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A Genetic System for *Clostridium ljungdahlii*: a Chassis for Autotrophic Production of Biocommodities and a Model Homoacetogen

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Methods for genetic manipulation of *Clostridium ljungdahlii* are of interest because of the potential for production of fuels and other biocommodities from carbon dioxide via microbial electrosynthesis or more traditional modes of autotrophy with hydrogen or carbon monoxide as the electron donor. Furthermore, acetogenesis plays an important role in the global carbon cycle. Gene deletion strategies required for physiological studies of *C. ljungdahlii* have not previously been demonstrated. An electroporation procedure for introducing plasmids was optimized, and four different replicative origins for plasmid propagation in *C. ljungdahlii* were identified. Chromosomal gene deletion via double-crossover homologous recombination with a suicide vector was demonstrated initially with deletion of the gene for FliA, a putative sigma factor involved in flagellar biogenesis and motility in *C. ljungdahlii*. Deletion of *fliA* yielded a strain that lacked flagella and was not motile. To evaluate the potential utility of gene deletions for functional genomic studies and to redirect carbon and electron flow, the genes for the putative bifunctional aldehyde/alcohol dehydrogenases, *adhE1* and *adhE2*, were deleted individually or together. Deletion of *adhE1*, but not *adhE2*, diminished ethanol production with a corresponding carbon recovery in acetate. The double deletion mutant had a phenotype similar to that of the *adhE1*-deficient strain. Expression of *adhE1* in *trans* partially restored the capacity for ethanol production. These results demonstrate the feasibility of genetic investigations of acetogen physiology and the potential for genetic manipulation of *C. ljungdahlii* to optimize autotrophic biocommodity production.

Homoacetogenic microorganisms have unique metabolic pathways and energy conservation mechanisms that could substantially enhance microbial strain design options for the production of fuels and other biocommodities. Furthermore, homoacetogens play an important role in the carbon cycle of a diversity of anaerobic environments (1, 2). However, the understanding of homoacetogen physiology and the development of a homoacetogenic microorganism as a chassis for the production of biocommodities have been limited by a lack of methods for genetic manipulation.

The homoacetogen *Clostridium ljungdahlii* has been proposed as a potential chassis for biocommodity production (3). Like other acetogens, *C. ljungdahlii* metabolizes sugars through the Embden-Meyerhof-Parnas pathway, and CO_2 released during glycolysis is fixed via the Wood-Ljungdahl pathway, which makes it possible to recover completely the carbon from sugars in organic end products (4, 5), i.e., $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 8H^+ + 8e^-$ and $2CO_2 + 8H^+ + 8e^- \rightarrow CH_3COOH + 2H_2O$. Depending on the growth conditions, other organic products such as ethanol and 2,3-butanediol are also generated (3, 6).

C. ljungdahlii can grow autotrophically using H_2 and/or CO as the electron donor, reducing CO_2 via the Wood-Ljungdahl pathway to produce organic products (3, 5, 6). The ability of *C. ljungdahlii* to use CO as an electron donor is significant because CO is a waste product of steel manufacturing and a major component of the syngas produced from the gasification of municipal waste and other organic feedstocks (7, 8). Biocommodity production using syngas as an intermediate is an attractive strategy because organic feedstocks, such as lignocellulosic biomass, municipal waste, and plastics, are difficult for microorganisms to degrade directly (9–11).

Furthermore, *C. ljungdahlii* was able to grow with electrons derived directly from an electrode as the electron donor coupled to reduction of CO_2 to produce acetate (12). Electrode-driven reduction of carbon dioxide via acetogenic microorganisms, known as microbial electrosynthesis (13, 14), is a strategy for conversion of CO_2 to organic commodities without a biomass intermediate. Biofilms of acetogenic microorganisms colonize surfaces of cathodes and directly convert CO_2 to organic products that are excreted from the cells. When microbial electrosynthesis is powered with electricity derived from solar technology, it is an artificial form of photosynthesis that converts CO_2 to desired products much more efficiently and in a more environmentally sustainable manner than biomass-based approaches (13, 15).

The development of *C. ljungdahlii* as a chassis for production of biocommodities will require strategies for genetic manipulation. Although heterologous gene expression by introduction of a plasmid in *C. ljungdahlii* was reported (3), the efficiency of plasmid transformation by the reported method was low (M. Köpke, personal communication). In general, genetic manipulation of clostridia has been difficult (8, 16–18). Limiting factors have been a strong restriction-modification system, high nuclease activity that can degrade foreign DNA, and the thick outer layers of these Gram-positive organisms. Even when the restriction-modification system barrier has been overcome by protecting DNA with *in*

