Clostridium ljungdahlii represents a microbial production platform based on syngas

Michael Köpke[a,1], Claudia Held[b,2], Sandra Hujer[a], Heiko Liesegang[b], Arnim Wiezer[b,3], Antje Wollherr[b], Armin Ehrenreich[b,2], Wolfgang Liebl[c], Gerhard Gottschalk[b], and Peter Dürre[a,4]

[a]Institut für Mikrobiologie und Biotechnologie, Universität Ulm, 89081 Ulm, Germany; [b]Laboratorium für Genomanalyse, Institut für Mikrobiologie und Genetik, Georg-August-Universität Göttingen, 37077 Göttingen, Germany; and [c]Technische Universität München, Abteilung Mikrobiologie, Wissenschaftszentrum Weihenstephan, 85354 Freising, Germany

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***Clostridium ljungdahlii* is an anaerobic homoacetogen, able to ferment sugars, other organic compounds, or CO_2/H_2 and synthesis gas (CO/H_2). The latter feature makes it an interesting microbe for the biotech industry, as important bulk chemicals and proteins can be produced at the expense of CO_2, thus combining industrial needs with sustained reduction of CO and CO_2 in the atmosphere. Sequencing the complete genome of *C. ljungdahlii* revealed that it comprises 4,630,065 bp and is one of the largest clostridial genomes known to date. Experimental data and in silico comparisons revealed a third mode of anaerobic homoacetogenic metabolism. Unlike other organisms such as *Moorella thermoacetica* or *Acetobacterium woodii*, neither cytochromes nor sodium ions are involved in energy generation. Instead, an Rnf system is present, by which proton translocation can be performed. An electroporation procedure has been developed to transform the organism with plasmids bearing heterologous genes for butanol production. Successful expression of these genes could be demonstrated, leading to formation of the biofuel. Thus, *C. ljungdahlii* can be used as a unique microbial production platform based on synthesis gas and carbon dioxide/hydrogen mixtures.**

biofuels | butanol | *Clostridium acetobutylicum* | genome sequence | Rnf-dependent energy conservation

Worldwide energy consumption is projected to grow by 57% until 2030 (1), with mobility being the major pillar of our industry-based society. Because fossil oil, coal, and gas are finite, the need for alternative fuels and industrial bulk substrates is obvious. Another driving force for searching for new energy compounds and technologies is the alarming effect of increasing CO_2 concentration in the atmosphere, due to burning of fossil resources. These emissions are related to an increase of greenhouse effects and thus to global warming. New models predict that even a switch to global cellulosic or algal bioenergy programs will cause an increase in warming potential by extensive use of fertilizers, resulting in higher nitrous oxide emissions (2, 3). Humankind must therefore derive new methods for the production of chemicals and energy. One way to address these problems is the biotechnological use of autotrophic bacteria that grow on CO_2 and CO and produce important bulk chemicals and biofuels. *Clostridium ljungdahlii* is such an organism. It was isolated for its ability to produce ethanol from synthesis gas (4), mostly a mixture of carbon monoxide and hydrogen. Syn(thesis)gas is a simple, abundant, and inexpensive substrate. It can easily be generated not only from natural gas and by gasification of coal and oil, but also from biomass, municipal waste, or by recycling of used plastics (5). A recent publication even proposes to use solar energy for conversion of CO_2 into syngas (6). Syngas has already been used as a major feedstock in the chemical industry for decades. However, the respective reactions require a set CO/H_2 ratio and expensive purification of the gases, as contaminants will poisen noble catalysts. Bacteria with the ability to ferment syngas are far more tolerant to such contaminations (7) and are thus already industrially used for production of the biofuel additive ethanol (Coskata, INEOS Bio, LanzaTech). However, a major limitation for large-scale application is the lack of genome knowledge and genetic systems that will allow construction of tailor-made strains, suitable for a whole variety of different production processes. The blueprint of the genome and development of tools for molecular biological handling now offers the possibility to use *C. ljungdahlii* as a novel biotechnological production platform based on syngas and CO_2/H_2.

Results and Discussion

General Features of the *C. ljungdahlii* Genome. Following the *Moorella thermoacetica* genome (8), the one of *C. ljungdahlii* is the second of a homoacetogen, which has been completely sequenced. With a size of 4.6 Mbp, it belongs to the largest clostridial genomes (Table S1). Genes encoding nine rRNA clusters and 72 tRNAs were detected. The genome contains 25.2% genes without a predicted function and a high percentage (77.4%) of the 4,198 coding sequences are encoded on the leading strand (Fig. S1). Comparative genome analysis with other clostridial genomes was carried out using the MUMmer user software package (9). Two putative prophage regions (CLJU_c03280–3800 and CLJU_c16550–17000) were detected. *C. ljungdahlii* does not contain *tat* genes, required for export of folded proteins with bound cofactors.

C. ljungdahlii is motile by flagella (4). The genes for flagella biosynthesis are arranged in two clusters (CLJU_c09470–610 and CLJU_c10180–450). Chemotaxis genes are located adjacent to one of these clusters (CLJU_c09390–460).

C. ljungdahlii forms endospores and contains the master regulator Spo0A (encoded by CLJU_c11220) for sporulation, but lacks the so-called phosphorelay (Spo0F and Spo0B), as all other clostridia (10). All genes encoding the sporulation-specific sigma factors SigH (CLJU_c41230), SigF (CLJU_c33950), SigE (CLJU_c12320), SigG (CLJU_c12330), and SigK (CLJU_c12260) are present.

In the following, only CDS involved in special metabolic features (Wood–Ljungdahl pathway, substrate utilization, energy conservation, intermediary, and N-metabolism) will be discussed.

Features of demethylation of methoxylated compounds are presented in Fig. S2.

Wood–Ljungdahl Pathway. In homoacetogenic bacteria, the Wood–Ljungdahl pathway is used to fix CO_2 or CO and convert it into acetyl-CoA (Fig. 1). Genes encoding all respective enzymes were detected. Formate dehydrogenase reduces CO_2 to formate that can

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[1]Present address: LanzaTech, 24 Balfour Road, Parnell, Auckland 1052, New Zealand.

[2]Present address: TU München, Abt. Mikrobiologie, Wissenschaftszentrum Weihenstephan, Emil-Ramann-Str. 4, 85354 Freising, Germany.

[3]Present address: Qiagen Hamburg GmbH, Königstr. 4a, 22767 Hamburg, Germany.

[4]To whom correspondence should be addressed. E-mail: peter.duerre@uni-ulm.de.

