

Lactose-Inducible System for Metabolic Engineering of *Clostridium ljungdahlii*

Areen Banerjee, Ching Leang, Toshiyuki Ueki, Kelly P. Nevin, Derek R. Lovley

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts, USA

The development of tools for genetic manipulation of *Clostridium ljungdahlii* has increased its attractiveness as a chassis for autotrophic production of organic commodities and biofuels from syngas and microbial electrosynthesis and established it as a model organism for the study of the basic physiology of acetogenesis. In an attempt to expand the genetic toolbox for *C. ljungdahlii*, the possibility of adapting a lactose-inducible system for gene expression, previously reported for *Clostridium perfringens*, was investigated. The plasmid pAH2, originally developed for *C. perfringens* with a *gusA* reporter gene, functioned as an effective lactose-inducible system in *C. ljungdahlii*. Lactose induction of *C. ljungdahlii* containing pB1, in which the gene for the aldehyde/alcohol dehydrogenase *AdhE1* was downstream of the lactose-inducible promoter, increased expression of *adhE1* 30-fold over the wild-type level, increasing ethanol production 1.5-fold, with a corresponding decrease in acetate production. Lactose-inducible expression of *adhE1* in a strain in which *adhE1* and the *adhE1* homolog *adhE2* had been deleted from the chromosome restored ethanol production to levels comparable to those in the wild-type strain. Inducing expression of *adhE2* similarly failed to restore ethanol production, suggesting that *adhE1* is the homolog responsible for ethanol production. Lactose-inducible expression of the four heterologous genes necessary to convert acetyl coenzyme A (acetyl-CoA) to acetone diverted ca. 60% of carbon flow to acetone production during growth on fructose, and 25% of carbon flow went to acetone when carbon monoxide was the electron donor. These studies demonstrate that the lactose-inducible system described here will be useful for redirecting carbon and electron flow for the biosynthesis of products more valuable than acetate. Furthermore, this tool should aid in optimizing microbial electrosynthesis and for basic studies on the physiology of acetogenesis.

Clostridium ljungdahlii is a promising chassis for the production of organic commodities and biofuels from syngas and carbon monoxide wastes (1–5) as well as for microbial electrosynthesis, a process in which electrical energy powers the microbial reduction of carbon dioxide to multicarbon organic compounds (6–9). One reason that *C. ljungdahlii* is an attractive catalyst for these processes is that it uses carbon dioxide as an electron acceptor for anaerobic respiration via the Wood-Ljungdahl pathway (7, 10–12). There is ample evidence to suggest that this is the most efficient pathway for the reduction of carbon dioxide to externally released organic products (7, 13, 14). Furthermore, *C. ljungdahlii* can be genetically manipulated (4, 15, 16). This provides the possibility of redirecting carbon and electron flux for the production of commodities other than acetate and ethanol, the primary products of wild-type *C. ljungdahlii* respiration (4) (Fig. 1). Acetyl coenzyme A (acetyl-CoA), the central intermediate in the Wood-Ljungdahl pathway, can potentially serve as a precursor for the enzymatic production of a wide diversity of organic compounds (4, 13, 17, 18).

Inducible gene expression systems are useful tools in metabolic engineering as well as studies of gene function (19). They are commonly employed for genetic complementation studies, for fine-tuning protein expression for physiological studies, to express toxic genes for counterselection, and/or to express a synthetic pathway for synthesis of desired products. A gene expression reporter system is also essential for evaluation of promoter strengths and other transcriptional regulatory components, such as operator sequences or the Shine-Dalgarno sequences, for metabolic engineering. Different induction systems in combination with various reporter genes have been developed for *Clostridium* species other than *C. ljungdahlii* (20–27). These have incorporated regulatory elements from sugar utilization pathways, such as xylose- or

lactose-inducible systems (22–25, 28), a radiation-inducible promoter (26), or the tetracycline efflux regulation system (20).