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vivo or *in vitro* methylation (19, 20), homologous recombination frequencies have been low, with single-crossover recombination as the predominant event (21–23). Consequently, not many *Clostridium* mutants have been produced in the last 20 years (21, 22, 24–26). However, with renewed interest in biotechnological applications of *Clostridium* species there have been renewed efforts to develop strategies for genetic modification, leading to such recent developments as the following: replicative plasmids for gene deletion (27); counterselection methods to improve the efficiency of gene deletion and to select for double-crossover events (28–31); the use of a promoterless antibiotic resistance cassette in conjunction with a constitutively expressed promoter to select for double-crossover events (32); the use of the *Bacillus subtilis recU* gene, which codes for resolvase, to increase homologous recombination frequencies (33–36); the use of the bacterial mobile group II intron as an alternative to homologous recombination to disrupt a gene (16, 37–40); and the use of antisense RNA to downregulate a target gene product (41, 42). Here we report on a more efficient electroporation protocol for *C. ljungdahlii* and demonstrate that chromosomal gene deletion is feasible for *C. ljungdahlii*. These results open windows for biotechnological applications of *C. ljungdahlii* and for investigation of basic acetogen physiology.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain NEB 10-beta [*araD139* Δ(*ara-leu*)*7697 fhuA lacX74 galK* (ϕ80 Δ(*lacZ*)*M15*) *mcrA galU recA1 endA1 nupG rpsL* (Str^r) Δ(*mrr-hsdRMS-mcrBC*)] (New England BioLabs) was used for general plasmid propagation and cloning. Plasmid DNA to be electroporated into *C. ljungdahlii* was isolated from *E. coli* strain NEB Express [*fhuA2 [lon] ompT gal sulA11 R*(*mcr-73*::miniTn*10*-Tet^s)*2 [dcm] R*(*zgb-210*::Tn*10*-Tet^s) *endA* Δ(*mcrC-mrr*)*114*::IS*10*] (New England BioLabs). *E. coli* strain ER2275 (*recA1 hsdR mcrBC*) was the host strain for *in vivo* methylation of plasmid DNA prior to transformation of *C. ljungdahlii* when the effect of *in vivo* methylation on transformation efficiency was evaluated, as previously described (3, 20). *E. coli* cells were cultivated in LB medium supplemented with the appropriate antibiotic, if necessary, at 37°C with shaking.

C. ljungdahlii DSM 13528 (ATCC 55383) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). For growth studies and phenotype characterization, *C. ljungdahlii* cells were grown anaerobically at 37°C in DSMZ 879 medium supplemented with 0.04% L-cysteine, 0.04% sodium sulfide, 0.1% sodium bicarbonate, and 5 g liter^{-1} fructose. For general propagation, cells were grown anaerobically at 37°C in PETC 1754 medium (American Type Culture Collection [ATCC]) supplemented with 1 mM L-cysteine (pH 7) and 5 g liter^{-1} fructose. The original protocol includes both cysteine and sodium sulfide as reductants, but we found no significant difference in growth if the sulfide was omitted.

Cells were grown on agar plates (1.5% Difco Noble agar) at 35°C in a heated anaerobic glove bag containing an N_2-CO_2-H_2 (83:10:7) atmosphere. The medium was either a modified reinforced clostridial medium (RCM) (43) supplemented with 5 mM L-cysteine (pH 7) or YTF medium (10 g liter^{-1} yeast extract, 16 g liter^{-1} Bacto tryptone, 4 g liter^{-1} sodium chloride, 5 g liter^{-1} fructose, pH 6) supplemented with 2 mM L-cysteine (pH 7).

For motility assays, *C. ljungdahlii* wild-type and mutant cells were spotted on YTF soft agar plates with 0.3% Difco Noble agar and incubated at 35°C in an anaerobic chamber.

C. ljungdahlii strains were maintained by freezing mid-log-phase cultures at −80°C with 10% dimethyl sulfoxide (DMSO) for long-term storage.

Thiamphenicol was dissolved in dimethylformamide (DMF). Clarithromycin was first dissolved in acidic MilliQ water (pH 2) and then adjusted to pH 6.5 with sodium hydroxide. Both antibiotic solutions were made anoxic by sparging and overlaying with an N_2 atmosphere.

DNA manipulation and construction of *E. coli-C. ljungdahlii* shuttle vectors. *C. ljungdahlii* genomic DNA was prepared with the Epicenter MasterPure DNA purification kit. All enzymes for DNA manipulation were purchased from New England BioLabs, unless stated otherwise. Qiagen *Taq* polymerase was used for all DNA amplifications, unless stated otherwise. Plasmids, PCR products, and DNA fragments from agarose gels were purified with Qiagen mini/midiprep, PCR purification, and gel extraction kits, respectively.

Plasmid pCL1 was constructed by replacing the kanamycin resistance cassette (EcoRI-PstI) of pIKM1 (44) with an EcoRI-SmaI-BamHI-SalI-PstI polylinker (see Table S1 in the supplemental material). Plasmids pQexp and pQint were generous gifts from G. M. Church (40). Plasmid pJIR750ai was purchased from Sigma. Plasmid pCL2 was constructed by digesting pJIR750ai with PvuI and religating the vector portion with T4 DNA ligase.

In vivo methylation of plasmid DNA was carried out when necessary as previously described (20). *E. coli* strain ER2275 harboring plasmid pAN1 for *in vivo* methylation was provided by E. T. Papoutsakis.

Preparation of electrocompetent *C. ljungdahlii* cells. The procedure for making *C. ljungdahlii* electrocompetent cells was modified from a protocol reported previously (3). All manipulations except centrifugation were carried out on ice in an anaerobic chamber. All buffers and centrifuge tubes were made ice cold and anoxic before use. All plasticware was placed in the anaerobic chamber at least 24 h before use in order to eliminate any residual oxygen. Competent cells were maintained in SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, 7 mM sodium phosphate, pH 6) with 10% DMSO at −80°C until use.