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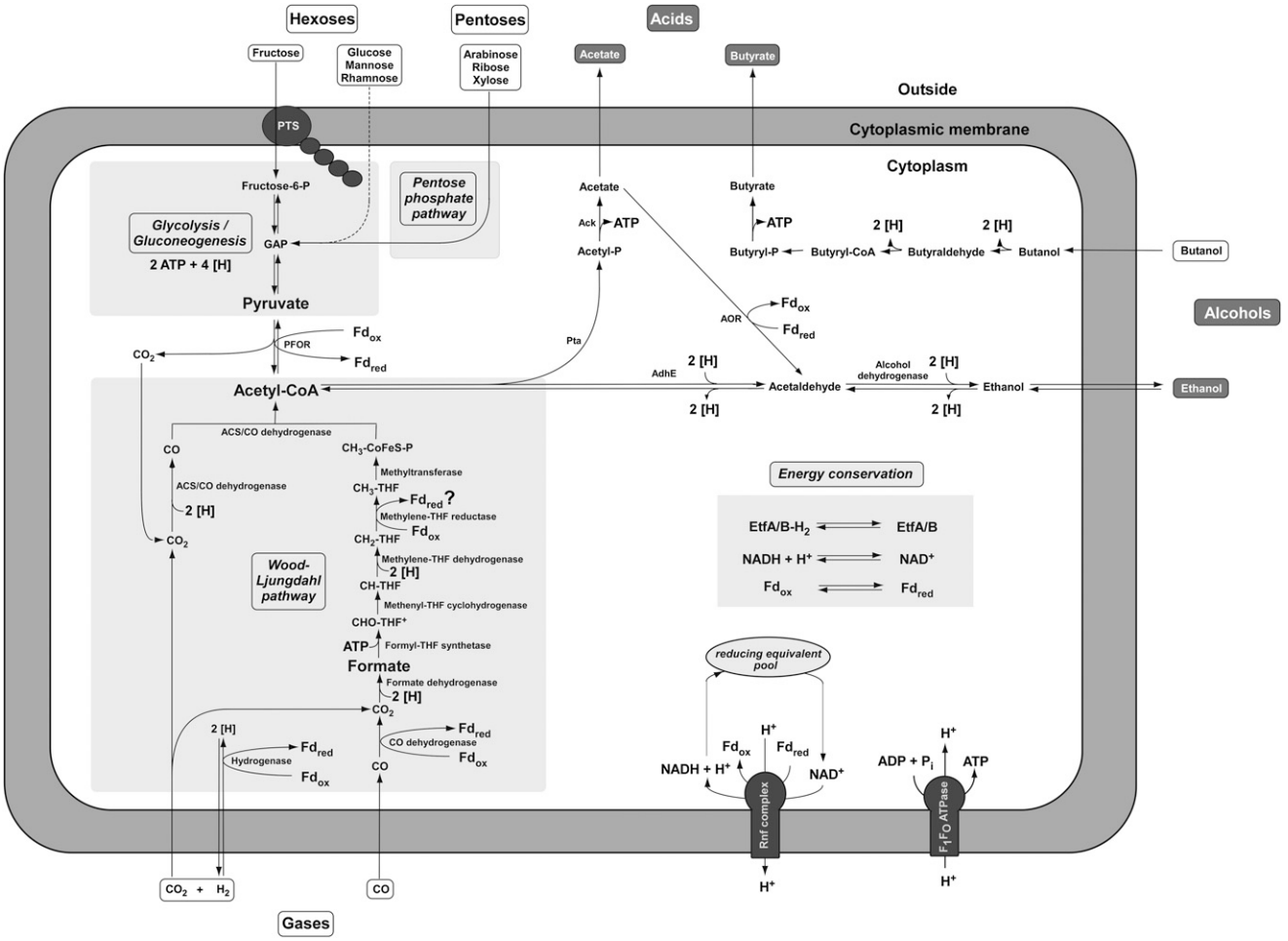

Fig. 1. Scheme of basic metabolic pathways and energy conservation in *C. ljungdahlii* when growing heterotrophically on sugars (hexoses or pentoses) or autotrophically on gases (CO or CO_2 + H_2). Glycolysis and pentose phosphate pathway for heterotrophic growth are underlaid in gray as well as the Wood–Ljungdahl pathway for autotrophic growth. The white boxes represent substrates and the dark gray boxes with white letters represent products. Single reactions do not represent stoichiometric fermentation balances. Ack, acetate kinase; ACS, acetyl-CoA synthase; AdhE, butyraldehyde/butanol dehydrogenase E; AOR, aldehyde oxidoreductase; Co-FeS-P, corrinoid iron-sulfur protein; Etf, electron-transferring flavoprotein; Fd, ferredoxin; GAP, glyceraldehyde-3-phosphate; PFOR, pyruvate:ferredoxin-oxidoreductase; Pta, phosphotransacetylase; PTS, PEP-dependent phosphotransferase system; THF, tetrahydrofolate.

be linked to tetrahydrofolate (THF) by a synthetase, yielding formyl-THF. The genome of *C. ljungdahlii* encodes three formate dehydrogenase genes. Two (CLJU_c08930 and CLJU_c06990) seem to be paralogs, of which CLJU_c06990 encodes a selenoprotein. The third also codes for a selenocysteine-containing enzyme (CLJU_c20040) and is linked to a gene of an electron transport protein with a [4Fe-4S] ferredoxin domain (CLJU_c20030). Formate dehydrogenases use diverse electron acceptors. The enzyme from *Clostridium pasteurianum*, being very similar to one of the *C. ljungdahlii* enzymes, uses ferredoxin in vitro as an electron acceptor.

Clostridium ljungdahlii is able to use CO as substrate. Whereas CO can be converted by the CO dehydrogenase activity of the acetyl-CoA synthase system, the genome encodes an additional CO dehydrogenase complex. It is nickel dependent (anaerobic type) and consists of a catalytic subunit (CooS) and a FAD/NAD^+-dependent oxidoreductase (CLJU_c09090–9110). All these enzymes are predicted to be soluble. The genome sequence of *C. ljungdahlii* also contains a cluster with similarity to genes of aerobic CO dehydrogenase complexes (CLJU_c23590–610) as well as of xanthine dehydrogenases. Because genes of purine biosynthesis (*purC*, CLJU_c23690) and of a putative nucleic acid-binding protein (CLJU_c23620) were found in the vicinity of this cluster, we assume the cluster encodes a xanthine dehydrogenase. This is further supported by the absence of the conserved active site loop of aerobic CO dehydrogenases (VAYRCSFR) in the respective *C. ljungdahlii* proteins (11).

During autotrophic growth, *C. ljungdahlii* uses hydrogenases to generate reducing equivalents from H_2 for CO_2 reduction. *C. ljungdahlii* contains four Fe-only hydrogenases and one NiFe-hydrogenase. The genes of two Fe-only hydrogenases are associated with those of NADH dehydrogenase subunits and Fe/S cluster-containing protein subunits. The gene product of CLJU_c37220 has similarity to hydrogenases in *Clostridium botulinum*, *Clostridium beijerinckii*, and *C. acetobutylicum* and is also associated with the NiFe-hydrogenase maturation factor HyaD, which inserts nickel in the hydrogenase. Other hydrogenase maturation-factor proteins (HypEDCF) (CLJU_c23060–090) involved in the insertion of nickel are also present. Only the NiFe-hydrogenase contains a transmembrane domain in the small subunit (CLJU_c28660–70).

C. ljungdahlii possesses neither genes encoding a formate: hydrogen–lyase complex as does *M. thermoacetica* nor the Ech hydrogenase complex as does *Methanosarcina barkeri*. There is also no heterodisulfide reductase present as in *M. thermoacetica* and *Desulfobacterium autotrophicum*, which use the Wood–Ljungdahl pathway for acetate oxidation.

C. ljungdahlii contains a methylene-THF reductase that consists of two subunits (encoded by CLJU_c37630 and CLJU_c37610), as the respective enzymes of *Clostridium formicoaceticum* and *M. thermoacetica* (12); the small subunit is supposed to possess

a zinc finger motif and very similar ORFs are found adjacent to the methylene-THF reductase genes in *Clostridium difficile*, *M. thermoacetica*, and several methanogens.

Ethanol Production and Utilization. *C. ljungdahlii* has several systems to either produce ethanol or to use it for growth. Utilization and generation of ethanol can proceed via acetyl-CoA and acetaldehyde using NAD^+-dependent acetaldehyde and ethanol dehydrogenases.

Genes of two such enzyme complexes form clusters on the genome. One contains two paralogs of a bifunctional acetaldehyde/ethanol dehydrogenase (*adhE*)(CLJU_c16510–20). Both genes are adjoining, indicating a gene duplication event. Such an arrangement is so far unique. An additional ethanol and an acetaldehyde dehydrogenase gene (CLJU_c11880, CLJU_c11960) are found among 15 genes encoding five microcompartment proteins being homologs to clustered ethanolamine-using genes (*eutN*) in *Salmonella typhimurium*. This group of genes also contains other orthologs of genes required for ethanolamine utilization (*eutTJQ*, *pduL*) and a gene that encodes a porin protein (CLJU_c11890). Because acetaldehyde is toxic to the cell, ethanol oxidation via acetaldehyde to acetyl-CoA is possibly performed in a subcellular, carboxysome-like compartment as in *Clostridium kluyveri*.