For example, an effective lactose-inducible system was developed in *C. perfringens* (23) and also functioned in *C. acetobutylicum* (28). It includes *bgaR*, which encodes a transcriptional regulator that, in the presence of lactose, binds to a promoter region, P_{bgaL} , of the gene *bgaL*. The *bgaR*- P_{bgaL} sequence provided an effective lactose-inducible promoter plasmid for lactose-dependent expression of a diversity of genes introduced downstream of the P_{bgaL} sequence (23). The *bgaR*- P_{bgaL} lactose-inducible plasmid system was cloned into a plasmid with a pIP404 origin of replication (23). This origin of replication functions well in *C. ljungdahlii* (15), suggesting that the *bgaR*- P_{bgaL} plasmid-based system might be directly transformed into *C. ljungdahlii* and serve as an inducible system for expression of genes for diverting carbon and electron flow to desired products.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Clostridium ljungdahlii* DSM13528 (ATCC 55383) from the German Collection of Microorganisms and Cell Cultures (DSMZ) was obtained from our laboratory culture

Received 8 November 2013 Accepted 2 February 2014

Published ahead of print 7 February 2014

Editor: F. E. Löffler

Address correspondence to Areen Banerjee, areenbanerjee@microbio.umass.edu, or Ching Leang, leang@microbio.umass.edu.

A.B. and C.L. contributed equally to this article.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.03666-13

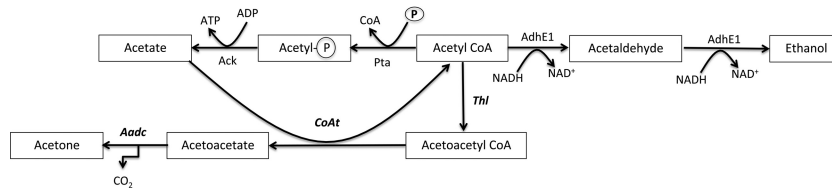


FIG 1 Pathways for indigenous formation of acetate and ethanol from acetyl-CoA and the synthetic pathway introduced for acetone (bold and italic) in *C. ljungdahlii*. For each mole of acetone formed, one acetyl-CoA is converted to acetate, yielding one ATP via substrate-level phosphorylation. The acetate does not accumulate but rather is recycled to form acetyl-CoA with CoA obtained from the conversion of acetoacetyl-CoA to acetoacetate, which is then decarboxylated to acetone. Abbreviations: Pta, phosphotransacetylase; Ack, acetate kinase; AdhE1, alcohol/aldehyde dehydrogenase; Thl, thiolase; CoAt, coenzyme A transferase; Aadc, acetoacetate decarboxylase.

collection. Cells were grown in liquid or agar-solidified medium under anaerobic conditions as previously described (15). For studies on ethanol production, cells were grown in DSMZ879 medium, whereas for acetone production, cells were grown in PETC 1754 medium (4, 15). It has been observed that *C. ljungdahlii* can produce ca. 60% more acetate in PETC 1754 medium than in DSMZ879, and the production of acetate is an important intermediary step in acetone production (29) (Fig. 1).

Escherichia coli strain NEB 10-beta [*araD139* Δ (*ara-leu*)7697 *fhuA lacX74 galK* (ϕ 80 Δ *lacZ M15*) *mcrA galU recA1 endA1 nupG rpsL* (Str^r) Δ (*mrr-hsdRMS-mcrBC*)] (New England Biolabs) was used for cloning and plasmid propagation. *E. coli* cells were cultivated with LB medium supplemented with appropriate antibiotics when necessary at 37°C with shaking. For introducing plasmids into *C. ljungdahlii* via electroporation, plasmids were isolated from *E. coli* strain NEB Express [*fhuA2* (*lon ompT gal sulA11 R* (*mcr-73::miniTn10-Tet^s*)2 (*dcm*) *R*(*zgb-210::Tn10-Tet^s*) *endA* Δ (*mcrC-mrr*)114::[S10] (New England Biolabs) as previously described (15).

DNA manipulation and plasmid construction. *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. Phusion DNA polymerase (New England Biolabs) was used for all DNA amplifications, except that colony PCR amplification was with Qiagen *Taq* polymerase. Plasmids, PCR products, and DNA fragments from agarose gel were purified with Qiagen mini/midi-prep, PCR purification, and gel extraction kits, respectively.

