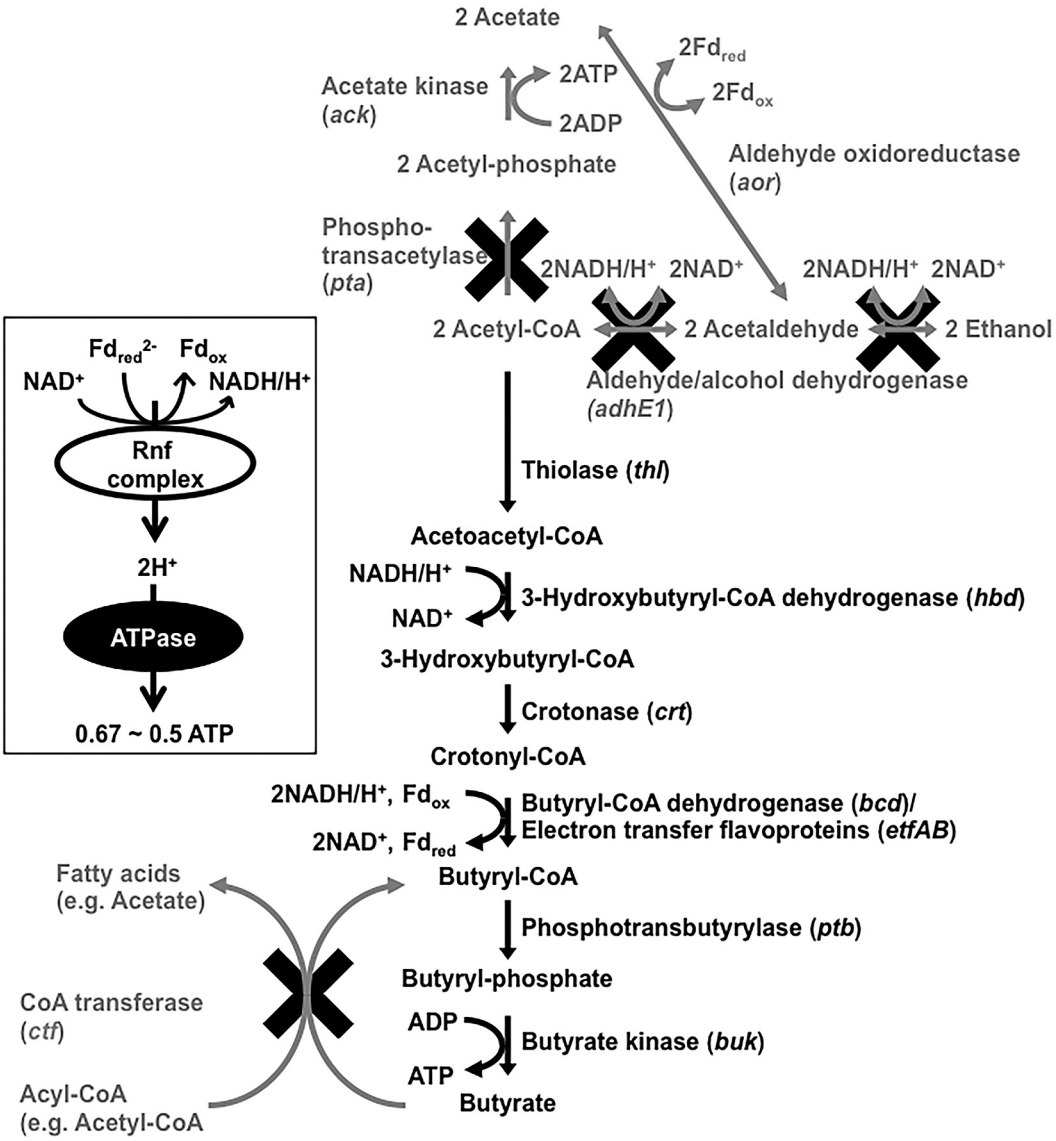

FIG 1 Engineering a pathway for butyrate synthesis. The *C. ljungdahlii* native pathways for acetate and ethanol formation are indicated in grey. The added butyrate pathway is in black. Genes encoding enzymes are in parentheses. The thick black "x" indicates pathways that were disrupted in this study.

be an effective acetogen chassis for the construction of strains that produce organic products with more than two carbons from carbon dioxide with high yields.

We chose butyrate for proof-of-concept studies. Butyrate is a food supplement that benefits colon function (24, 25) and is also used in food flavorings (26, 27). Butyrate is a feedstock for production of cellulose acetate butyrate (28), which is a thermoplastic that has a wide range of applications, such as paints (29) and polymers, such as poly(3-hydroxybutyrate)/cellulose acetate butyrate (30). Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) was synthesized from butyrate using an engineered strain of *Ralstonia eutropha* (31). Furthermore, butyrate is a precursor to butanol, which has value as a fuel (32, 33).

Current industrial production of butyrate relies on chemical synthesis from petroleum. Another potential biological route is fermentation of sugars (34–38). Several strains naturally produce high titers of butyrate from sugars, and butyrate production has been enhanced with genetic engineering or adaptation approaches (37–45).

However, producing butyrate from carbon dioxide with waste gases (1) or renewable electricity (5) as the energy source may be more environmentally sustainable and does not consume high-quality biomass feedstocks that may be more appropriately consumed as food. Therefore, we evaluated the possibility of engineering *C. ljungdahlii* to produce butyrate from carbon dioxide.

RESULTS AND DISCUSSION

Initial engineering of *C. ljungdahlii* for butyrate production: strain B1. As previously reported (46, 47), wild-type cells grew with either H_2, CO, or fructose as the electron donor, with acetate as the primary end product (Fig. 1 and 2). Growth rates and yields were highest with fructose, intermediate with CO, and lowest with H_2.