C. ljungdahlii might use one mechanism for ethanol (and butanol, see below) consumption and the other for ethanol production. In addition to these specific ethanol dehydrogenases, genes of several other Fe- and Zn-dependent alcohol dehydrogenases of unknown specificity are present.

C. ljungdahlii also contains two genes encoding an aldehyde oxidoreductase (AOR) (CLJU_c20110 and CLJU_c20210). The respective enzymes catalyze acetate reduction to acetaldehyde with reduced ferredoxin. This might also have implications in energy metabolism under conditions of surplus reducing equivalents (first formation of ATP by acetate production, then formation of acetaldehyde and ethanol) (Fig. 1).

Substrate Utilization. *C. ljungdahlii* can grow heterotrophically on fructose, glucose (after adaptation), gluconate, arabinose, ribose, xylose, erythrose, threose, formate, pyruvate, malate (pH change required), fumarate, ethanol, arginine, aspartate, glutamate, histidine, serine, choline, citrulline, guanine, hypoxanthine, and xanthine (3, 13, 14).

Accordingly, its genome encodes the complete set of enzymes for the Embden–Meyerhof–Parnas (EMP) pathway as well as the enzymes for gluconeogenesis to glucose-6-phosphate. The EMP pathway genes are organized in two clusters: 6-phosphofructokinase (CLJU_c03250) and pyruvate kinase (CLJU_c03260) in one, triosephosphate isomerase (CLJU_c39130), glyceraldehyde-3-phosphate dehydrogenase (CLJU_c39150), phosphoglycerate kinase (CLJU_c39140), phosphoglycerate mutase (CLJU_c39120), and enolase (CLJU_c39110) in the second one. The other genes are located individually on the genome. Fructose is taken up by two predicted fructose- or mannitol-specific phosphotransferase (PTS) systems (encoded by CLJU_c20590 and CLJU_c26050–70) and can be further converted by 1-phosphofructokinase (encoded by CLJU_c20600) to 1,6-bisphosphofructose. Pyruvate, resulting from glycolysis or taken up as a substrate, is converted to acetyl-CoA, CO_2, and reduced ferredoxin by pyruvate:ferredoxin-oxidoreductase (two copies are found, encoded by CLJU_c09340 and CLJU_c29340). Acetyl-CoA leads to both, ethanol (see above) and acetate formation (by phosphotransacetylase and acetate kinase, encoded by CLJU_c12770 and CLJU_c12780). Genes required for butyrate or butanol synthesis such as *crt* (encoding crotonase) and *bcd* (encoding butyryl-CoA dehydrogenase) are not present.

The genome of *C. ljungdahlii* codes for enzymes involved in arabinose and xylose metabolism as well as for all enzymes of the oxidative part of the pentose–phosphate pathway. Although the organism can use gluconate, no key enzymes of an Entner–Doudoroff pathway could be identified. However, *C. ljungdahlii* may convert D-gluconate via 6-phosphogluconate to D-ribulose-6-phosphate, which can be channeled into the oxidative branch of the pentose–phosphate pathway. The genes for gluconate metabolism are clustered on the chromosome together with a high-affinity gluconate transporter (CLJU_c11600).

Several purines such as guanine, hypoxanthine, and xanthine are fermented. Two potential xanthine dehydrogenases are encoded by clusters CLJU_c23880–910 and CLJU_c29920–40 (in addition to cluster CLJU_c23590–610 mentioned before). The genome of *C. ljungdahlii* contains two clusters of selenocysteine-containing glycine/betaine reductases (CLJU_c27770–840 and CLJU_c27290–360), which are very similar to each other. These enzyme complexes are probably responsible for reduction of glycine and betaine, respectively, to acetate. Glycine is an intermediate in clostridial purine degradation, being reduced to acetate, ammonia, and ATP by glycine reductase and acetate kinase (15). Subunits A and B of both glycine/betaine reductases are selenoproteins. Next to one of the glycine/betaine reductase clusters (CLJU_c27770–840) the genes for selenocysteine biosynthesis are located. There are two clusters of genes (CLJU_c27720–40 and CLJU_c07460–80) for selenocysteine biosynthesis and incorporation. One cluster is located next to a tRNA gene that possibly represents a $tRNA^{Sec}$. The second cluster contains additionally a selenocysteine lyase (CLJU_27730).

C. ljungdahlii has three possibilities for nitrogen assimilation as predicted from the genome. First, it could use ammonia by directly incorporating it into amino acids by use of a glutamine synthetase (encoded by CLJU_c41950) and a glutamine:2-oxoglutarate aminotransferase (encoded by CLJU_c17370–80). Second, the organism is predicted to fix molecular nitrogen using a molybdenum-dependent nitrogenase (encoded by CLJU_c04930–50). Third, the organism is able to assimilate nitrate presumably by using a *Clostridium perfringens*-type fermentative nitrate reductase (encoded by CLJU_c23710–30).

Intermediary Metabolism. At least two enzymes were found that interconvert C_3 and C_4 compounds: pyruvate carboxylase (encoded by CLJU_c37390) and PEP-carboxykinase (encoded by CLJU_06210). *C. ljungdahlii* operates a branched TCA "cycle," like most anaerobic prokaryotes. Citrate is formed by a (Re)-citrate synthase (CLJU_c06610). Genes encoding enzymes converting citrate to 2-oxoglutarate are clustered (CLJU_c06610–30). The other TCA branch allows formation of fumarate from oxaloacetate by malate dehydrogenase (encoded by CLJU_c05920) and fumarate hydratase (encoded by CLJU_c40590–600). No genes for succinate dehydrogenase, 2-oxoglutarate synthase, and succinyl-CoA synthetase were found. Thus, 5-aminolevulinate as a precursor for tetrapyrroles can only be formed by glutamyl-tRNA synthase, glutamyl-tRNA reductase, and glutamate-1-semialdehyde 2,1-aminomutase (encoded by CLJU_c13390, CLJU_c04480, and CLJU_c04490, respectively). However, no genes for heme biosynthesis could be identified on the genome. Therefore, *C. ljungdahlii* is apparently unable to synthesize cytochromes.

The organism has numerous proteins requiring cobalamin as cofactor and can synthesize vitamin B_{12} via the anaerobic pathway. Most genes are located in a large cluster (CLJU_c31820–32020), conserved among many clostridia. Several cobalt transport systems are found in the genome (e.g., CLJU_c40730–50, CLJU_c22300–20, and CLJU_c28590–600).

Other cofactors predicted to be synthesized are folate, riboflavin, NAD^+, CoA, and thiamine, but this has not been experimentally verified. Interestingly, many genes for biotin biosynthesis are missing, so that biotin has to be taken up as by *C. kluyveri*. No genes for the biosynthesis of quinones were found.

Energy Conservation. ATP can be generated during heterotrophic growth by substrate level phosphorylation during glycolysis and combined phosphotransacetylase/acetate kinase reactions.