The plasmids pAH2 (containing the *gusA* reporter gene fused in frame to the first 13 codons of the *cpe* gene and its ribosomal binding site of *C. perfringens*, which was controlled by *bgaR-P_{bgaL}*; chloramphenicol resis-

tance) and pKRAH1 (containing *bgaR-P_{bgaL}* and polylinker; chloramphenicol resistance) were obtained from Stephen B. Melville (Virginia Tech).

The *C. ljungdahlii adhE1* (CLJU_c16510), and *adhE2* (CLJU_c16520) genes were amplified with primer sets AdhE1ForiSD-AdhE1compBmR1 and AdhE2ForiSD-AdhE2compBmR, respectively (Table 1), digested with XbaI and BamHI, and then inserted into the XbaI- and BamHI-digested pKRAH1. The resulted plasmids were designated pB1 and pB2, respectively. The plasmids contained the original *C. ljungdahlii* ribosome-binding sites.

In order to construct the acetone synthetic pathway (Fig. 1), the *C. acetobutylicum thl* gene, encoding thiolase (acetyl-CoA acetyltransferase), the *ctfA* and *ctfB* genes, encoding butyrate acetoacetate CoA transferase, and the *adc* gene, which encodes acetoacetate decarboxylase, were amplified with primer sets thlP1BamHI-thlP2Xma, ctfABP1Xma-ctfABP2EcoRI, and adcP1EcoRI-adcP2SalI, respectively (Table 1). The genes contained the original ribosome-binding site from *C. acetobutylicum*. The PCR products of the *thl*, *ctfAB*, and *adc* genes were digested with BamHI-XmaI, XmaI-EcoRI, and EcoRI-SalI, respectively, and cloned and assembled into the corresponding sites of pBluescript II KS(-) (Stratagene), resulting in pTU1. In order to clone the acetone synthetic pathway to pKRAH1, pTU1 was digested with SacII and SalI and ligated into SacII-SalI-digested pKRAH1. The resultant plasmid was designated pB3.

Lactose-inducible gene expression. To determine if pAH2 functioned as a lactose-inducible system in *C. ljungdahlii*, *C. ljungdahlii* cells containing pAH2 (with the transcription of the *gusA* reporter gene controlled by the *bgaR-P_{bgaL}*) or pAH2-MCS (control plasmid with promoterless *gusA* gene only) (23) were grown to early mid-log phase (optical

TABLE 1 Primers used in this study

Name	Sequence	Enzyme
AdhE1ForiSD	AATCTAGATTTAAGGGAGGAAAGCATATGAAAGTTACAAACGTAGAAG	XbaI
AdhE1compBmR1	ATGGATCCAATTACTTTTCTTCATCTTCTAC	BamHI
AdhE2ForiSD	AATCTAGATTTAAGGGAGGAAAGCATATGAAGGTAACAAAGGTAAC	XbaI
AdhE2compBmR1	ATGGATCCGTCCTTTTTGTTTACTATATTC	BamHI
CathlP1	TCTGGATCCGTATCAAAATTTAGGAGGTTAG	BamHI
CathlP2	TCTCCCGGGCTAGCACTTTTCTAGCAATATTGC	XmaI
CactfABP1	TCTCCCGGGAAAAGGAGGGATTAATAATGAACTC	XmaI
CactfABP2	TCTGAATTCTAAACAGCCATGGGTCTAAG	EcoRI
CaadcP1	TCTGAATTCCAGGAAGGTGACTTTTTATGTTAAAGG	EcoRI
CaadcP2	TCTGTGACGTTTAGAAAAGAAATACTATGAAAC	SalI
ClPptaP1	TCTGAATTCCATTTGTCAACTATAGATG	EcoRI
ClPptaP2	TCTGGTACCTTTAAATTTAACACAAAATTACACACAC	KpnI
ErmB-AfeI	AGCGCTCCTTAGAAGCAAACCTTAAG	AfeI
ErmB-NheI	GCTAGCGACCTCTTTAGCTTCTTGG	NheI
adhE1F2	ACCAGATCCAACCCCTTGCTA	
adhE1R2	TGAGCCACCACCAACTGATA	
recAF2	ATGTGGATGCCATTTCAACA	
recAR2	TGCCACCGTAGCTTACCTG	