Electrocompetent *C. ljungdahlii* cells were prepared from cultures that were freshly inoculated from frozen stocks and then transferred twice in PETC liquid medium. PETC medium can be replaced with YTF medium without significant loss of transformation efficiencies. About 15 to 16 h before preparation of competent cells, a mid- to late-log-phase culture was transferred into two serum bottles containing 100 ml fresh PETC medium supplemented with 40 mM DL-threonine (final optical density at 600 nm [OD_{600}], ~0.004). After overnight growth at 37°C, early-log-phase cells (OD_{600}, ~0.2 to 0.3; 200 ml, 3×10^7 to 3.5×10^7 cells/ml) were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cells were washed twice with 200 ml of SMP wash buffer and resuspended in the same buffer at a final concentration of ~10^{10} to 10^{11} cells/ml. Antifreezing buffer (60% DMSO–40% SMP, pH 6) was added to the competent cells at one-fifth of the final resuspension volume to achieve a final concentration of 10% DMSO. The resulting competent cells (25 μl/tube) were stored at −80°C for future use. The competence of these frozen competent cells remained stable for about 1 month.

Electrotransformation procedures for *C. ljungdahlii*. All procedures were carried out in an anaerobic chamber. Electrocompetent *C. ljungdahlii* cells (25 μl) were quickly transferred on ice from a −80°C freezer to an anaerobic chamber. After thawing on ice (about 1 min), the cells were mixed with 1 to 5 μg DNA and transferred to a prechilled, 0.1-cm-gap Gene Pulser cuvette (Bio-Rad). Cells were pulsed at 0.625 kV with resistance at 600 Ω and a capacitance of 25 μF by using a Gene Pulser Xcell microbial electroporation system (Bio-Rad). Immediately after the pulse, cells were recovered with 0.5 ml of fresh PETC medium, transferred to a pressure tube containing 10 ml of PETC medium supplemented with 5 mM L-cysteine (pH 7), and incubated at 37°C. The electroporated cells were allowed to recover at 37°C until their cell densities were higher than immediately after the electroporation (about 9 to 12 h). Five-milliliter volumes of the outgrowth cultures or appropriately diluted cultures were mixed with 20 ml of RCM molten agar (1.5%) containing an appropriate antibiotic and poured into a petri dish. RCM medium can be replaced with YTF medium without significant loss of transformation efficiencies. After the agar mixtures were solidified, plates were incubated upside down

in a secondary container with a petri dish containing palladium pellets in order to eliminate any residual oxygen.

Verification of the presence of plasmids in *C. ljungdahlii* transformants was carried out by either of two methods: (i) restriction analysis of plasmid DNA isolated from an *E. coli* strain transformed with plasmid DNA isolated from *C. ljungdahlii* transformants or (ii) colony PCR analysis of *C. ljungdahlii* transformants to detect the *catP* gene (primer sequences are listed in Table S1 in the supplemental material).

Construction of a mutant allele to disrupt the *fliA* gene. All primers are listed in Table S1 in the supplemental material. The gene *fliA* (CLJU_c10410) was replaced with the gene *ermC*, such that the coding region from 83 Lys to 220 Val was deleted. DNA fragments containing the upstream (883 bp) and downstream (882 bp) regions of *fliA* were amplified by PCR with chromosomal DNA as a template. The *ermC* cassette was amplified by PCR with pCL1 as a template. The PCR products were cloned separately in a plasmid, and their sequences were confirmed. The upstream region of the *fliA* gene, the downstream region of the *fliA* gene, and the *ermC* gene were prepared by digesting the plasmids with XbaI and EcoRI, HindIII and XhoI, and EcoRI and HindIII, respectively, and were cloned in the XbaI and XhoI sites of pBluescript II KS(−) (Stratagene). The plasmid (pBuTU-ΔfliA-ermC) thus constructed was electroporated into the wild-type *C. ljungdahlii* strain as described above. Transformants resistant to clarithromycin were selected as described above. The genotypes were examined by PCR amplification with primers fliA-1 and fliA-4.

Construction of mutant alleles to disrupt *adhE1*, *adhE2*, or both. The coding region from 327 Glu to 685 Pro of the gene *adhE1* (CLJU_c16510) was replaced with the gene *ermC*. Upstream (977 bp) and downstream (1,024 bp) regions of *adhE1* were amplified by PCR with wild-type genomic DNA as a template. The coding region of the *ermC* gene was amplified by PCR with pCL1 as a template. The SacI site within the multiple-cloning sites in pBluescript II KS(−) was deleted by digestion of the vector with SacI, filling in with Klenow fragment, and vector ligation. The resulting vector, designated pBuKsII-SacI, was sequenced to confirm that the SacI site was absent. The upstream and downstream regions and the *ermC* coding region were digested with XbaI and SacI, HindIII and XhoI, and SacI and HindIII, respectively, and were cloned into the XbaI and XhoI sites of pBuKsII-SacI.

The coding region from 34 Val to 775 Arg of *adhE2* (CLJU_c16520) was replaced with the gene *ermC*. For construction of a mutant allele to disrupt *adhE2*, the upstream (977 bp) and downstream (997 bp) regions of *adhE2* were amplified by PCR. The *ermC* cassette was amplified with the same primers as for the *fliA* mutant allele. The three pieces were digested with the respective restriction enzymes and cloned into pBluescript II KS(−) as described above.

For disruption of both *adhE1* and *adhE2*, the coding region from 34 Val of *adhE1* to 775 Arg of *adhE2* was replaced with the gene *ermC*. The 904-bp upstream region of *adhE1* and the 997-bp downstream region of *adhE2* were amplified by PCR and the mutant allele was constructed as described above.