Until now, two types of homoacetogenic metabolism were known (16). Organisms such as *Acetobacterium woodii* establish a Na^+ gradient, which is converted into ATP by a Na^+-specific

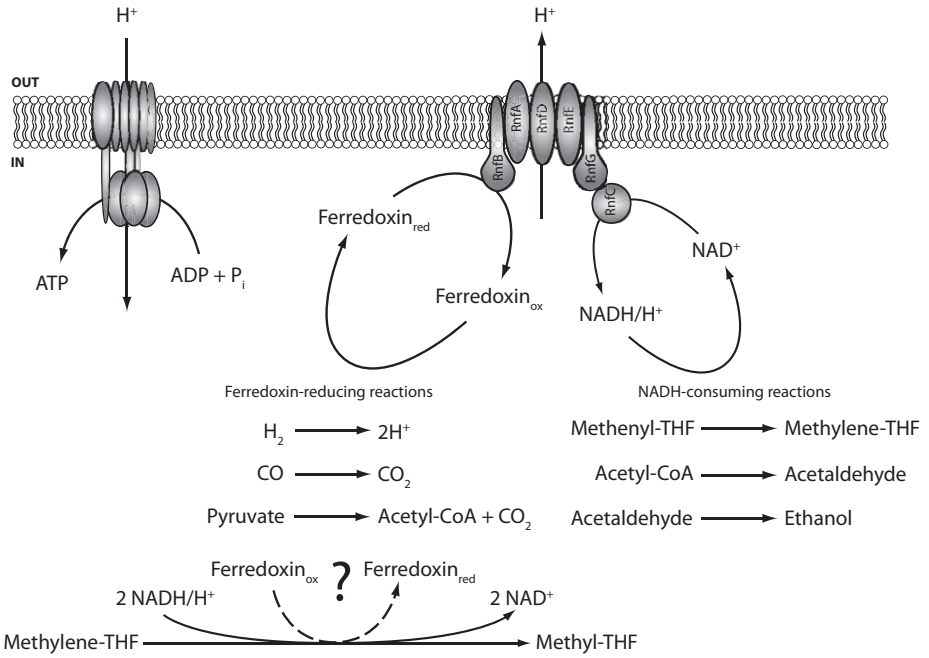

Fig. 2. Rnf complex in *C. ljungdahlii.*

ATPase. Bacteria such as *Clostridium aceticum*, *M. thermoacetica*, and *Moorella thermoautotrophica* possess cytochromes and quinones and use them to generate a proton gradient, leading to ATP formation by a F_1F_0-type ATPase. Genes coding for an H^+-translocating ATPase were detected in *C. ljungdahlii*, but a Na^+-translocating ATPase is not present as judged from the lack of a Na^+-liganding amino acid motif (17, 18). In addition, growth dependence of *C. ljungdahlii* for sodium ions was tested on fructose, syngas, and CO_2/H_2. The original medium contained 38.2 mM Na^+. By using potassium salts, the sodium ion concentration was diminished to 14, 7, 3.5, and 1.4 mM. No change in growth pattern was observed when *C. ljungdahlii* was grown in these media. Similar experiments with *A. woodii* had revealed that a Na^+ concentration below 5 mM caused severe growth defects, and no growth was possible below 2.5 mM Na^+ (19). Thus, *C. ljungdahlii* does not belong to the sodium-type homoacetogens. On the other hand, it also cannot generate a proton gradient via cytochromes and quinones, due to a lack of these compounds. However, *C. ljungdahlii* contains genes encoding the six subunits of the Rnf-complex (*rnfCDGEAB*, CLJU_c11360–410), RnfA, -D, and -E being predicted integral membrane proteins (Fig. 2). Subunits C and B have two ferredoxin domains with [4Fe-4S]-clusters. Genes for the Rnf-

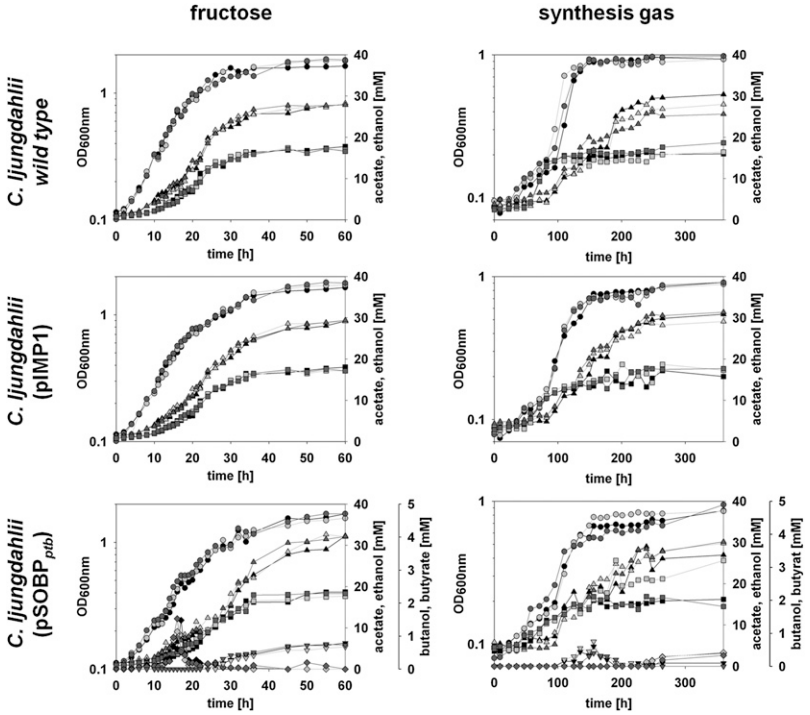

Fig. 3. Growth experiments with *C. ljungdahlii* wild type, *C. ljungdahlii* (pIMP1), and *C. ljungdahlii* ($pSOBP_{ptb}$) in 1,000-mL flasks with 200 mL PETC media and fructose or 0.8 bar synthesis gas. OD_{600nm} (circles) and concentration of acetate (triangles up), ethanol (squares), butanol (triangles down), and butyrate (diamonds) were measured in three different cultures (black, dark gray, and light gray).

complex are present in many clostridial species including *Clostridium tetani* and *C. kluyveri*. In contrast, no *rnf* genes were found in the genome of *M. thermoacetica*, which is producing a proton gradient via cytochromes and quinones (8). In *C. kluyveri*, the Rnf complex was shown to play an important role in energy metabolism by coupling electron flow from reduced ferredoxin to NAD^+ to proton translocation (20–22). Thus, *C. ljungdahlii* represents a third mode of homoacetogenic metabolism by presumably generating a proton gradient via the Rnf system. We propose that during autotrophic growth *C. ljungdahlii* uses reduced ferredoxin (stemming from oxidation of hydrogen) to generate a proton gradient and NADH, which is consumed by CO_2 reduction and ethanol formation (Fig. 2).

Under heterotrophic conditions, the Rnf complex might be a means of additional energy conservation. The reduced ferredoxin from the pyruvate: ferredoxin–oxidoreductase reaction will be needed for CO_2 refixation and therefore cannot be used in the Rnf complex. However, the reduction step from methylene-THF and NADH to methyl-THF and NAD^+ (catalyzed by methylene-THF reductase) is highly exergonic ($\Delta E_o' = -200$ mV) and irreversible under physiological conditions ($\Delta G_0' = -22$ kJ/mol) (23). In analogy to the butyryl-CoA dehydrogenase/EtfAB complex in butyrate-forming clostridia (20), we therefore postulate that electrons from NADH are bifurcated in this reaction to 5,10-methylene-THF and ferredoxin$^-$ and a second NADH delivers the second electron to complete formation of 5-methyl-THF and Fd^{2-}. The reduced ferredoxin then will transfer the electrons to NAD^+ at the Rnf complex, regenerating the second NADH and forming a proton gradient. Thus, ATP can be formed in addition to substrate-level phosphorylation. Further support for this proposal comes from enzymatic characterization, showing that methylene-THF reductases of homoactogens contain flavin (12, 24), thus enabling an easy coupling to the electon-transfer-flavoproteins (EtfA/B) required for the process (20) and being present in five sets (see below) in the genome of *C. ljungdahlii*.