In order to redirect carbon and electron flow to the production

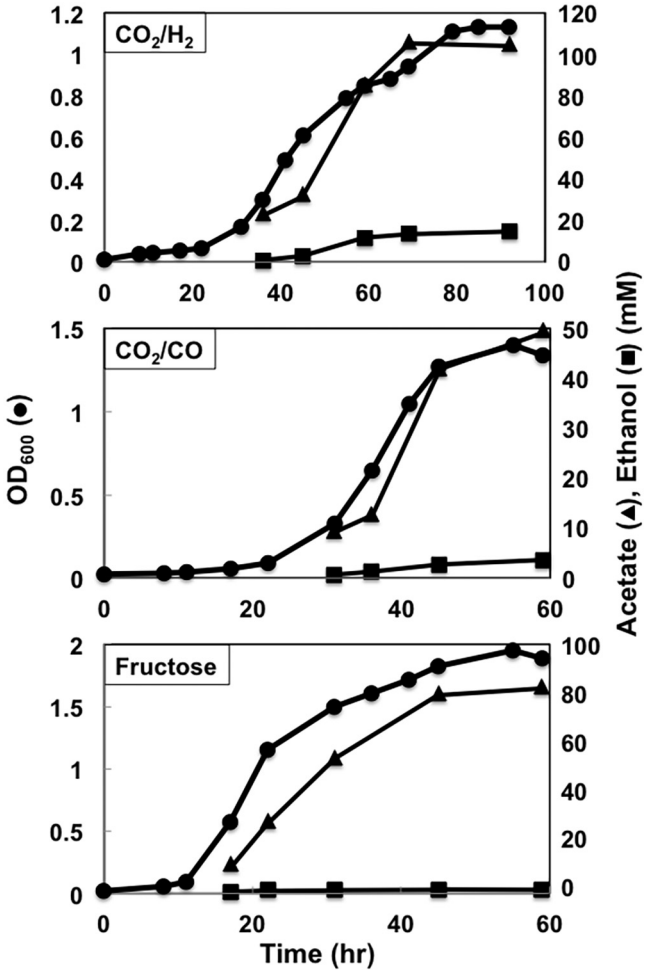

FIG 2 Growth profiles of the *C. ljungdahlii* wild-type strain. Data are representative of duplicate cultures.

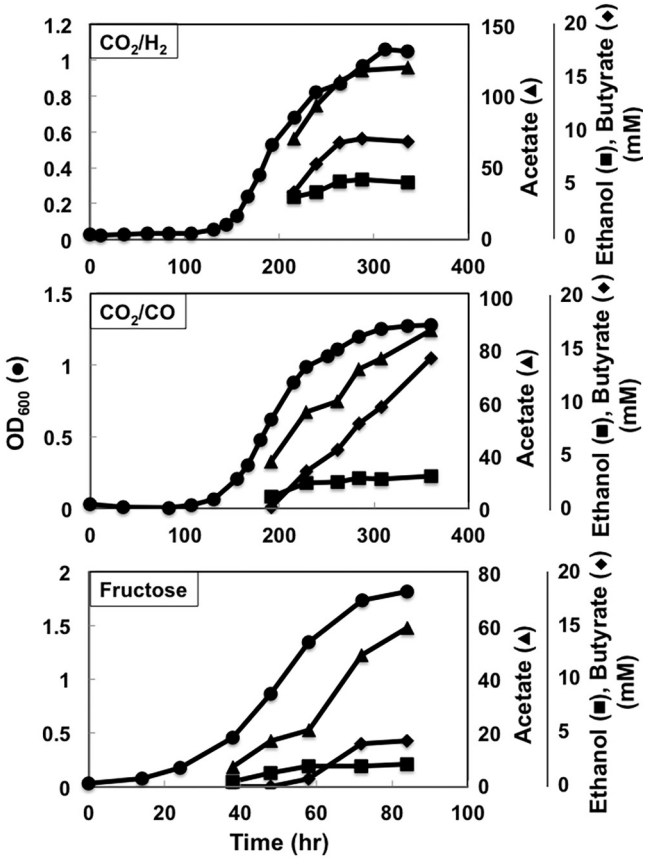

FIG 3 Growth profiles of strain B1. Data are representative of duplicate cultures.

of butyrate, the following genes from *Clostridium acetobutylicum* were introduced into *C. ljungdahlii*: *thl*, which encodes thiolase for the synthesis of acetoacetyl-coenzyme A (CoA) from acetyl-CoA; *hbd*, which encodes 3-hydroxybutyryl-CoA dehydrogenase for the synthesis of 3-hydroxybutyryl-CoA from acetoacetyl-CoA; *crt*, which encodes crotonase for the synthesis of crotonyl-CoA from 3-hydroxybutyryl-CoA; *bcd*, which encodes butyryl-CoA dehydrogenase for the synthesis of butyryl-CoA from crotonyl-CoA; *etfA* and *etfB*, which encode electron transfer flavoproteins that form an enzyme complex with butyryl-CoA dehydrogenase for the synthesis of butyryl-CoA; *ptb*, which encodes phosphotransbutyrylase for the synthesis of butyryl phosphate from butyryl-CoA; and *buk*, which encodes butyrate kinase for synthesis of butyrate from butyryl phosphate (Fig. 1). The genes *thl*, *crt*, *bcd*, *etfA*, *etfB*, and *hbd* were on one plasmid, and the genes *ptb* and *buk* were on a second plasmid (see Fig. S1 and S2 in the supplemental material). The expression of all eight genes was under the control of the putative promoter of the *pta* gene from *C. ljungdahlii*, which is considered to be highly expressed under a diversity of conditions. This strain was designated B1.

Both acetate synthesis via the Pta/Ack pathway and butyrate synthesis via the Ptb/Buk pathway result in ATP production (Fig. 1). The conversion of acetyl-CoA to acetate is expected to yield 1 ATP via substrate-level phosphorylation, whereas only 0.5 ATP is expected to be derived from each acetyl-CoA converted to butyrate (Fig. 1). However, the conversion of crotonyl-CoA to butyryl-CoA catalyzed by the complex of Bcd and EtfAB is considered to be coupled with energy generation via the Rnf complex and ATP synthase (Fig. 1) to yield ca. 0.5 ATP (48, 49), but the stoichiometry of the proton gradient and ATP generation has not been clarified for *C. ljungdahlii*. Butyrate synthesis from acetyl-CoA requires additional reducing equivalents in the form of NADH for Hbd to reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA (50) and for Bcd to reduce crotonyl-CoA to butyryl-CoA (51).

When grown with H_2 as the electron donor, strain B1 produced butyrate as well as acetate and ethanol over time (Fig. 3 and 4). Acetate remained the primary product, with minor production of ethanol, but the butyrate produced (8.5 mM) accounted for 13% of the carbon and 16% of the electrons appearing in acetate. More butyrate was produced with CO as the electron donor than with H_2 (Fig. 3 and 4), and the proportion of carbon and electrons in butyrate compared to that in acetate, 25% and 29%, respectively, was also greater. Butyrate production was lowest during growth on fructose, with only slightly more butyrate than ethanol produced (Fig. 3 and 4). When grown with H_2 or CO as the electron donor, strain B1 had longer lag periods and lower growth rates than the wild-type strain, but the final cell yields were comparable to those of the wild-type strain with all three substrates.