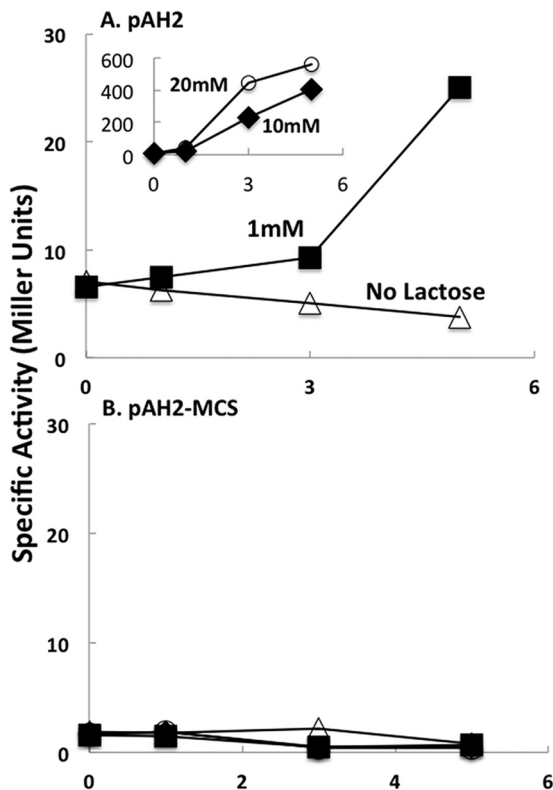


FIG 2 Induction of β -glucuronidase activity with 1, 10, or 20 mM lactose in the *C. ljungdahlii* strain containing plasmid pAH2 (A) or the control pAH2-MCS (B). Cells were grown in DSMZ879 medium supplemented with 5 g/liter fructose. Data are means for duplicates and are representative of two independent experiments. x axis, time (in hours).

density at 600 nm [OD_{600}] of ~ 0.3) and induced with different concentrations of lactose. One milliliter of cells was collected, snap-frozen, and kept at -80°C until assayed.

The transcript abundance of *adhE1* in the strain containing pB1 was determined in cells grown to an OD_{600} of 0.3 with or without induction with lactose (1 mM). The cell suspension were mixed with two volumes of RNAprotect reagent (Qiagen) and then pelleted. RNA was extracted from the samples with the Ribopure RNA extraction kit (Ambion, Life Technologies), and cDNA was generated from these using the Transplex Complete WTA kit (Sigma-Aldrich). Quantitative PCR (qPCR) was performed with *adhE1* primer pair adhE1F2-adhE1R2 using an ABI7500 real-time

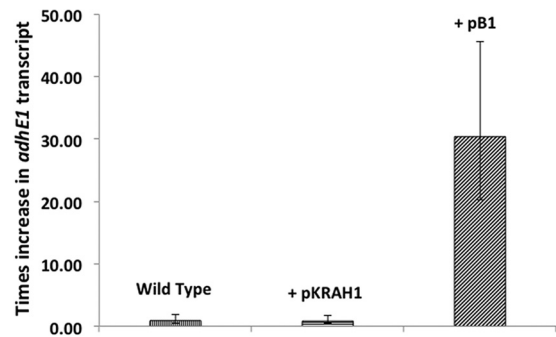


FIG 3 Lactose-induced expression of *adhE1*. Transcript abundance determined by qRT-PCR was normalized to expression of the housekeeping gene *recA*. Total RNA was isolated from fructose-grown mid-log-phase cells. Data are the means and standard errors from triplicate assays on two biological replicates.

PCR system (Applied Biosystems). *recA* primer pair recAF2-recAR2 was used to quantify transcripts of *recA* as a control housekeeping gene.

Analytical techniques. β -Glucuronidase activity was assayed as previously described (23). Acetate was measured via high-performance liquid chromatography (HPLC) as previously described (8). Ethanol and acetone were measured with a gas chromatograph as previously described (30). Cell growth was monitored using a Genesys 2 spectrophotometer (Spectronic Instruments, Rochester, NY) at 600 nm. The β -glucuronidase assays were carried out with cells at early mid-log phase, and specific activity was calculated according to protocols described previously (31).