DNA Transfer. To develop a genetic system for *C. ljungdahlii*, the *Clostridium–Escherichia coli* shuttle vector pIMP1 was used (25). Our goal was to combine production of the biofuel butanol, having superior qualities to ethanol (26–28), with the autotrophic growth of *C. ljungdahlii* on syngas. Plasmid $pSOBP_{ptb}$ was constructed (Fig. S3), containing the *C. acetobutylicum* butanol synthesis pathway genes *thlA*, *hbd*, *crt*, *bcd*, *adhE*, and *bdhA* (encoding thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase, butanol/butyraldehyde dehydrogenase, and butanol dehydrogenase, respectively) under control of the phosphotransbutyrylase operon promoter P_{ptb} and the acetoacetate decarboxylase terminator T_{adc}. All were amplified by PCR from *C. acetobutylicum* and cloned into pIMP1, using *E. coli* as a host. However, in various *E. coli* strains no butanol production could be detected. This inability probably depends on the Bcd enzyme. Inui et al. (29) showed that this enzyme requires two electron-transferring flavoproteins (EtfA/B), which were not present on $pSOBP_{ptb}$, for activity in *E. coli*. Nevertheless, we tried to transform $pSOBP_{ptb}$ in *C. ljungdahlii*. Successful electroporation was achieved using a modified protocol for *Clostridium tyrobutyricum* (30), which is a close relative of *C. ljungdahlii*.

Heterologous Gene Expression in *C. ljungdahlii*. Clarythromycin- and thiamphenicol-resistant transformants were used for growth experiments (Fig. 3). Recombinant *C. ljungdahlii* was able to produce small amounts of butanol. Five pairs of genes encoding electron transferring flavoproteins (EtfA/B) were identified in the genome sequence of *C. ljungdahlii* (CLJU_c21580–90, CLJU_c13880–90, CLJU_c39400–10, CLJU_c20330–40, and CLJU_c40220–30). One set (CLJU_c21580–90) has high homology to that of *C. acetobutylicum* and could possibly conduct activity of Bcd. A maximum amount of about 2 mM butanol was detected in batch cultures of *C. ljungdahlii* ($pSOBP_{ptb}$) during midgrowth phase (OD_{600nm} ~0.6), providing proof of principle. However, at the end of growth, almost no butanol (0–0.2 mM) was detected. Instead, small amounts of butyrate (0.6–0.8 mM) were measured (Fig. 3), indicating that *C. ljungdahlii* can metabolize 1-butanol to butyrate. Further experiments revealed that *C. ljungdahlii* could not use various amounts (5, 10, 20, 28, or 50 mM) of butanol as a sole carbon source, but, in combination with another carbon source, could convert butanol to butyrate. After growth of wild-type *C. ljungdahlii* on fructose supplemented with 5, 10, or 20 mM butanol, only 2.6, 5.5, and 13.7 mM butanol, respectively, remained at the end of growth, whereas 1.3, 2.7, and 3.6 mM butyrate, respectively, were produced (besides acetate and ethanol). As mentioned before, several aldehyde and alcohol dehydrogenase genes were identified in the genome, one set of which is probably responsible for butanol consumption. Inactivation of respective dehydrogenase genes (by the universal *Clostridium* gene knock-out system) (31) will allow sustained butanol production. $pSOBP_{ptb}$ can be further improved to achieve a higher butanol yield: The *bdhA* gene could be replaced with *bdhB*, whose gene product has a higher specificity for butyraldehyde (32). Moreover, plasmid analysis revealed a dyad symmetry upstream of the *bdhA* and *adhE* genes. These dyad symmetries are fragments of the deduced *thlA* and *bdhA* terminators (in each case part of the stem loop, but not the poly-A region) and could possibly decrease transcription downstream of the *thlA* gene, i.e., of the *bdhA* and *adhE* genes (Fig. 4). Nevertheless, butanol production by *C. ljungdahlii* indicates that *bdhA* and *adhE* are still transcribed. This could be confirmed by Northern blot experiments. However, the level of transcription was significantly diminished (Fig. 4), so that removal of this dyad symmetry would certainly enhance butanol production. A DNA probe from the acetate kinase gene of *C. ljungdalii* was used as positive control in the Northern blot experiments, showing that *ack* is strongly transcribed (Fig. 4). Using the promoter of this operon for expression would mean a further enhancement of butanol synthesis.

Concluding Remarks. The synthesis capabilities of *C. ljungdahlii* from CO and CO_2 are not limited to biofuels, but can be expanded to virtually every compound, for which biological pathways exist or will be newly constructed (synthetic biology). Thus, this biotechnological approach will reduce dependency on crude oil, will fulfill industrial needs, and, by doing so, could contribute to reducing the atmospheric greenhouse effect, if the hydrogen required stems from a non-CO_2-producing process.

Materials and Methods

Bacterial Strains and Growth Conditions. Bacterial strains used are listed in Table S2. Organisms were cultivated in liquid or solid (1–1.5% agar) media at 37 °C. Growth was monitored by measuring the optical density at 600 nm (OD_{600nm}). *C. ljungdahlii* was grown anaerobically in PETC medium (American Type Culture Collection (ATCC) medium 1754) and *C. acetobutylicum* in 2× YT medium (33), respectively. The *E. coli* strains used as intermediate hosts (XL1-blue, DH5a, JM109, SURE) and for in vivo methylation of plasmids (ER2275) (22) were grown aerobically in LB medium (34). Growth experiments with *E. coli* were carried out in TM3a/glucose medium (35) under aerobic, semiaerobic, and anaerobic conditions. Appropriate antibiotics were used for *C. ljungdahlii* (5 μg/mL clarythromycin, 20 μg/mL thiamphe-

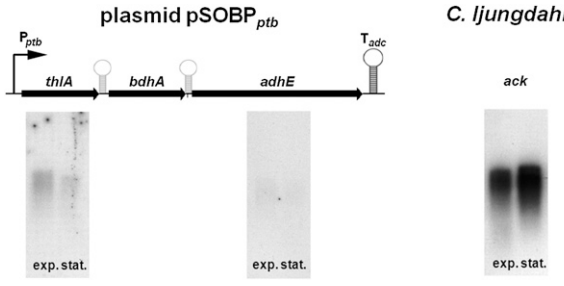

Fig. 4. Northern blots of recombinant *C. ljungdahlii* ($pSOBP_{ptb}$). Probes used: *thlA* and *adhE* from *C. acetobutylicum* and *ack* from *C. ljungdahlii*. Samples were taken from exponential (exp.) and stationary (stat.) growth phases.

nicol) and *E. coli* (100 μg/mL ampicillin, 250 μg/mL erythromycin, 30 μg/mL chloramphenicol, 50 μg/mL spectinomycin).

Plasmids. All plasmids used in this study are listed in Table S2. For construction of pSOBP$_{ptb}$ (Fig. S3), the respective regions were PCR amplified from genomic DNA of *C. acetobutylicum* and *catP* from plasmid pJIR750, respectively, using standard techniques. Primers contained restriction sites for further cloning and are listed in Table S3.

Sequencing, Gene Prediction, and Annotation. Recently described procedures were followed (36, 37) with 66,585 sequences analyzed.

Transformation. Transformation of *E. coli* was performed according to Inoue et al. (38) or Dower et al. (39). *C. ljungdahlii* was electroporated using a protocol for *C. tyrobutyricum* (30). Modifications: For preparation of competent cells a 50-mL culture of *C. ljungdahlii* in early exponential growth phase was used to inoculate 50 mL fresh medium containing 40 mM DL-threonine. Incubation time was 8–12 h (OD_{600nm} 0.3–0.7). For electroporation 0.1–1.5 μg methylated plasmid DNA was used. After the pulse, the cells were transferred into 5 mL prewarmed medium in a hungate tube and incubated at 37 °C until growth was visible (measured in hungate tubes in a photometer). Aliquots of the transformants were inoculated into 5 mL liquid medium and spread onto antibiotic-containing plates to develop mutant colonies.

Nucleic Acid Isolation. Plasmid DNA from *E. coli* and *C. ljungdahlii* was isolated using the peqGOLD plasmid miniprep kit I (PEQLAB Biotechnologie). Because the quality of the isolated plasmid DNA from *C. ljungdahlii* was not sufficient for all molecular applications, plasmid verification was carried out by PCR or by retransformation into *E. coli* ("plasmid rescue"). Genomic DNA from *C. acetobutylicum* and from *C. ljungdahlii* was isolated according to Bertram and Dürre (40). RNA from *C. ljungdahlii* was isolated according to Oelmüller et al. (41).