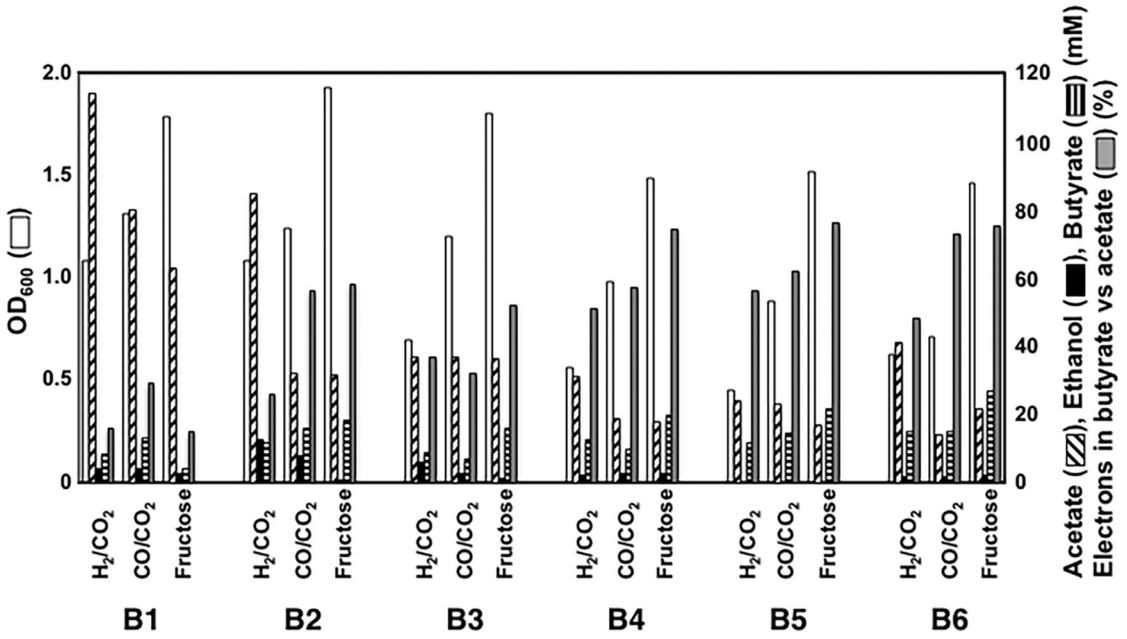

FIG 4 Summary of yields of biomass (OD_{600}), acetate (mM), ethanol (mM), butyrate (mM), and electrons appearing in butyrate versus acetate (%). Data are means from duplicate cultures.

Enhancing Crt expression through ribosome binding site modification: strain B2. Proteomic analysis demonstrated that with the exception of crotonase (Crt), all of the heterologous enzymes were expressed at levels comparable to those for highly expressed native proteins, such as phosphotransacetylase (Pta) and acetate kinase (Ack) (Fig. 5A). However, the transcript abundance of *crt* was comparable to that of other heterologous genes (Fig. 5B). These results suggested that the low abundance of the Crt protein resulted from inefficient translation of *crt* transcripts. The distance between the putative ribosome binding site (RBS) and translation initiation codon of *crt* was shorter than those for the other heterologous genes (Fig. 6A). Therefore, a *crt* sequence in which the length between the putative RBS and translation initiation codon was increased and substituted for the original *crt* sequence in constructing strain B2. Western blot analysis revealed that strain B2 produced substantially more Crt than strain B1 (Fig. 6B). Strain B2 produced more butyrate with all three electron donors (Fig. 4 and 7), suggesting that the low level of Crt was a bottleneck in butyrate production in strain B1.

Integration of butyrate pathway genes into chromosome: strain B3. Chromosomal integration of the genes for butyrate synthesis may be preferable to maintaining the genes on plasmids, to avoid the need to add antibiotics to retain plasmids and to reduce the cellular metabolic burden (52, 53). A fragment containing the genes *ptb* and *buk* was integrated at the *adhE1* locus on the chromosome with double-crossover homologous recombination, but a longer fragment containing *thl-crt-bcd-etfB-etfA-hbd* could not be integrated in this manner (data not shown). However, the DNA fragment of *thl-crt-bcd-etfB-etfA-hbd-ptb-buk* (*crt* with the modified RBS), under the control of the putative *pta* promoter region, was successfully integrated into the putative *pta* promoter region via single-crossover homologous recombination (see Fig. S3 in the supplemental material).

Compared with strain B2, which had the same genes expressed on plasmids, strain B3 grew faster but with a lower final biomass yield when H_2 was the electron donor, (Fig. 8). A final butyrate yield by strain B3 was slightly lower than that by strain B2, but butyrate production compared to acetate production was improved (Fig. 4). In

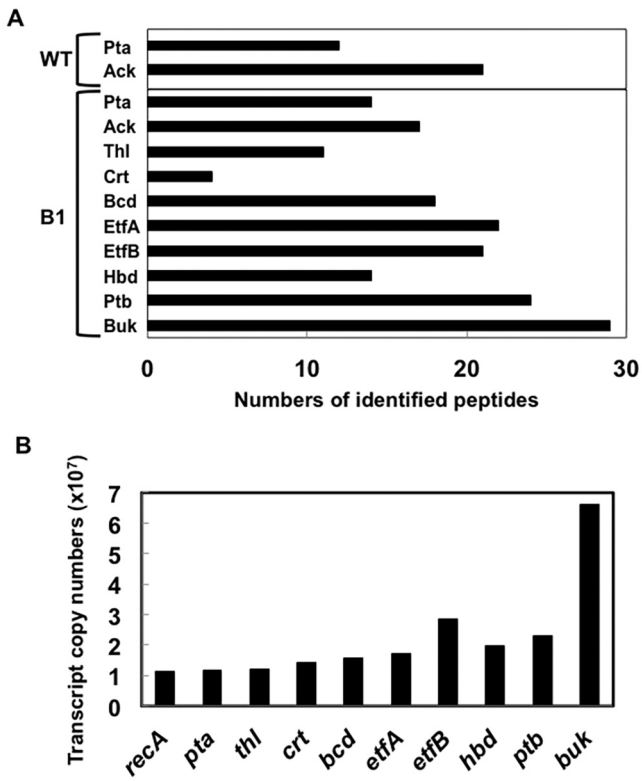

FIG 5 Proteomic and transcriptomic analyses of strain B1. (A) Proteomic analysis. Numbers of identified peptides are shown. The wild type (WT) and strain B1 were analyzed. (B) Transcriptomic analysis. Transcript abundance in strain B1 is presented as transcript copy numbers ($\times 10^7$).