Expression of *adhE1* in *trans*. The complete coding sequence of *adhE1* was amplified with Phusion DNA polymerase (New England BioLabs). The *adhE1* coding sequence was digested with NdeI and BamHI and inserted into the NdeI and BamHI sites of the expression vector pMTL83152 (45) to generate plasmid pMTL83152-*adhE1*. The *adhE1* gene was then sequenced to confirm that no mutations were introduced during amplification. The pMTL83152-*adhE1* plasmid was then electroporated into either the *adhE1* or the *adhE1 adhE2* deletion mutants. The presence of the plasmid was confirmed by PCR and plasmid preparation.

Analytical techniques. Acetate was measured via high-performance liquid chromatography (HPLC) as previously described (12). Ethanol was measured with a gas chromatograph as previously described (46). Cell growth was monitored using a Genesys 2 spectrophotometer (Spectronic Instruments, Rochester, NY) at 600 nm. Cell numbers were determined by epifluorescence microscopy using acridine orange staining (47).

Transmission electron microscopy. Both wild-type and *fliA* deletion mutant cells grown in PETC medium were placed on 400-mesh carbon-coated copper grids, incubated for 5 min, and then stained with 2% uranyl acetate. Cell appendages were observed using a Tecnai 12 transmission electron microscope at an accelerating voltage of 100 kV. Images were taken digitally with the Teitz TCL camera system.

RESULTS AND DISCUSSION

Growth on solid medium. Conditions for growth of *C. ljungdahlii* on solidified media were evaluated in order to obtain single colonies of *C. ljungdahlii* with a high plating efficiency for isolation of mutant strains. The PETC medium (ATCC 1754) that was used routinely for maintenance of *C. ljungdahlii* cultures was unsuitable for sustaining growth on solidified medium. On average, fewer than 5 colonies were obtained when ~100 cells were plated on the PETC agar medium. The poor plating efficiencies might be due to a shift in pH caused by differences in gas compositions between the culture tube containing the liquid medium (100% CO_2) and the anaerobic chamber (10% CO_2, 7% H_2, and 83% N_2), where the plates were incubated. In contrast, the plating efficiency with RCM medium was ~30%. Furthermore, if cells were added to molten agar (~45°C), plating efficiencies were 72% ± 10% (mean ± standard deviation; $n = 3$). Single colonies were visible within 3 days.

The MICs of thiamphenicol and clarithromycin were determined with the molten agar plating method. The growth of ca. 10^8 cells on solid medium was inhibited by thiamphenicol (5 μg/ml) or clarithromycin (4 μg/ml).

Development of an electroporation protocol and identification of *E. coli-C. ljungdahlii* shuttle vectors. A transformation protocol for *C. ljungdahlii* was recently reported (3, 48). However, the reported protocol did not consistently yield transformants with the *E. coli-Clostridium* shuttle vector pIMP1 (M. Köpke, personal communication). After the evaluation of the protocol, several changes were made, including changes in (i) the pH of the wash buffer, (ii) the density of competent cells, and (iii) the electroporation procedures (Table 1). With this revised protocol, transformants were consistently obtained with plasmid pCL1, a derivative of pIMP1 (0.2 transformants/μg DNA) (Table 2). However, the transformation efficiency was still poor, possibly due to the plasmid not being stably maintained.

In order to determine whether plasmids known to be stably maintained in other *Clostridium* species (49) might be more effectively propagated in *C. ljungdahlii*, plasmids pQexp (40) and pJIR750ai (38), with origins of replication from pAMβ1 and pIP404, respectively, were tested. Both plasmids were more efficiently transformed than pIMP1 or pCL1 (Table 2). The transformation efficiencies for pQexp and pJIR750ai were 0.7 and 7 transformants/μg plasmid DNA, respectively (Table 2). The presence of these plasmids in *C. ljungdahlii* was confirmed with restriction analysis and/or colony PCR (Fig. 1). These results indicate that replication origins from pQexp and pJIR750ai are functional in *C. ljungdahlii*.

The transformation protocol was further optimized with pCL2, which was constructed from pJIR750ai by deleting the region for the group II intron insertion that is not required for its propagation in *C. ljungdahlii*. One modification in the optimized protocol (Table 1) was to change the growth phase at which cells were harvested to prepare competent cells, because it is known that growth phase can affect competence and the optimal growth

TABLE 1 Changes in the optimized transformation protocol for *C. ljungdahlii*

Step or parameter	Original procedure (3, 48)	Optimized procedure
Preparation of competent cells		
Growth phase for harvesting (OD_{600})	0.3–0.7	0.2–0.3
pH of wash buffer	7.4	6
Resuspension buffer	SMP[a] (pH 7.4)	SMP[a] (pH 6) with 10% DMSO[b]
Cell density of competent cells	~80× concentrated from the original cultures	10^{10}–10^{11} cells/ml (~1,000× concentrated)
Freeze-thaw	No	Yes
Thawing of cells	No	1 min on ice
Preparation of plasmids		
In vivo methylation	Yes (20)	No
E. coli strain for plasmid preparation	K[c] strain (ER2275)	B[c] strain (NEB Express[d])
Electroporation procedures		
Cell vol (μl)	600	25
Preincubation with plasmid	5 min on ice	No
Amount of plasmid DNA (μg)	0.1–1.5	1–5
Electroporation cuvette gap (cm)	0.4	0.1
Electric pulse	2.5 kV, 600 Ω, 25 μF	0.625 kV, 600 Ω, 25 μF
Recovery	5 ml PETC[e]; 37°C, until clear growth occurs	10 ml PETC; 37°C, 9–12 h
Plating	Liquid cultures on a solid agar plate	Liquid cultures mixed with molten agar
Antibiotic concn (μg/ml)	Clarithromycin (5), thiamphenicol (20)	Clarithromycin (4), thiamphenicol (5)