Northern Blots. RNA (~10 μg) was fractionated by denaturing agarose gel electrophoresis in a 1% agarose gel. The RNA was transferred to a Biodyne A Transfer nylon membrane (Pall) by vacuum blotting, using 5× SSC buffer (750 mM NaCl, 75 mM sodium citrate × 2 H_2O; pH 11.5). The RNA was covalently bound to the membrane with an UV crosslinker (GE Healthcare Europe) at 254 nm and an energy density of 120 mJ/cm^2.

DNA Probes. DNA probes were PCR amplified from pSOBP$_{ptb}$. The *thlA* probe was amplified with primers pSOB-thlA-Sonde-F and pSOB-thlA-Sonde-R and the *adhE* probe with pSOB-adhE-Sonde-F and pSOB-adhE-Sonde-R. An acetate kinase *ack* probe from *C. ljungdahlii* was amplified from genomic DNA with primers Clj-ack-Sonde-F and Clj-ack-Sonde-R (positive control). DNA probes were labeled with 50 μCi [α-^{32}P] γ-ATP (GE Healthcare) using the HexaLabel Plus DNA labeling kit (Fermentas).

Hybridization. Hybridization of labeled DNA probes with membrane-bound RNA was carried out according to Thomas (42). For detection, the membrane was exposed on a Cronex 5 X-ray film (Agfa), which was developed with a Curix 60 processor (Agfa).

Detection of Metabolites. Ethanol, acetone, acetate, 1-butanol, acetoin, and butyrate (listed in the order of their elution) were quantified gaschromatographically as described previously (43). Modification: GC oven temperature profile: 130 °C for 1 min, 130–200 °C with 4 °C increase per minute, 200 °C for 3 min.

ACKNOWLEDGMENTS. Work was supported by grants from the Bundesministerium für Bildung und Forschung GenoMik and GenoMikPlus projects (Competence Network Göttingen).

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Corrections

CHEMISTRY

Correction for "Depression of reactivity by the collision energy in the single barrier H + CD_4 → HD + CD_3 reaction," by Weiqing Zhang, Yong Zhou, Guorong Wu, Yunpeng Lu, Huilin Pan, Bina Fu, Quan Shuai, Lan Liu, Shu Liu, Liling Zhang, Bo Jiang, Dongxu Dai, Soo-Ying Lee, Zeng Xie, Bastiaan J. Braams, Joel M. Bowman, Michael A. Collins, Dong H. Zhang, and Xueming Yang, which appeared in issue 29, July 20, 2010, of *Proc Natl Acad Sci USA* (107:12782–12785; first published July 6, 2010; 10.1073/pnas.1006910107).

The authors note that author name Zeng Xie should have appeared as Zhen Xie. The online version has been corrected. The corrected author line appears below.

Weiqing Zhang[a,1], Yong Zhou[a,1], Guorong Wu[a], Yunpeng Lu[b], Huilin Pan[a], Bina Fu[a], Quan Shuai[a], Lan Liu[a], Shu Liu[a], Liling Zhang[b], Bo Jiang[a], Dongxu Dai[a], Soo-Ying Lee[b], Zhen Xie[c], Bastiaan J. Braams[d], Joel M. Bowman[e], Michael A. Collins[f], Dong H. Zhang[a,2], and Xueming Yang[a,2]

www.pnas.org/cgi/doi/10.1073/pnas.1011320107

MICROBIOLOGY

Correction for "*Clostridium ljungdahlii* represents a microbial production platform based on syngas," by Michael Köpke, Claudia Held, Sandra Hujer, Heiko Liesegang, Arnim Wiezer, Antje Wollherr, Armin Ehrenreich, Wolfgang Liebl, Gerhard Gottschalk, and Peter Dürre, which appeared in issue 29, July 20, 2010, of *Proc Natl Acad Sci USA* (107:13087–13092; first published July 2, 2010; 10.1073/pnas.1004716107).

The authors note that, due to a printer's error, the sequence deposition number was not published with the manuscript. The data have been deposited in the GenBank database (accession no. CP001666).

www.pnas.org/cgi/doi/10.1073/pnas.1010816107

PROFILE

Correction for "Profile of Ronald Levy," by Beth Azar, which appeared in issue 29, July 20, 2010, of *Proc Natl Acad Sci USA* (107:12745–12746; first published July 12, 2010; 10.1073/pnas.1008810107).

The editors note that Philip Downey should have been listed as the author of the Profile. This information has been updated online.

www.pnas.org/cgi/doi/10.1073/pnas.1011244107

NEUROSCIENCE

Correction for "SH3TC2/KIAA1985 protein is required for proper myelination and the integrity of the node of Ranvier in the peripheral nervous system," by Estelle Arnaud, Jennifer Zenker, Anne-Sophie de Preux Charles, Claudia Stendel, Andreas Roos, Jean-Jacques Médard, Nicolas Tricaud, Joachim Weis, Ueli Suter, Jan Senderek, and Roman Chrast, which appeared in issue 41, October 13, 2009, of *Proc Natl Acad Sci USA* (106:17528–17533; first published September 29, 2009; 10.1073/pnas.0905523106).

The authors note that Henning Kleine and Bernhard Luscher should be added to the author list between Nicolas Tricaud and Joachim Weis. Henning Kleine should be credited with performing research. Bernhard Luscher should be credited with analyzing data. The online version has been corrected. The corrected author and affiliation lines, and author contributions appear below.

Estelle Arnaud[a], Jennifer Zenker[a,b], Anne-Sophie de Preux Charles[a], Claudia Stendel[c], Andreas Roos[d], Jean-Jacques Médard[a], Nicolas Tricaud[c], Henning Kleine[e], Bernhard Luscher[e], Joachim Weis[f], Ueli Suter[c], Jan Senderek[c,d,f], and Roman Chrast[a,1]

[a]Department of Medical Genetics and [b]Graduate Program in Neurosciences, University of Lausanne, CH-1005 Lausanne, Switzerland; [c]Institute of Cell Biology, Eidgenössische Technische Hochschule (ETH) Zurich, CH-8093 Zurich, Switzerland; Institutes of [d]Human Genetics and [f]Neuropathology, Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen University, 52074 Aachen, Germany; and [e]Institute of Biochemistry and Molecular Biology, Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen University, 52057 Aachen, Germany

Author contributions: E.A., J.S., and R.C. designed research; E.A., J.Z., A.-S.d.P.C., C.S., A.R., J.-J.M., H.K., N.T., and R.C. performed research; E.A., J.Z., A.-S.d.P.C., C.S., A.R., B.L., J.W., U.S., J.S., and R.C. analyzed data; and E.A., N.T., J.W., U.S., J.S., and R.C. wrote the paper.