RESULTS AND DISCUSSION

Evaluation of the *C. perfringens* lactose-inducible promoter system in *C. ljungdahlii*. In order to determine if the *bgaR*- P_{bgaL} lactose-inducible system developed for *C. perfringens* (23) would function in *C. ljungdahlii*, it was transformed with the plasmid pAH2. This plasmid contains a β -glucuronidase reporter gene fusion downstream of the *bgaR*- P_{bgaL} sequence, comprised of the ribosomal binding site and 13 codons of the 5' end of the *cpe* gene (encoding CPE, a *C. perfringens* enterotoxin) fused in frame with *gusA*, an *E. coli* gene for β -glucuronidase (23).

The addition of lactose to fructose-grown, mid-log-phase cells of *C. ljungdahlii* containing the pAH2 plasmid increased the expression of β -glucuronidase (Fig. 2). Within 5 h of induction, a substantial increase in *gusA* expression was observed with the addition of 10 or 20 mM lactose (Fig. 2A, inset). However, even with only 1 mM lactose, expression of *gusA* was increased 8-fold

TABLE 2 Plasmids used in this study

Name	Description	Reference or source
pAH2	Lactose-inducible promoter system upstream of <i>gusA</i> reporter gene	23
pKRAH1	Lactose-inducible promoter system with multiple cloning sites	23
pAH2-MCS	The <i>bgaR</i> gene and the promoter region (KpnI-PstI) were replaced by multiple-cloning sites KpnI-ApaI-XhoI-SalI-ClaiI-HindIII-EcoRV-EcoRI-SmaI-BamHI-XbaI-NotI-SacII-NdeI-PstI.	This work
pB1	pKRAH1 containing the <i>adhE1</i> gene downstream of the lactose-inducible promoter	This work
pB2	pKRAH1 containing the <i>adhE2</i> gene downstream of the lactose-inducible promoter	This work
pB3	pKRAH1 containing the synthetic acetone operon downstream of the lactose-inducible promoter	This work
pBKS(-)	pBluescript II KS(-)	Stratagene
pTU1	pBKS(-) containing synthetic acetone operon	This work
pCL2	<i>E. coli</i> - <i>Clostridium ljungdahlii</i> shuttle vector	15
pJIR-ermB Ppta	pJIR-ermB containing the <i>C. ljungdahlii</i> <i>pta</i> promoter region	This work
pJIR-ermB	The <i>catP</i> gene was replaced with <i>ermB</i> gene in pCL2	This work

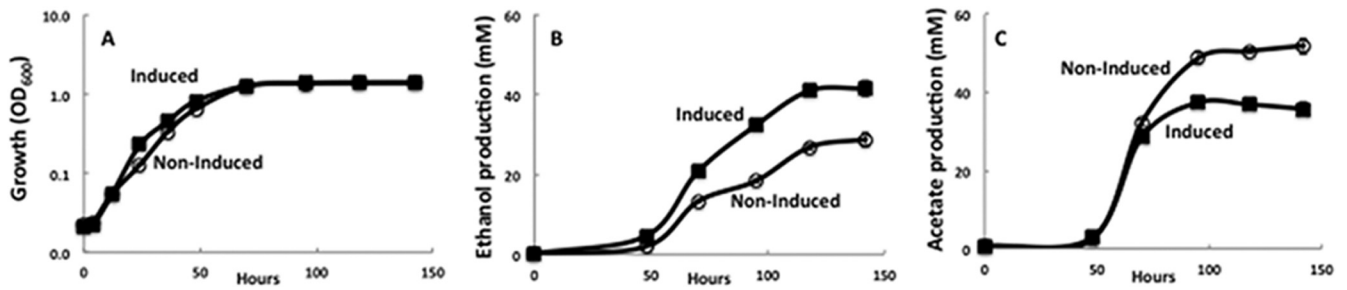


FIG 4 Cell growth and production of ethanol and acetate by the *C. ljungdahlii* wild-type strain containing the plasmid pB1 under fructose fermentation growth conditions in DSMZ879. (A) Cell growth. (B) Ethanol production. (C) Acetate production. Data are the means and standard deviations for triplicate cultures. Where no error bar is seen, the bar is smaller than the size of the symbol.