[a] SMP, 270 mM sucrose, 1 mM $MgCl_2$, and 7 mM phosphate buffer.
[b] DMSO, dimethyl sulfoxide.
[c] K strain, Dcm^+ Dam^-; B strain, Dcm^- Dam^+.
[d] New England Biolabs, Ipswich, MA.
[e] PETC, American Type Culture Collection (ATCC) PETC 1754 medium.

phase must be decided empirically (50). Furthermore, the cells were frozen prior to electroporation, which may have weakened the cell wall structure, and the 10% DMSO in the storage buffer may facilitate DNA-cell membrane contact (50). The methods for isolating plasmid DNA for electroporation were also modified (Table 1). With the optimized conditions, the transformation efficiency of pCL2 was $1.73 \times 10^4 \pm 0.57 \times 10^4$/μg DNA ($n = 5$) (Table 2). The transformation efficiency of pCL1 using the final optimized conditions was 1.1 ± 0.1/μg DNA ($n = 3$) (Table 2). Similar transformation efficiency was also obtained for a plasmid (pCL1-catP) that has the same origin of replication as pCL1 but carries the same antibiotic resistance gene as pCL2 (data not shown), suggesting that the poor transformation efficiency of pCL1 was not due to selections with different antibiotics. The competence of these frozen competent cells remains consistent for about 1 month (data not shown).

After the optimization of the transformation protocol with pCL2, plasmids from the pMTL80000 modular system (45) with origins of replication other than pAMβ1 and pIP404 were made available to us. The pBP1 and pCB102 origins of replication were functional in *C. ljungdahlii*, whereas the pCD6 origin of replication was not. The transformation efficiency for plasmids with either a pBP1 or pCB102 origin of replication was somewhat lower than that of pCL2 (Table 2).

In vivo or *in vitro* methylation of plasmid DNA to be transformed stimulates the transformation efficiency in some *Clostridium* species (19, 20), and this procedure was employed in *C. ljungdahlii* in the previously reported protocol (3). However, *in vivo*

TABLE 2 Transformation of *C. ljungdahlii* with different plasmids

Plasmid	Origins of replication (*E. coli*, *Clostridium*)[a]	Antibiotic resistance gene(s)[b]	Transformation efficiency (transformants/μg DNA), mean ± SD (*n*)	
			First optimization[c]	Final protocol
pCL1	pMB1, pIM13	*bla*, *ermC*	0.2 ± 0 (5)	1.1 ± 0.1 (3)
pQexp	pMB1, pAMβ1	*ermB*	0.7 ± 0.5 (3)	14.9 ± 4.9 (6)
pJIR750ai	pMB1, pIP404	*catP*	7 (1)	ND[d]
pCL2	pMB1, pIP404	*catP*	ND	$(1.7 \pm 0.6) \times 10^4$ (5)
pMTL82151[e]	ColE1, pBP1	*catP*	ND	$(3.8 \pm 0.2) \times 10^3$ (3)
pMTL83151[e]	ColE1, pCB102	*catP*	ND	$(3.1 \pm 1.8) \times 10^3$ (3)

[a] All plasmids are *E. coli-Clostridium* shuttle vectors. *E. coli* origins of replication, pMB1 and ColE1; *Clostridium* origins of replication, pIM13, pAMβ1, pIP404, pBP1, and pCB102.
[b] *bla*, ampicillin resistance; *ermC* or *ermB*, clarithromycin/erythromycin resistance; *catP*, thiamphenicol/chloramphenicol resistance.
[c] The first optimization included the changes of wash buffer pH, cell density of the competent cells, and electroporation procedures (details are listed in Table 1).
[d] ND, not determined.
[e] The plasmid became available after the optimized protocol was developed.

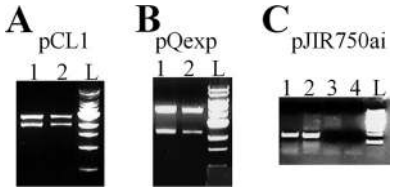

FIG 1 Restriction and PCR analyses of various plasmid DNAs from *C. ljungdahlii*. (A) Plasmid pCL1. Plasmid DNA was digested with HindIII and analyzed by agarose gel electrophoresis. Lane 1, plasmid preparation from *E. coli* which was transformed with a plasmid preparation from a *C. ljungdahlii* transformant; lane 2, plasmid used to transform *C. ljungdahlii*; lane L, 1-kb DNA ladders (New England BioLabs). (B) HindIII-digested pQexp. Lane 1, plasmid preparation from *E. coli* which was transformed with a plasmid preparation from a *C. ljungdahlii* transformant; lane 2, plasmid used to transform *C. ljungdahlii*; lane L, 1-kb DNA ladders. (C) Colony PCR amplification of the *catP* gene from pJIR750ai. Lane 1, a *C. ljungdahlii* transformant; lane 2, plasmid preparation from *E. coli* as the positive control; lane 3, *C. ljungdahlii* genomic DNA as a negative control; lane 4, no DNA as a negative control for the PCR amplification.

methylation had a negative impact on the transformation efficiency in *C. ljungdahlii* with our protocols (data not shown). For *C. thermocellum*, transformation efficiency was increased when plasmid DNA was isolated from an *E. coli* Dcm^- Dam^+ strain (e.g., the B strain) instead of a Dcm^+ Dam^- (K) strain (51, 52). This was also the case for *C. ljungdahlii* (data not shown). The B and K strains differ in their endogenous methylation sites. The endogenous adenine methylation at GATC sequences is abolished in the K strain, whereas the cytosine methylation at CCWGG sequences is abolished in the B strain. Therefore, methylation plays a role in transformation of foreign DNA in *C. ljungdahlii*. Analysis of the *C. ljungdahlii* genome sequence identified homologs of a type I restriction-modification system (CLJU_c03310-03320-03330). However, the specificity of the *C. ljungdahlii* restriction-modification system has yet to be characterized, and elucidation of the restriction-modification system may further improve the transformation efficiency.