www.pnas.org/cgi/doi/10.1073/pnas.1010446107

CORRECTIONS

Supporting Information

Köpke et al. 10.1073/pnas.1004716107

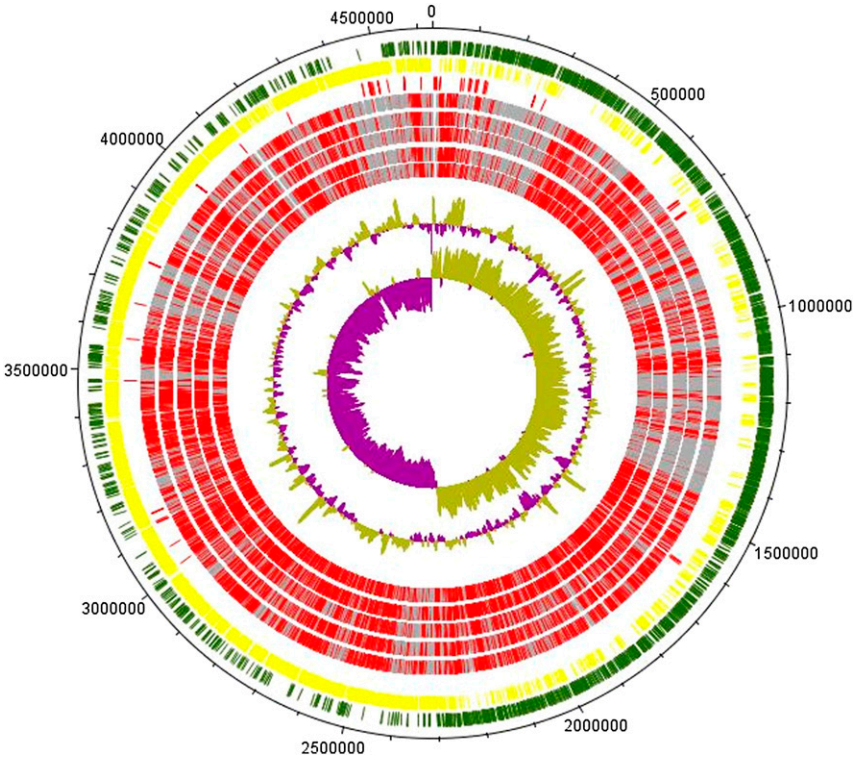

Fig. S1. Circular representation of the *C. ljungdahlii* DSM 13528 chromosome. Rings from the outside to the inside: scale (in base pairs), protein-encoding sequences (green or yellow), rRNA and tRNA (red), comparison of the genes with homologs in *C. kluyveri, Clostridium novyi, C. beijerinckii, C. tetani,* and *C. acetobutylicum* (gray areas are specific to *C. ljungdahlii*), GC content variation (higher values on the outside), GC-skew.

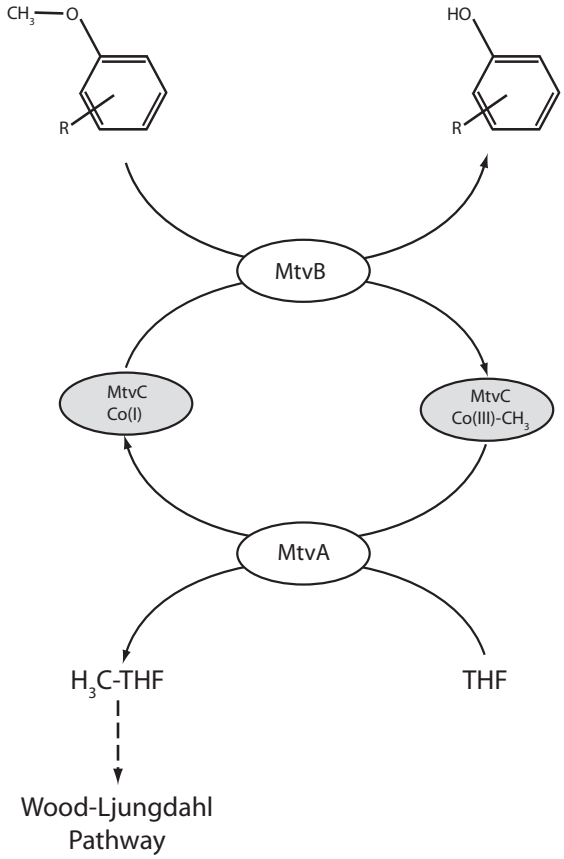

Fig. S2. Possible system for demethylation of methoxylated compounds in *C. ljungdahlii*. *C. ljungdahlii* contains several genes that are homologs of genes involved in demethylation of methoxylated phenylacrylates in *M. thermoacetica*. The methyl transferases are composed of the three proteins MtvA, MtvB, and MtvC. There are five homologs of MtvB that catalyze the demethylation reaction; four of them are clustered with homologs of MtvC and MtvA. MtvC is a corrinoid protein that transfers the methyl group to MtvA. There are eight homologs of MtvC present, and 16 genes that are homologs to *mtvA*, whose gene product catalyzes the transfer of the methyl group from the cobalamin of MtvC to THF. Most of these ORFs are clustered on the *C. ljungdahlii* genome in groups with at least one counterpart of each of the three genes of the demethylation system.

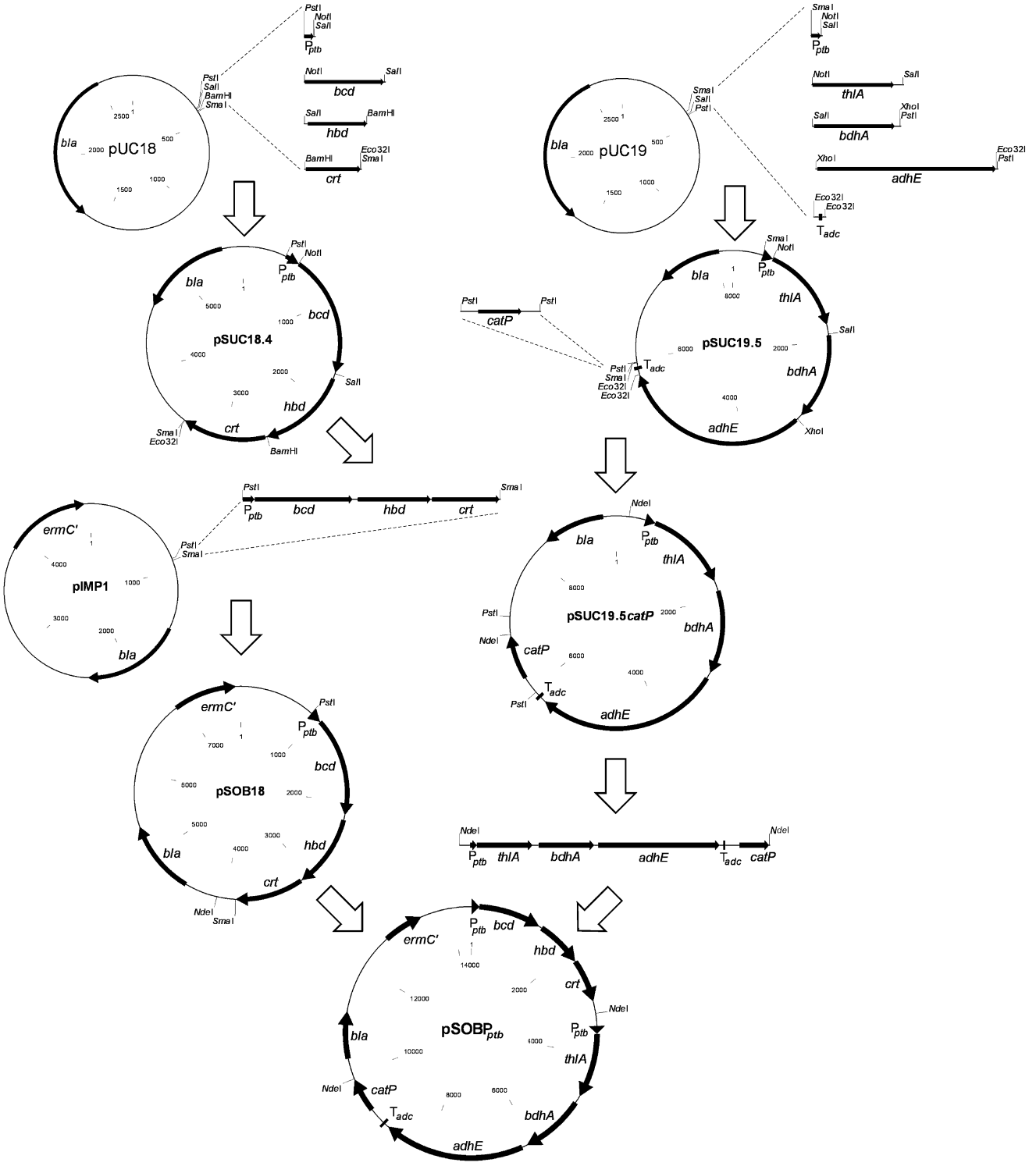

Fig. S3. Construction of plasmid pSOBP*ptb*.