(Fig. 2A). This pattern of expression of *gusA* was similar to that reported by Hartman et al. (23). In the absence of the lactose inducer, some low enzyme activities can be detected (Fig. 2A), and potential explanations for this could be that (i) the regulator, BgaR, transiently binds to the promoter region and initiates minimal transcription of the *gusA* gene or (ii) other transcription regulators might nonspecifically bind to the *C. perfringens* P_{bgaL} promoter region. *C. ljungdahlii* utilizes not only fructose but also other sugars, including xylose, arabinose, ribose, and glucose (32). The utilization of different sugars is likely to be tightly regulated, and it is possible that one or more transcription regulators, such as a regulator for arabinose metabolism (33), may bind to the P_{bgaL} promoter region. The finding that the lactose-inducible system functioned well in *C. ljungdahlii*, coupled with previous reports of

the effectiveness of this system in both *C. perfringens* (23) and *C. acetobutylicum* (28), suggests that this inducible system may function well in a diversity of *Clostridium* species.

Diverting carbon flux to ethanol production with the lactose-inducible system. In order to determine if carbon and electron flow in *C. ljungdahlii*, could be altered with the lactose-inducible system, the impact of expressing genes for the aldehyde/alcohol dehydrogenases AdhE1 and AdhE2 was investigated. The plasmid pB1 was constructed with *adhE1* and the original ribosome-binding site from wild-type *C. ljungdahlii* inserted downstream of *bgaR*-P_{bgaL} (Table 2). When the fructose-grown strain of *C. ljungdahlii* containing pB1 was induced with 1 mM lactose in DSMZ879 medium, the transcript abundance of *adhE1* was 30-fold higher than that in the wild-type strain containing no plasmid