Gene replacement via homologous recombination. In order to expand the available genetic tools for *C. ljungdahlii*, genetic modification of the *C. ljungdahlii* chromosome was examined with the improved transformation protocol described above. Homologous recombination was evaluated with the *fliA* gene (CLJU_c10410), which encodes a putative sigma factor of RNA polymerase known to control the expression of genes involved in flagellar biosynthesis and motility in other bacteria (53, 54). Deletion of *fliA* was expected to result in the loss of flagella and motility in *C. ljungdahlii*.

Initial attempts to obtain a *fliA* deletion mutant with introduction of a linear DNA fragment prepared by digesting the plasmid pBuTU-ΔfliA-ermC with XhoI were not successful. No transformants were obtained. Transformants were obtained only with the introduction of the intact plasmid pBuTU-ΔfliA-ermC into *C. ljungdahlii* competent cells. PCR analysis demonstrated both single- and double-crossover homologous recombination (Fig. 2B). The frequencies of the double-crossover events were ca. 30%. This result demonstrated that a strategy for mutagenesis via homologous recombination with a suicide vector was possible.

Transmission electron microscopy revealed that wild-type cells produced multiple flagella, whereas the mutant did not (Fig. 3A). Deletion of the *fliA* gene did not affect cell growth in the PETC liquid medium with fructose. However, when the wild-type and the mutant cells were spotted onto YTF soft agar plates, the mutant cells were nonmotile, whereas the wild-type cells were motile and formed a larger growth zone than the mutant cells (Fig. 3B). These results demonstrated that it was possible to create a deletion mutant of *C. ljungdahlii* by double-crossover homologous recombination.

Identification of a bifunctional aldehyde/alcohol dehydrogenase that contributes to ethanol production in *C. ljungdahlii*. In order to determine whether carbon and electron flow could be altered by gene deletion, the possibility of diminishing ethanol production was evaluated for proof of concept. CLJU_c16510 and CLJU_c16520, designated *adhE1* and *adhE2*, respectively, have

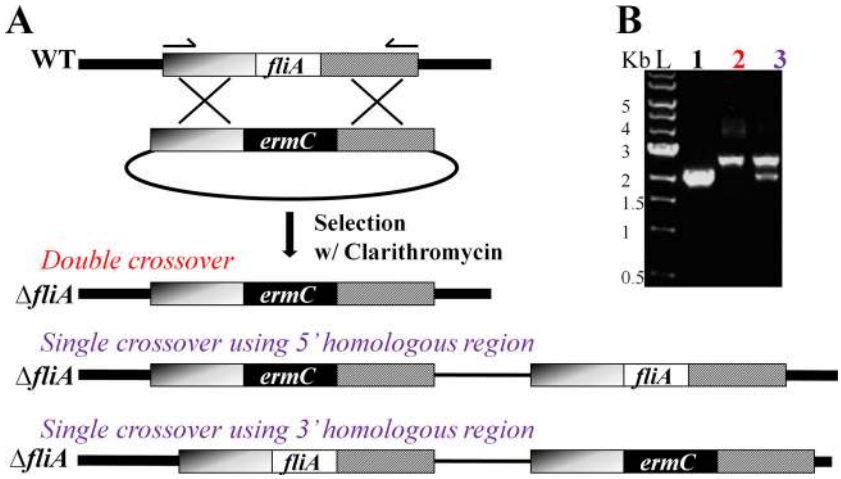

FIG 2 Construction of a *fliA* deletion mutant. (A) Schematic diagram showing the mutagenesis strategy employed to construct the *fliA* deletion mutant of *C. ljungdahlii*. Homologous recombination via a suicide vector, pBuTU-ΔfliA-ermC, resulted in three possible genotypes: double crossover, single crossover at the 5′ homologous region, and single crossover at the 3′ homologous region. (B) PCR analysis of representative *fliA* deletion mutants. Genotypes were verified by PCR amplification with distal primers indicated by arrows in panel A. The PCR product from the wild-type locus (lane 1) is smaller than that from the mutant locus (lanes 2 and 3) because the replaced region in the *fliA* gene is smaller than the *ermC* gene. Lane L, 1-kb DNA ladders (New England BioLabs); lane 1, wild type; lane 2, double-crossover *fliA* mutant; lane 3, single-crossover *fliA* mutant.