A

Gene	Sequence
crt	TTT**AGGAGG**ATTAGTCATG
crt-M	g**agAGGA**GGATTAGTCATG
pta	A**AGGGAG**GAAATGAACATG
ack	**AGGAGG**AATATTAAACATG
thl	c**AGGAGG**TTAGTTAGAATG
hbd	c**AGGGAG**GTCTGTTTAATG
bcd	T**AGGAGG**TAAGTTTATATG
etfA	T**AGGAGG**GATTTTTCAATG
etfB	TA**AGGAGG**TTAAGAGGATG
ptb	**AGGGAG**TGTACGACCAGTG
Buk	GT**GGAGGA**ATGTTAACATG

B

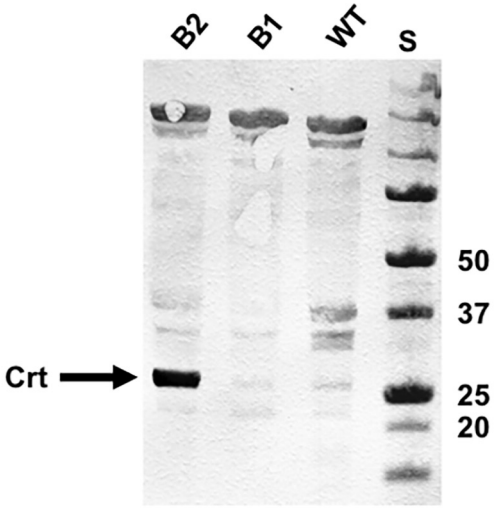

FIG 6 Modification of the ribosomal binding site (RBS) of the *crt* gene. (A) Comparison of putative RBSs. Putative RBSs are indicated in bold. Putative translation initiation codons are underlined. Lowercase indicates sequence from restriction sites used for cloning. "*crt*-M" represents the modified sequence used in strain B2. (B) Western blot analysis for the Crt protein. Cell extracts prepared from the wild-type strain (WT), strain B1, and strain B2 were analyzed using an antibody against Crt. "S" represents protein standards, whose sizes are shown in kDa.

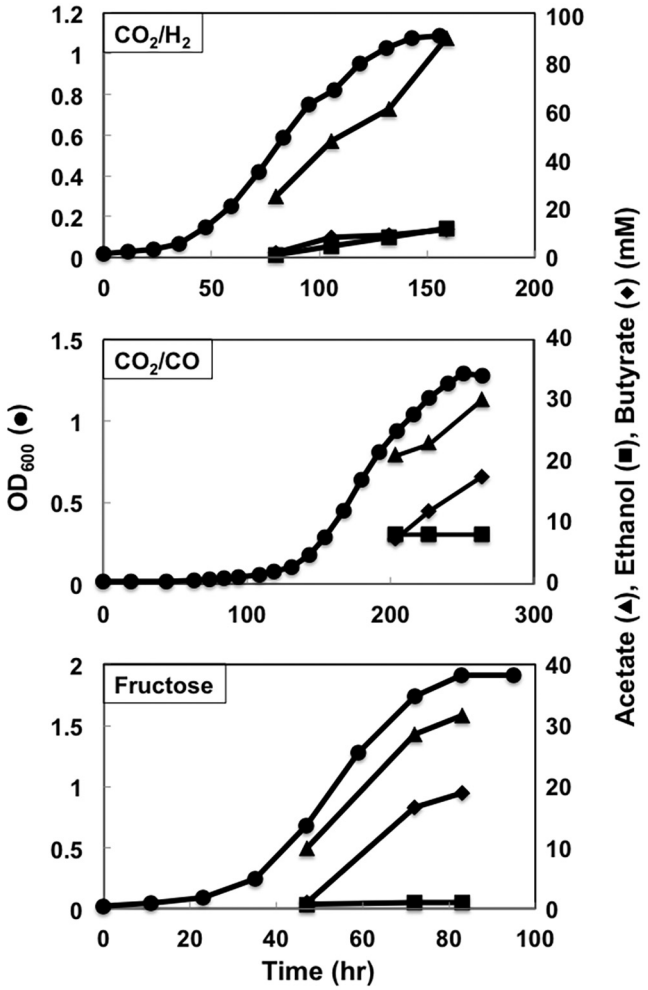

FIG 7 Growth profiles of strain B2. Data are representative of duplicate cultures.

contrast, strain B3 grew faster than strain B2 with a similar final biomass yield on CO, and butyrate production was lower than that of strain B2. With fructose as the substrate, strain B3 grew faster than strain B2, but yields of biomass, acetate, and butyrate were comparable. In addition to acetate and butyrate, strain B3 produced ethanol at lower levels under all growth conditions (Fig. 8).

Inactivating Pta-dependent acetate synthesis: strain B4. In an attempt to further enhance the diversion of carbon and electron flux from acetate production to butyrate synthesis, Pta, which is thought to catalyze the first step in the conversion of acetyl-CoA to acetate (Fig. 1), was disrupted with a single-crossover homologous recombination, which simultaneously integrated the butyrate pathway genes (see Fig. S4 in the supplemental material). The Cre-lox system, which allows reuse of an antibiotic resistance gene (54), was applied to *C. ljungdahlii* strain engineering (see Fig. S5, S6, and S7). This strain was designated B4.

Strain B4 grew slower than strain B3 with H_2 as the electron donor but with a comparable final cell yield (Fig. 9). Surprisingly, strain B4 produced acetate at amounts comparable to those for strain B3 (Fig. 4), despite the fact that the expected primary route for acetate synthesis was disrupted. Strain B4 produced slightly more butyrate and less acetate than strain B3 during growth with CO or fructose (Fig. 4 and 9), but acetate production remained substantial (Fig. 4 and 9), suggesting that there are one or more unknown pathways for acetate synthesis in *C. ljungdahlii*.