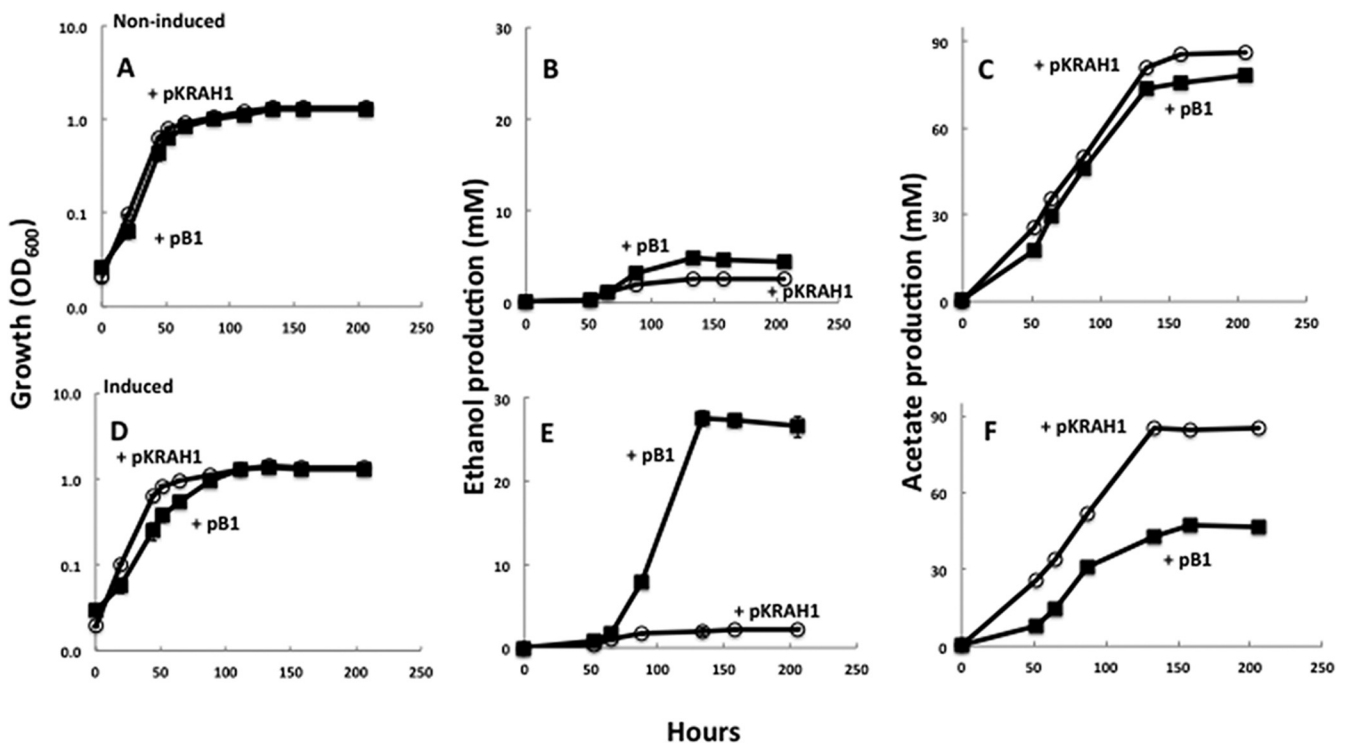


FIG 5 Cell growth and production of ethanol and acetate by the *C. ljungdahlii* *adhE1/adhE2*-deficient mutant containing either plasmid pKRAH1 or pB1 under fructose fermentation growth condition in DSMZ879. (A and D) Cell growth; (B and E) ethanol production; (C and F) acetate production. (A, B, and C) Noninduced samples; (D, E, and F) samples induced with 1 mM lactose. Data are the means and standard deviations for triplicate cultures. Where no error bar is seen, the bar is smaller than the size of the symbol.