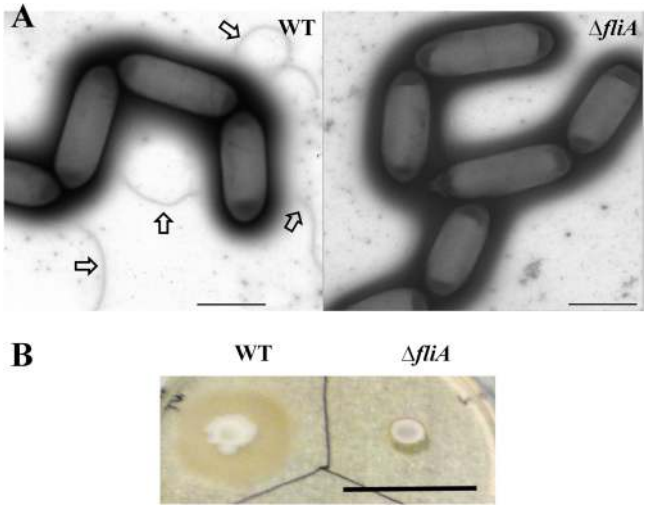

FIG 3 Characterization of the *fliA* deletion mutant. (A) Production of flagella. Transmission electron micrographs of negatively stained *C. ljungdahlii* wild-type cells (left panel) and the *fliA* deletion mutant (right panel) showed that the mutant did not produce any flagella, whereas the wild-type cells produced flagella as indicated by arrows. Scale bars in both panels represent 2,000 nm. (B) Motility assay. The *fliA* deletion mutant was not motile when spotted on a YTF soft agar plate on which the wild-type cells were motile and thus exhibited a larger growth zone. The scale bar represents 3 cm.

been annotated to code for bifunctional aldehyde/alcohol dehydrogenases (3) and thus have the potential to promote ethanol production. These genes are located next to each other and possibly are transcribed from the same promoter. Therefore, care was taken when designing the mutagenesis cassette to avoid polar effects. The mutagenesis cassette for deletion of *adhE1* was designed to introduce two stop codons (TAA and TAA) after the sequence for amino acid 326 (Leu), followed by a promoterless *ermC* and then the downstream homologous region. This ensured that *adhE2* was transcribed from the original promoter sequence. The mutagenesis cassettes for deletion of either *adhE2* or both *adhE1* and *adhE2* were designed as described above for deletion of *fliA* because polar effects were not a concern. All three mutants were isolated from agar plates supplemented with fructose and clarithromycin. Double-crossover events for all three mutants were confirmed by PCR analysis (data not shown).

The *adhE2* deletion mutant grew on fructose as well as the wild type and produced similar amounts of ethanol and acetate (Fig. 4; Table 3). Deleting *adhE1*, either alone or in combination with *adhE2*, slightly diminished the growth yield, increased the doubling time, and significantly inhibited ethanol production while increasing acetate production (Fig. 4; Table 3). The amounts of ethanol produced by the *adhE1* deletion strain and the *adhE1 adhE2* double deletion strain were similar. In both strains, ethanol production was decreased to ca. 15% of that in the wild type (Table 3). Within the error of the measurements, the decreased carbon recovery in ethanol in the *adhE1* deletion or the *adhE1 adhE2* double deletion strain could be accounted for by an increase in carbon recovery in acetate over that produced by the wild type (Table 3). The slightly decreased cell yields and increased doubling times in the absence of AdhE1 or both AdhE1 and AdhE2 may suggest that these mutants were not able to dispose of reducing equivalents as efficiently as the wild type.