Inactivating Pta-dependent acetate synthesis and AdhE1-dependent ethanol synthesis: strain B5. In an attempt to divert the NAD(P)H being consumed for ethanol production toward the production of butyrate, the ethanol pathway in strain B4 was inactivated by disrupting the *adhE1* gene, which was shown (18) to encode the major bifunctional aldehyde/alcohol dehydrogenase for ethanol production, via single-crossover homologous recombination (Fig. 1; see also Fig. S8 in the supplemental material), creating strain B5. Strain B5 produced significantly less ethanol than strain B4, slightly improving butyrate production during growth on CO or fructose but not H_2 (Fig. 4 and 10).

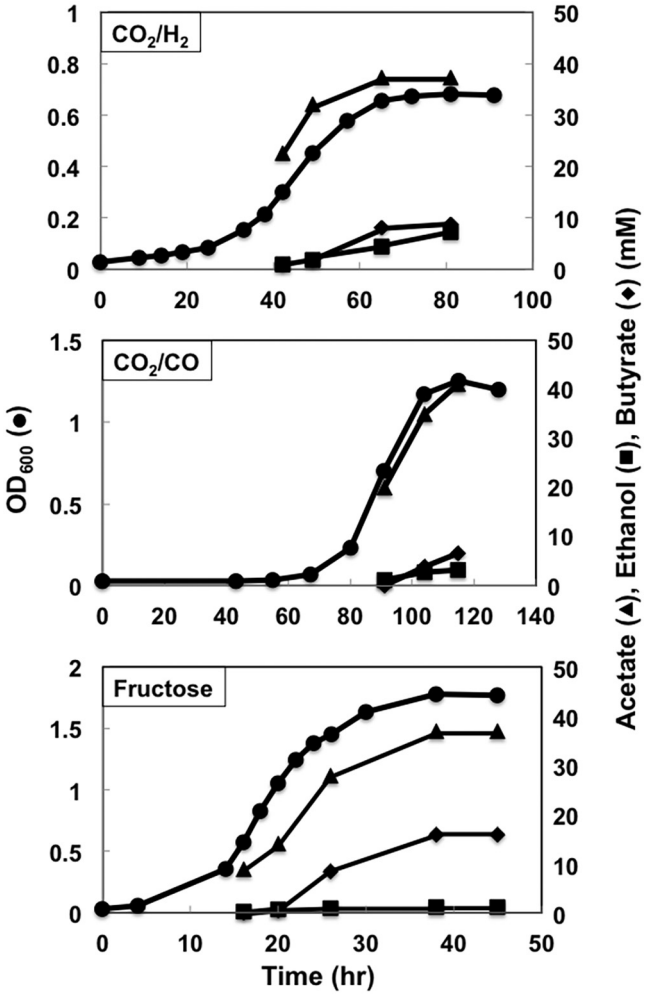

FIG 8 Growth profiles of strain B3. Data are representative of duplicate cultures.

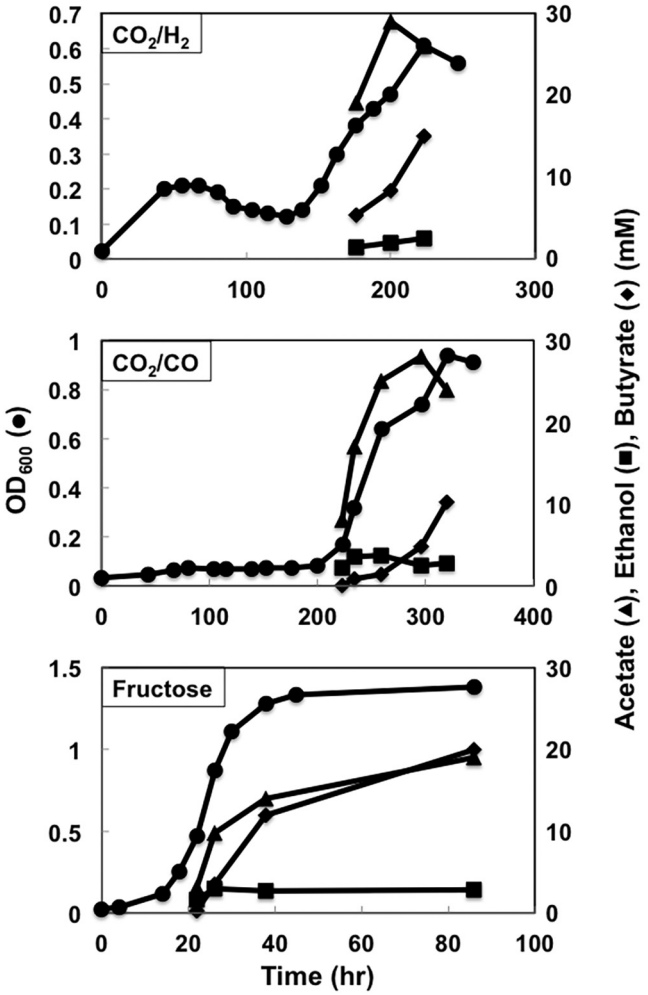

FIG 9 Growth profiles of strain B4. Data are representative of duplicate cultures.

Inactivating Pta-dependent acetate synthesis and CoA transferase: strain B6. It was considered that a CoA transferase might convert butyrate back to butyryl-CoA with simultaneous production of acetate or other fatty acids (Fig. 1). In an attempt to avoid this possibility, the gene Clju_c39430, which encodes a homolog of CoA transferases, was disrupted via single-crossover homologous recombination (see Fig. S8 in the supplemental material) in strain B4, yielding strain B6. Acetate continued to be produced in strain B6, but this strain was the best butyrate-producing strain in terms of butyrate yield under all three growth conditions tested (Fig. 4 and 11). Carbon and electron yields in butyrate were 42% and 48% (H_2), 68% and 73% (CO), and 71% and 75% (fructose), respectively.

Implications. The results demonstrate that it was possible through genetic manipulation to redirect carbon and electron flow to the production of butyrate from carbon dioxide in *C. ljungdahlii*. However, further genetic manipulation will be required before the goal of producing butyrate as the sole product of metabolism will be achieved.