Table S1. Comparison of *C. ljungdahlii* genome features to those of other clostridial genomes

	C. l.	*C. a.*	*C. b.*	*C. d.*	*C. k.*	*C. t.*	*M. t.*
Genome size (bp)	4,630,065	4,132,880	6,000,632	4,298,133	4,023,800	2,873,333	2,628,784
G + C content of DNA (%)	31	31	29	29	32	29	56
DNA replicons	1	2	1	2	2	2	1
ORFs total	4,198	3,848	5,020	3,757	3,912	2,432	2,615
ORFs with assigned functions (% of total ORFs)	3,141 (75)	2,350 (61)	3,883 (77)	3,211(85)	1,240 (32)	1,989 (82)	1,866 (76)
rRNA cluster	9	11	14	11	7	6	1
tRNAs	72	73	94	87	61	54	51

C. l., *Clostridium ljungdahlii*; *C. a.*, *Clostridium acetobutylicum*; *C. b.*, *Clostridium beijerinckii*; *C. d.*, *Clostridium difficile* 630; *C. k.*, *Clostridium kluyveri*; *C. t.*, *Clostridium tetani*; *M. t.*, *Moorella thermoacetica.*

Table S2. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Clostridium acetobutylicum		
DSM 792	Wild type	DSMZ*
Clostridium ljungdahlii		
DSM 13528	Wild type	DSMZ*
Escherichia coli		
DH5α	F$^-$ φ80d*lacI*^{q}*Z*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(r_K^-, m_K^+) *phoA supE*44 λ$^-$ *thi*-1 *gyrA*96 *relA*1	(1)
ER2275	*trp-31 his-1 tonA2 rpsL104 supE44 xyl-7 mtl-2 metB1* e14$^-$ Δ(*lac*)*U169 endA1 recA1* R(*zbgZ10*::Tn*10*) Tc^s Δ(*mcr-hsd-mrr*)*114*::1510 [F′*proAB traD36 laq1*^{q} Δ*M15 zzf*::mini Tn*10* (Km^r)]	(2)
JM109	Δ*(lac-proAB) glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17* [F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15*]	(3)
SURE	*e14– (McrA–)* Δ*(mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5* (Km^r) *uvrC* [F′*proAB lacI*^{q}*Z*Δ*M15 Tn10* (Tet^r)]	Stratagene
WL3	*adhC81 fadR adhE* [F$^+$ *mel supF*]	(4)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q}*Z*Δ*M15 Tn10* (Tet^r)]	Stratagene
Plasmids		
pANS1	6.2 kbps; Sp^r (*spcE*), pACYC184/p15A *ori* (*rep*), φ*3tI*	This study
pCR4-TOPO	4.0 kbps; Ap^r (*bla*), Km^r (*aph*), pMB1/ColE1 *ori* (*rep*), *lac*POZα-*ccdB*	Invitrogen
pIMP1	4.7 kbps; Ap^r (*bla*), Em^r (*ermC′*), pMB1/ColE1 *ori* (*rep*), pIM13 *ori* (*repL*)	(5)
pJIR750	6.6 kbps; Cm^r (*catP*), pMB1/ColE1 *ori* (*rep*), pIP404 *ori* (*rep*), *lac*POZ′	(6)
pSOB18ptb	7.7 kbps; pIMP1, P_{ptb}, *bcd*, *hbd, crt*	This study
pSOBP$_{ptb}$	14.3 kbps; pIMP1, Cm^r (*catP*), P_{ptb}, *bcd*, *hbd*, *crt*, P_{ptb}, *thlA*, *bdhA, adhE*, T_{adc}	This study
pSUC18.2	3.9 kbps; pUC18, P_{ptb}, *bcd*	This study
pSUC18.3	4.9 kbps; pUC18, P_{ptb}, *bcd*, *hbd*	This study
pSUC18.4	5.7 kbp; pUC18, P_{ptb}, *bcd*, *hbd*, *crt*	This study
pSUC19.2	4.1 kbp; pUC19, P_{ptb}, *thlA*	This study
pSUC19.3	5.3 kkps; pUC19, P_{ptb}, *thlA*, *bdhA*	This study
pSUC19.4	8.0 kbps; pUC19, P_{ptb}, *thlA*, *bdhA*, *adhE*	This study
pSUC19.5	8.1 kbps; pUC19, P_{ptb}, *thlA*, *bdhA adhE*, T_{adc}	This study
pSUC19.5catP	9.3 kbps; pUC19, Cm^r (*catP*), P_{ptb}, *thlA*, *bdhA adhE*, T_{adc}	This study
pUC18	2.7 kBp; Ap^r (*bla*), pMB1/ColE1 *ori* (*rep*), *lac*POZ′	(7)
pUC19	2.7 kBp; Ap^r (*bla*), pMB1/ColE1 *ori* (*rep*), *lac*POZ′	(7)
pUC18ptb	2.9 kbps; pUC18, P_{ptb}	This study
pUC19ptb	2.9 kbps; pUC19, P_{ptb}	This study

Apr, ampicillin resistance gene; Cmr, chloramphenicol resistance gene; Emr, erythromycin resistance gene; Kmr, kanamycin resistance gene; Spr, spectinomycin resistance gene; *ccdB*, CcdB toxin gene; φ*3tI*, *Bacillus subtilis* phage Φ3TI methyltransferase gene.
*Deutsche Sammlung von Mikroorganismen und Zellkulturen.

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Table S3. Primers

Primer name	Sequence (5′ → 3′)
adhEfXhoI	ATAACTCGAGAGAAAGAAGTGTATATTTATG
adhEr	GGTTCTGCAG CCCGGG GATATCGAAATATGAAGGTTTAAGGTTG
bcdNotf	ATAAGCGGCCGCATGGATTTTAATTTAACAAG
bdhArXhoIPstI	GGTTCTGCAGATCTCGAGAGTGAGGCGTTAGAAC
Bubcdr	TGGCCGACGTCGACTTAACCTCCTTAAATTATC
BubdhAf	ACGCGTCGACTAGGAGGTAAGAAGTATGC
Bucrtf	CGGGATCCTAGGAGGATTAGTCATGGG
Bucrtr	TCCCCCGGG GATATCCCTATCTATTTTTGAAGCC
Buhbdfneu	ACGCGTCGACGGTGTAAATGCATTGATAG
Buhbdr	GCGGATCCTTATTTTGAATAATCGTAG
Buptbf	AACTGCAGCAGAAAGTATAATGAG
Buptbf19f	TCCCCCGGGCAGCAGAAAGTATAATGAG
Butermf	AATAGATATCGAACTTAGACCCATGGCTG
Butermr	AATAGATATCCTAGCTCTCACATTCTTGC
ButhlAr	TGGCCGACGTCGACGAAATTGAACTGTCTTTAG
catpPstIf	AAAACTGCAGCGAGTGAAAAAGTGTCCCTAGCG
catpPstIr	AACCCTGCAG CATATGGTTACAGACAAACCTGAAGTTAA
Clj-ack-Sonde-F	CAGACAATGCCAGATTATGC
Clj-ack-Sonde-R	TCACTGCTTACTCCTGATAC
pSOB-adhE-Sonde-F	GTCGGACAGTCAGCTTATAC
pSOB-adhE-Sonde-R	GGACCAACATTCTCGGAAAC
pSOB-thlA-Sonde-F	GTTTGTGGTTCAGGACTTAG
pSOB-thlA-Sonde-R	TAGGGTGCTCATCTGTATC
ptbrNotSal	TGGCCGACGTCGACATGCGGCCGCCACTCCCTTTTACTATTTAATT
thlANotIf	ATAAGCGGCCGCATGAAAGAAGTTGTAATAGC

Bases representing restriction enzyme cutting sites are underlined.