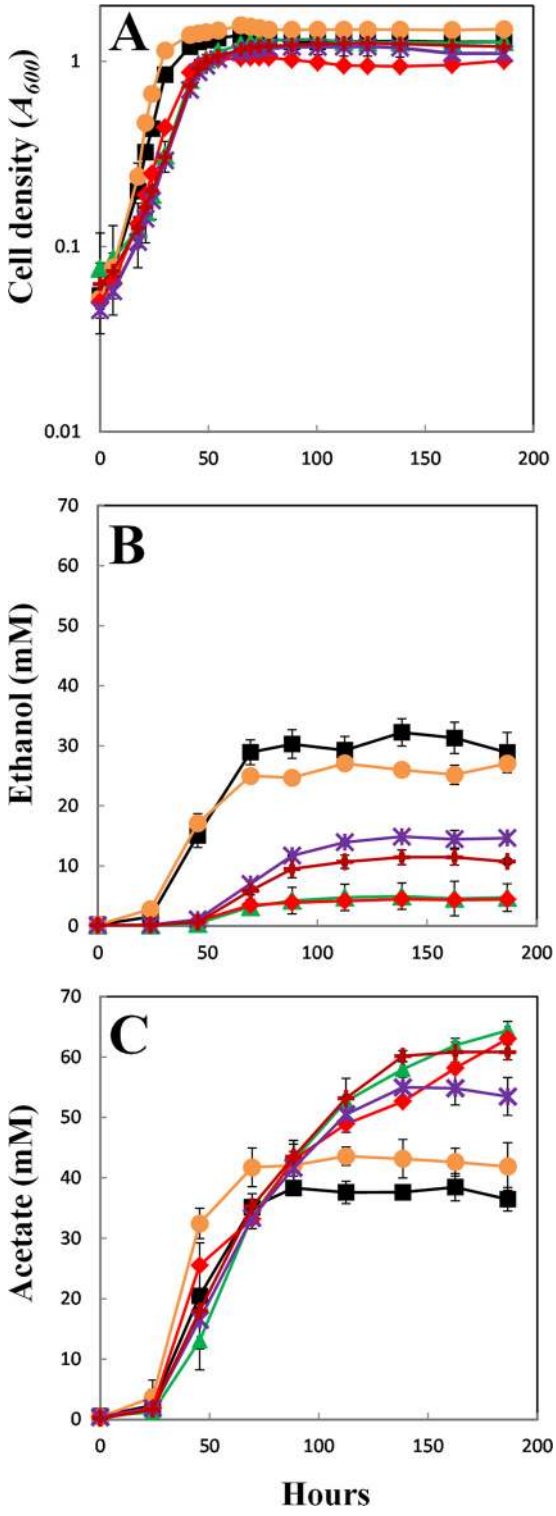

FIG 4 Cell growth and production of ethanol and acetate by the *C. ljungdahlii* wild-type strain, bifunctional aldehyde/alcohol dehydrogenase deletion mutants (Δ*adhE1*, Δ*adhE2*, and Δ*adhE1* Δ*adhE2*), and the complemented strains (Δ*adhE1/adhE1* and Δ*adhE1* Δ*adhE2/adhE1*). (A) Cell growth (A_{600}). (B) Ethanol production (mM). (C) Acetate production (mM). Symbols: filled squares, wild type; green triangles, Δ*adhE1*; orange circles, Δ*adhE2*; red diamonds, Δ*adhE1* Δ*adhE2*; purple crosses, Δ*adhE1/adhE1*; and maroon crosses, Δ*adhE1* Δ*adhE2/adhE1*. Data are the means ± standard deviations of quadruplicates.

TABLE 3 Production of acetate, ethanol, and biomass by the wild type, bifunctional aldehyde/alcohol dehydrogenase gene deletion mutants, and complemented strains

Parameter	Value for strain[a]:					
	WT	Δ*adhE1*	Δ*adhE2*	Δ*adhE1* Δ*adhE2*	Δ*adhE1*/*adhE1*	Δ*adhE1* Δ*adhE2*/*adhE1*
Ethanol production (mM)	28.9 ± 3.4	4.7 ± 2.3	27.1 ± 0.5	4.4 ± 0.6	14.7 ± 0.4	11.5 ± 1.3
Acetate production (mM)	38.5 ± 2.3	64.4 ± 1.5	41.9 ± 3.9	63.0 ± 1.8	53.4 ± 3.1	60.8 ± 1.7
Cell yield (A_{600})	1.4 ± 0.1	1.3 ± 0.2	1.5 ± 0.1	1.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.1
Doubling time (h)	5.8 ± 0.2	8.4 ± 0.4	5.7 ± 0.1	7.3 ± 0.2	8.7 ± 0.5	9.5 ± 1.0

[a] Data are the means ± standard deviations of quadruplicates. WT, wild-type *C. ljungdahlii* cells; Δ*adhE1*, *adhE1* deletion mutant; Δ*adhE2*, *adhE2* deletion mutant; Δ*adhE1* Δ*adhE2*, *adhE1* and *adhE2* double deletion mutant; Δ*adhE1*/*adhE1*, *adhE1* deletion mutant with the *adhE1* gene expressed in *trans*; Δ*adhE1* Δ*adhE2*/*adhE1*, *adhE1* and *adhE2* double deletion mutant with the *adhE1* gene expressed in *trans*.

When *adhE1* was expressed in *trans* under the control of a *C. acetobutylicum* thiolase gene promoter on a plasmid, ethanol production in the *adhE1*-deficient strain was tripled, with a corresponding decrease in acetate production, maintaining the carbon balance in ethanol and acetate comparable to that for the wild type and the uncomplemented *adhE1*-deficient strain (Table 3; Fig. 4). In a similar manner, expressing *adhE1* in *trans* in the strain deficient in both *adhE1* and *adhE2* increased ethanol production and decreased acetate production, with an overall carbon recovery in acetate and ethanol comparable to that for the wild type (Fig. 4; Table 3). The longer doubling time of the complemented strain may be due to the addition of thiamphenicol to the medium, which was required in order to maintain the plasmid carrying *adhE1*.

These results suggest that AdhE1 is an important bifunctional aldehyde/alcohol dehydrogenase in *C. ljungdahlii*. This finding demonstrates that it is now possible to elucidate gene function in *C. ljungdahlii* via genetic manipulations. The results suggest that AdhE2 does not play a role in ethanol production, at least under the conditions evaluated, even though AdhE2 shares 88% identity with AdhE1. This finding emphasizes the importance of functional genomic studies in order to understand cellular metabolism. The results also demonstrate that it is possible to redirect carbon and electron flow in *C. ljungdahlii* with a gene deletion without significantly impairing overall growth. This indicates that the metabolic engineering of *C. ljungdahlii* is now feasible.

Conclusions. The ability to effectively express foreign genes in *C. ljungdahlii* and to delete genes via homologous recombination is expected to substantially promote the development of this organism as a chassis for the production of biocommodities. For example, the wild-type *C. ljungdahlii* can produce multiple organic products from acetyl coenzyme A (acetyl-CoA), the central intermediate in the Wood-Ljungdhal pathway. Efficient production of desired commodities from acetyl-CoA will require disabling these competing pathways. As demonstrated in this study, deletion of the ethanol production pathway led to increased production of acetate in *C. ljungdahlii*. The homologous recombination method described here should also be applicable for introducing desired metabolic genes or reporters into the chromosome.

Furthermore, the ability to genetically manipulate *C. ljungdahlii* should aid in basic studies of homoacetogen physiology. Although important aspects of the physiology of acetogens have been elucidated with elegant biochemical investigations, much is still unknown about mechanisms for energy conservation and regulation of gene expression in these organisms (1, 55, 56). Tools for facile genetic manipulation of *C. ljungdahlii* should enhance further investigation of homoacetogenesis.

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