The continued production of acetate when *pta* is disrupted was previously observed in *C. acetobutylicum*, which has a fermentative metabolism (55). A potential alternative route for acetate production in *C. ljungdahlii*, even during autotrophic growth, is conversion of acetyl-CoA to acetate via an acetaldehyde intermediate (15). This could yield energy to support cell growth and maintenance through the Rnf complex and ATP synthase. Conversion of acetyl-CoA to acetaldehyde and acetaldehyde to acetate is thought to proceed with oxidation of NADH to NAD^+ and reduction of ferredoxin, respectively (Fig. 1) (15). Proton gradients generated with the reactions coupled with the reduction of NAD^+ and the oxidation of ferredoxin via the Rnf complex result in ATP synthesis by ATP synthase (48, 49). Therefore, net ATP synthesis may be possible via this route of acetate production. ATP generated in this manner would yield ca. 0.5 ATP, which would be comparable to ATP generation via butyrate production (Fig. 1).

A previous study demonstrated that deleting *adhE1*, which encodes a bifunctional aldehyde/alcohol dehydrogenase, prevented ethanol formation from acetyl-CoA, presumably by eliminating acetaldehyde formation (18). However, strain B5, in which both the *pta* gene and the *adhE1* gene were inactivated, still produced acetate. There are other homologs for aldehyde dehydrogenase in the *C. ljungdahlii* genome (Clju_c11960, Clju_c39730, and Clju_c39840). Therefore, deletion of one or more of these genes may be required in order to completely eliminate acetate produc-

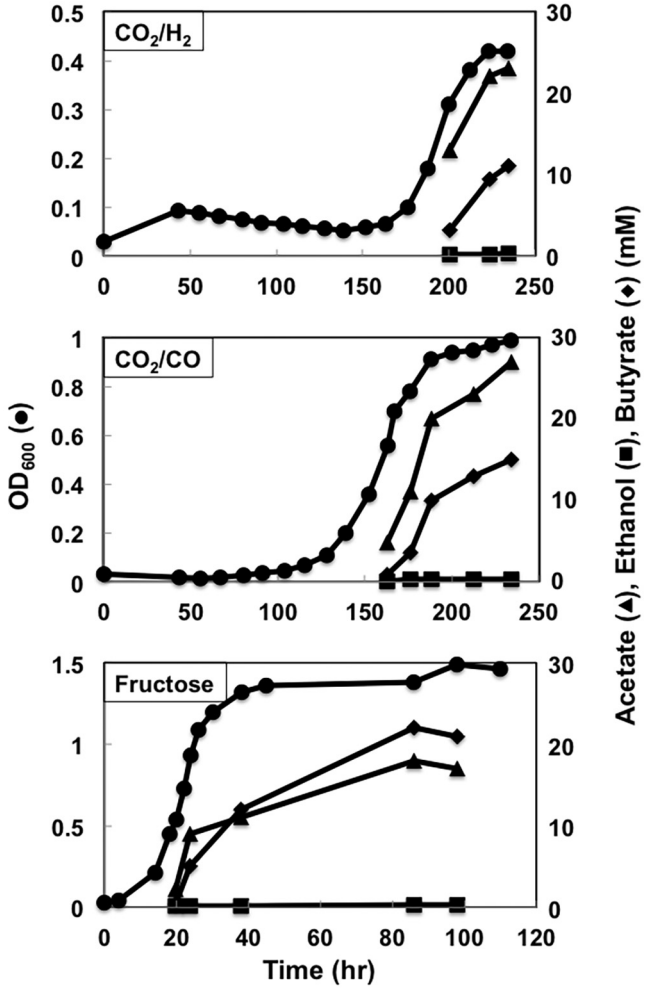

FIG 10 Growth profiles of strain B5. Data are representative of duplicate cultures.

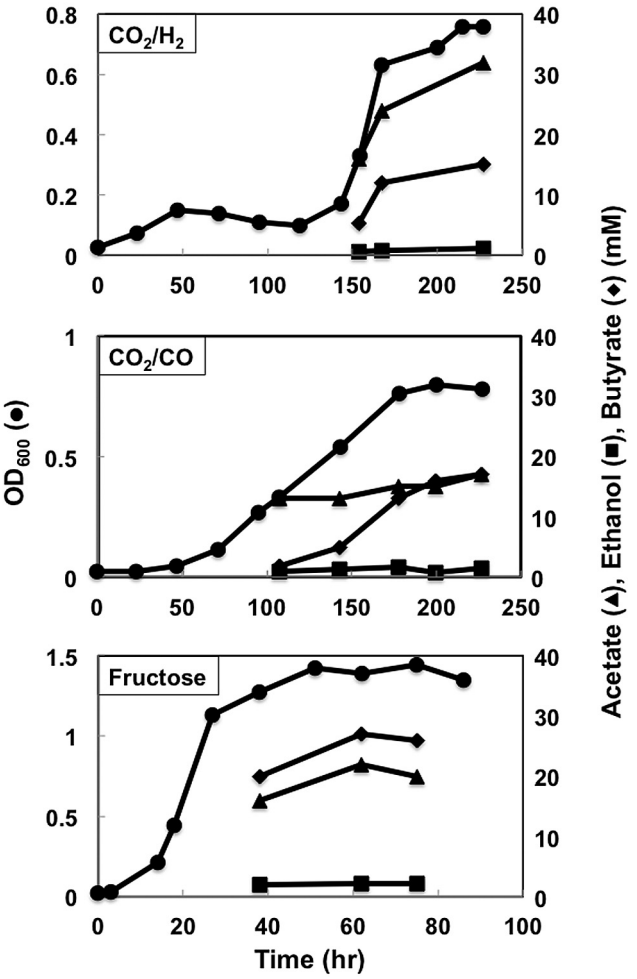

FIG 11 Growth profiles of strain B6. Data are representative of duplicate cultures.

tion. Deletion of many genes in the same strain is currently not possible because there are only three antibiotic resistance markers that have been identified, severely limiting the number of genes that can be deleted if they are not colocalized on the chromosome. We adopted the Cre-lox system in order to permit simultaneous disruption of three genes, but this method leaves the recombination site on the chromosome, and thus they can be used only once in a strain. Negative- or counterselection markers, such as *pyrF* (56), *galK* (57), and *mazF* (58), which can be used repeatedly and have been applied to other *Clostridium* species, would be useful, but attempts to apply them to *C. ljungdahlii* have not yet been successful (unpublished data).

Another possibility for the unexpected acetate production is that the enzyme phosphotransbutyrylase (Ptb) introduced in the synthetic butyrate pathway may also act on acetyl-CoA, as previously described for the Ptb purified from *C. acetobutylicum* (59). The specificity for acetyl-CoA was only 1.6% of that for butyryl-CoA, but even low reactivity with acetyl-CoA, which is likely to be a more abundant metabolite, could be significant. Acetate production via this pathway could be favored because it would yield more ATP and have lower reducing equivalent demands than the desired production of butyrate. When the appropriate tools are available, deleting *ack* may eliminate this possibility because acetate kinase is essential for this potential pathway. Substituting *bukII*, which encodes another butyrate kinase that is more specific for butyrate synthesis (37), for *buk* in the *ptb-buk* operon might also improve butyrate yields.

These and previous (16, 18) findings that carbon and electron flow associated with the Wood-Ljungdahl pathway in *C. ljungdahlii* can be genetically redirected suggest that *C. ljungdahlii* may be an effective chassis for the production of organic commodities from carbon monoxide-containing waste gases (11, 15) or from carbon dioxide via electrosynthesis (9). Further development of the genetic tools needed to accomplish this goal is under way.

MATERIALS AND METHODS

Strains and growth conditions. *C. ljungdahlii* DSM 13528 (ATCC 55383) from the German Collection of Microorganisms and Cell Cultures was the parent strain for butyrate production engineering. When fructose served as the carbon and energy source, cultures were grown in PETC 1754 medium (100% CO_2 in the headspace) with 28 mM fructose as described previously (18). For growth with CO as the electron donor, the fructose was omitted from the PETC 1754 medium and CO was added to the headspace at 20 lb/in^2. When H_2 served as the electron donor, the cells were grown in DSMZ medium 879 with the fructose omitted and with a

headspace of H_2-CO_2 (80%/20%). H_2-CO_2 (80%/20%) was further added to the headspace at 20 lb/in^2. DSMZ medium 879 was used to maintain the CO_2 partial pressure since the original contains N_2-CO_2 (80%/20%) in the headspace. Cultures were grown in anaerobic pressure tubes (27 ml) containing either 10 ml (fructose grown) or 5 ml (CO or H_2 grown) of medium. Cultures grown on CO or H_2 were shaken at 100 rpm.

Escherichia coli NEB 5-alpha and NEB Express (New England Biolabs) were used for plasmid preparation and grown as instructed.

Construction of butyrate pathway genes in plasmids (strains B1 and B2). *C. acetobutylicum* genes necessary for synthesis of butyrate from acetyl-CoA (Fig. 1) were amplified by PCR. The amplified genes were *thl* (NCBI GenBank; CA_C2873), *crt* (NCBI GenBank; CA_C2712), *bcd* (NCBI GenBank; CA_C2711), *etfB* (NCBI GenBank, CA_C2710), *etfA* (NCBI GenBank; CA_C2709), *hbd* (NCBI GenBank; CA_C2708), *buk* (NCBI GenBank; CA_C3075), and *ptb* (NCBI GenBank; CA_C3076). Primers used for the PCR are listed in Table S1 in the supplemental material. PCR products were cloned in pCR-Blunt II-TOPO (Life Technologies). The NdeI-PstI DNA fragment containing *etfA* was cloned at the NdeI (in *etfB*) and PstI sites in pCR-Blunt II-TOPO containing *bcd* and *etfB*. The KpnI-XhoI DNA fragment containing *thl* was cloned at the KpnI and XhoI sites in pBluescript II KS(−) (Stratagene). Then, the XhoI-EcoRI DNA fragment containing *crt* was cloned at the XhoI and EcoRI sites in pBluescript II KS(−) containing *thl*. The EcoRI-PstI DNA fragment containing *bcd*, *etfB*, and *etfA* was cloned at the EcoRI and PstI sites in pBluescript II KS(−) containing *thl* and *crt*. The PstI-BamHI DNA fragment containing *hbd* was cloned at the PstI and BamHI sites in pBluescript II KS(−) containing *thl*, *crt*, *bcd*, *etfB*, and *etfA* (see Fig. S1A). The KpnI-BamHI DNA fragment containing *thl*, *crt*, *bcd*, *etfB*, *etfA*, and *hbd* was cloned at the KpnI and BamHI sites in an expression vector, pJe-p (see Fig. S2). The BamHI-SalI DNA fragment containing *ptb* and *buk* was cloned at the BamHI and SalI sites in pM6-p (see Fig. S2). pJe-p and pM6-p were constructed by placing the putative promoter for the *pta* gene from *C. ljungdahlii* in pJe (16) and pMTL82151 (a gift from N. P. Minton) (60), respectively. pJe is a derivative of pCL2 (18) and contains an erythromycin resistance gene instead of the chloramphenicol resistance gene. The putative *pta* promoter was prepared by PCR using primers listed in Table S1. PCR products were cloned in pCR-Blunt II-TOPO. The EcoRI-KpnI DNA fragment containing the putative *pta* promoter was cloned at the EcoRI-KpnI sites in these vectors. Transformation of these plasmids was conducted as described previously (18).

Integration of butyrate pathway genes on chromosome (strain B3). The BamHI DNA fragment from pCR-Blunt II-TOPO containing *ptb* and *buk* as described above (a BamHI site is located downstream of *buk* in the vector) was cloned at the BamHI site of pBluescript II KS(−) containing *thl*, *crt*, *bcd*, *etfB*, *etfA*, and *hbd* (see Fig. S1B in the supplemental material). The *crt* gene with the modified RBS was used. The SalI site remains at the 3′ end of *buk* upstream of the BamHI site. A resultant plasmid containing *ptb* and *buk* in the same direction with *thl*, *crt*, *bcd*, *etfB*, *etfA*, and *hbd* was used for subsequent plasmid preparation. The *ermC* gene was amplified by PCR with primers listed in Table S1 and pCL1 (18). PCR products were cloned in pCR-Blunt II-TOPO. The SpeI-NotI fragment containing *ermC* was cloned at the SpeI and NotI sites in pBluescript II KS(−) containing *thl*, *crt*, *bcd*, *etfB*, *etfA*, *hbd*, *ptb*, and *buk* (see Fig. S2). The resultant plasmid was used to integrate the butyrate pathway genes at the *pta* promoter region on the chromosome (see Fig. S2).

Construction of Cre-lox system for *C. ljungdahlii*. A loxP cassette was constructed as follows. Oligonucleotides loxP1-T and lox-P1-B (see Table S1 in the supplemental material) were phosphorylated, annealed, and cloned at the EcoRI site in the plasmid pNEB193 (New England Biolabs). A resultant plasmid with the loxP1 sequence in the proper orientation was used for cloning the phosphorylated and annealed oligonucleotides loxP2-T and loxP2-B at the HindIII site. A resultant plasmid with the loxP2 sequence in the proper orientation was used for the subsequent cloning. The *catP* gene was amplified by PCR with primers listed in Table S1 and pMTL82151 (60). PCR products were cloned in pCR-Blunt II-TOPO. The BglII-SacII DNA fragment of the *catP* gene was cloned at the BglII and SacII sites in pNEB containing loxP1 and loxP2. Then, the *ermC* gene was amplified by PCR with primers listed in Table S1 and pCL1 (18). PCR products were cloned in pCR-Blunt II-TOPO. The SphI-HindIII DNA fragment of *ermC* was cloned at the SphI and HindIII sites in pNEB containing loxP1, loxP2, and *catP* (see Fig. S4).

A *cre* gene with codon usage optimized for *Clostridium* was synthesized by GenScript (see Fig. S5 in the supplemental material). The KpnI-BamHI DNA fragment of *cre* was cloned at the KpnI and BamHI sites in an expression vector, pJKe-p. For the construction of pJKe-p, a kanamycin resistance gene (*kan*) with codon usage optimized for *Clostridium* was synthesized by GenScript (see Fig. S6). The *kan* gene was fused with the putative promoter region of *ermB* (P*ermB*) from pQexp (61). P*ermB-kan* was amplified by PCR with primers listed in Table S1. PCR products were cloned in pCR-Blunt II-TOPO. The ClaI-HpaI DNA fragment of P*ermB-kan* replaced the ClaI-HpaI DNA fragment including *catP* in pJIR750ai (Sigma). pJKe-p was constructed by placing the *pta* promoter at the EcoRI-KpnI sites in the resultant plasmid, pJKe, as described above.

Inactivation of *pta* gene and integration of butyrate pathway genes (strain B4). The KpnI-SalI DNA fragment containing *thl*, *crt*, *bcd*, *etfB*, *etfA*, *hbd*, *ptb* and *buk* from the pBluescript II KS(−) containing *thl*, *crt*, *bcd*, *etfB*, *etfA*, *hbd*, *ptb*, and *buk* as described above was cloned at the KpnI-SalI sites in pNEB containing loxP1, loxP2, *catP*, and *ermC* as described above. The internal region of the *pta*-coding sequence (*pta*-int) was amplified by PCR with primers listed in Table S1 in the supplemental material. PCR products were cloned in pCR-Blunt II-TOPO. The SpeI-KpnI DNA fragment of *pta*-int was cloned at the SpeI and KpnI sites in the pNEB containing loxP1, loxP2, *catP*, *ermC*, and the butyrate pathway genes (see Fig. S4). The resultant plasmid was used to integrate the butyrate pathway genes at the internal region of the *pta*-coding sequence on the chromosome, which resulted in disruption of *pta* (see Fig. S4).

After integration of the above plasmid on the chromosome DNA, the vector region and *catP* were excised by Cre (see Fig. S4 and S7 in the supplemental material). Competent cells of this strain were prepared as described previously (18) and transformed with the plasmid containing *cre* with the following modification. After electroporation and subsequent outgrowth in the presence of erythromycin, kanamycin was added to the outgrowth culture at the final concentration of 20 μg/ml and further incubated overnight to facilitate recombination at the loxP sites by Cre. The outgrowth cultures were then plated normally in the presence of erythromycin and kanamycin. Absence of the vector region and *catP* and presence of the butyrate genes were verified by PCR analysis (see Fig. S7).

Inactivation of *adhE1* gene (strain B5). The *adhE1* gene was disrupted by single-crossover homologous recombination at the internal region of the *adhE1*-coding sequence (see Fig. S8 in the supplemental material). The internal region of the *adhE1*-coding sequence (*adhE1*-int) was amplified by PCR using primers listed in Table S1. PCR products were cloned in pCR-Blunt II-TOPO. The SpeI-KpnI DNA fragment of *adhE1*-int was cloned at the SpeI and KpnI sites in the pNEB containing *catP*. The resultant plasmid was used to disrupt *adhE1* on the chromosome of strain B4, in which *pta* was inactivated by inserting the butyrate pathway genes at the internal region of the *pta*-coding sequence on the chromosome.

Inactivation of *ctf* gene (strain B6). The *ctf* gene (Clju_c39430) was disrupted as described for *adhE1* (see Fig. S8 in the supplemental material). The internal region of the *ctf*-coding sequence (*ctf*-int) was amplified by PCR with primers listed in Table S1. PCR products were cloned in pCR-Blunt II-TOPO. The SpeI-KpnI DNA fragment of *ctf*-int was cloned at the SpeI and KpnI sites in pNEB containing *catP*. The resultant plasmid was used to disrupt *ctf* on the chromosome of strain B4.

Proteomic analysis. Protein expression profiles of *C. ljungdahlii* wild-type and B1 strains grown on fructose were analyzed at the Proteomics and Mass Spectrometry Facility, University of Massachusetts Medical School. Cells were harvested from cultures at a log phase. Each protein sample (5 μg) was run in to a gel. The gel slice was cut out, washed thoroughly, and digested with trypsin. Each digested sample was analyzed

in triplicate by using nano-liquid chromatography–tandem mass spectrometry (nano-LC-MS/MS).

Reverse transcription-quantitative PCR (qRT-PCR). Total RNA was prepared from the fructose-grown B1 strain by using the RiboPure-Bacteria kit (Life Technologies) as instructed. cDNA was prepared with random nonamers by using the Enhanced Avian RT First Strand synthesis kit (Sigma) as instructed. PCR was conducted with primers listed in Table S1 in the supplemental material as described previously (62).

Western blot analysis. Cell extracts were prepared from the fructose-grown wild-type, B1, and B2 strains by disruption with sonication. Cell extracts were loaded on an SDS-PAGE gel. Western blot analysis was carried out with an antibody against the peptide DISEMKEMNTIERG, from amino acid residues 67 to 80 of the Crt protein. The peptide and the antibody were prepared by GenScript.

Analytical techniques. Growth of cells was monitored by measuring the optical density at 600 nm (OD_{600}). Acetate and butyrate were measured by high-performance liquid chromatography (9), and ethanol was measured by gas chromatography (63).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01636-14/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.
Figure S5, PDF file, 0.1 MB.
Figure S6, PDF file, 0.1 MB.
Figure S7, PDF file, 0.1 MB.
Figure S8, PDF file, 0.1 MB.
Table S1, PDF file, 0.04 MB.

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