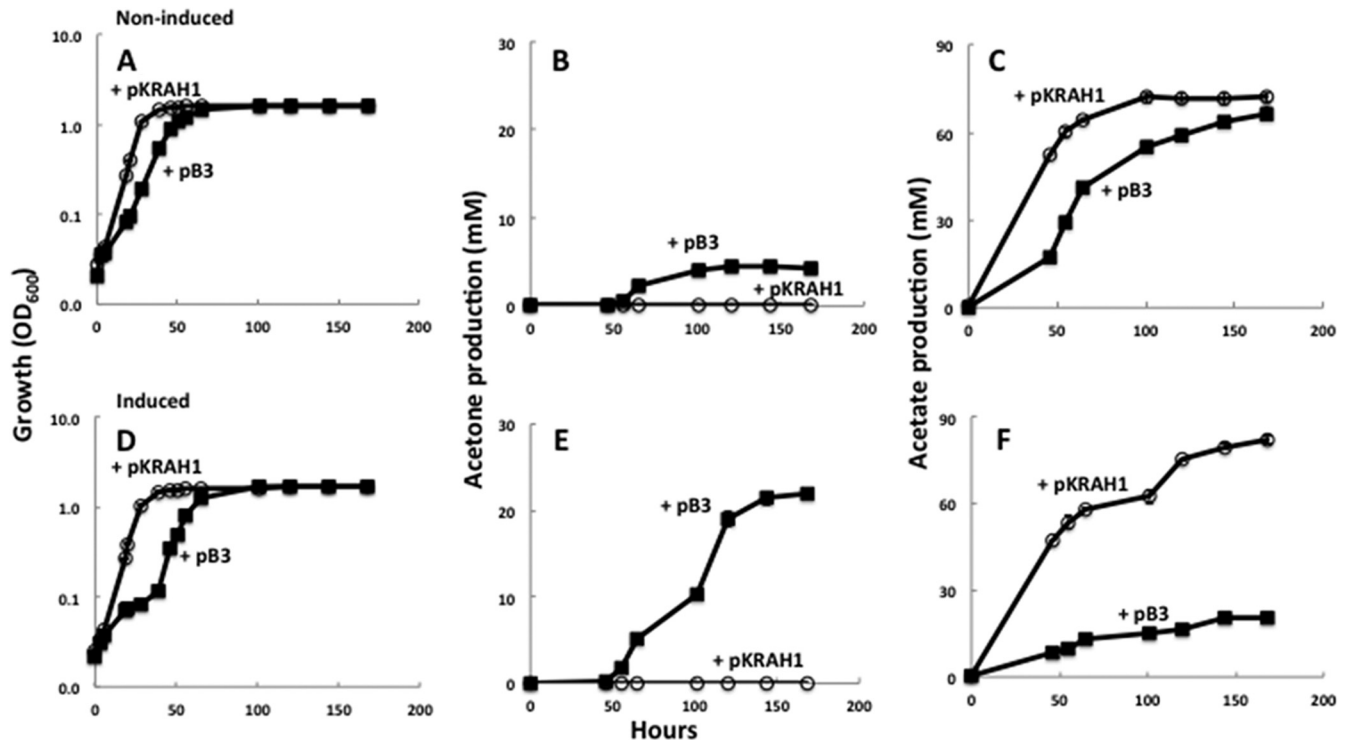


FIG 6 Cell growth and production of acetone and acetate by the *C. ljungdahliae* wild-type strain containing either plasmid pKRAH1 or pB3 under fructose fermentation growth condition in PETC1754. (A and D) Cell growth; (B and E) acetone production; (C and F) acetate production. (A, B, and C) noninduced samples; (D, E, F) samples induced with 1 mM lactose. Data are the means and standard deviations for triplicate cultures. Where no error bar is seen, the bar is smaller than the size of the symbol.

or the wild-type strain containing the control plasmid pKRAH1, which did not display any increase in expression of the *adhE1* gene after induction with lactose (Fig. 3). These results suggested that induction with 1 mM lactose was sufficient to substantially increase the expression of the *adhE1* in the *bgaR*-*P_{bgaL}* system.

Higher transcript abundance of *adhE1* in lactose-induced cells containing pB1 was associated with higher ethanol production than in the cells with the control plasmid (Fig. 4). The increase in ethanol was accompanied by a corresponding decrease in acetate. These results suggest that one factor limiting carbon and electron flux to ethanol was simply transcript abundance of *adhE1*. Diverting carbon and electron flow from acetate production to ethanol production had no significant impact on the cell growth rate or final cell yield, suggesting that energy yields were similar.

In order to further evaluate factors controlling ethanol production, studies were conducted with a previously described (15) strain of *C. ljungdahliae* which is deficient in ethanol production because *adhE1* and *adhE2* were deleted. When a fructose-grown *adhE1/adhE2*-deficient strain containing pB1 was induced with 1 mM lactose with the same medium as mentioned above, there was substantially more ethanol production than with cultures that were not induced with lactose or the control strain under either condition (Fig. 5). The increase in ethanol production in induced cultures was accompanied by a corresponding decrease in acetate production (Fig. 5). The amount of ethanol produced in the lactose-induced *adhE1/adhE2*-deficient strain of *C. ljungdahliae* with pB1 was comparable to that in wild-type cells (Fig. 4), suggesting that the lactose-inducible promoter system can be used for complementation studies.

In previous studies, the deletion of *adhE2* had no effect on ethanol production in *C. ljungdahliae* (15). It was considered that one possible explanation for this is that transcript abundance for *adhE2* was low during autotrophic as well as fermentative growth (M. Aklujkar and C. Leang, unpublished data). To determine whether increasing expression of *adhE2* would enhance ethanol production, the *adhE2* coding region and its own Shine-Dalgarno sequence were cloned downstream of the *bgaR*-*P_{bgaL}* induction system, yielding plasmid pB2 (Table 2), which was transformed into the *adhE1/adhE2*-deficient strain of *C. ljungdahliae*. However, the strain harboring pB2 produced levels of ethanol and acetate comparable to those in a strain without the plasmid (data not shown). These results suggest that the previous observation that deleting *adhE2* had no impact on ethanol production (15) may mean either that *adhE2* does not encode an effective aldehyde/alcohol dehydrogenase or that posttranscriptional factors limit expression and/or activity of the enzyme.

Producing acetone with a lactose-inducible construct. The possibility of constructing an inducible system for the synthesis of a product that *C. ljungdahliae* does not naturally produce (acetone) (Fig. 1) with enzymes encoded by heterologous genes was investigated. A synthetic operon for acetone production was constructed, which included genes from *C. acetobutylicum* for the enzymes thiolase (acetyl-CoA acetyltransferase), butyrate acetoacetate CoA transferase, and acetoacetate decarboxylase. The genes were placed under the control of the *bgaR*-*P_{bgaL}* inducible promoter system in pB3. The plasmid contained the *C. acetobutylicum* ribosome-binding site.

Even without the addition of the lactose inducer, the strain

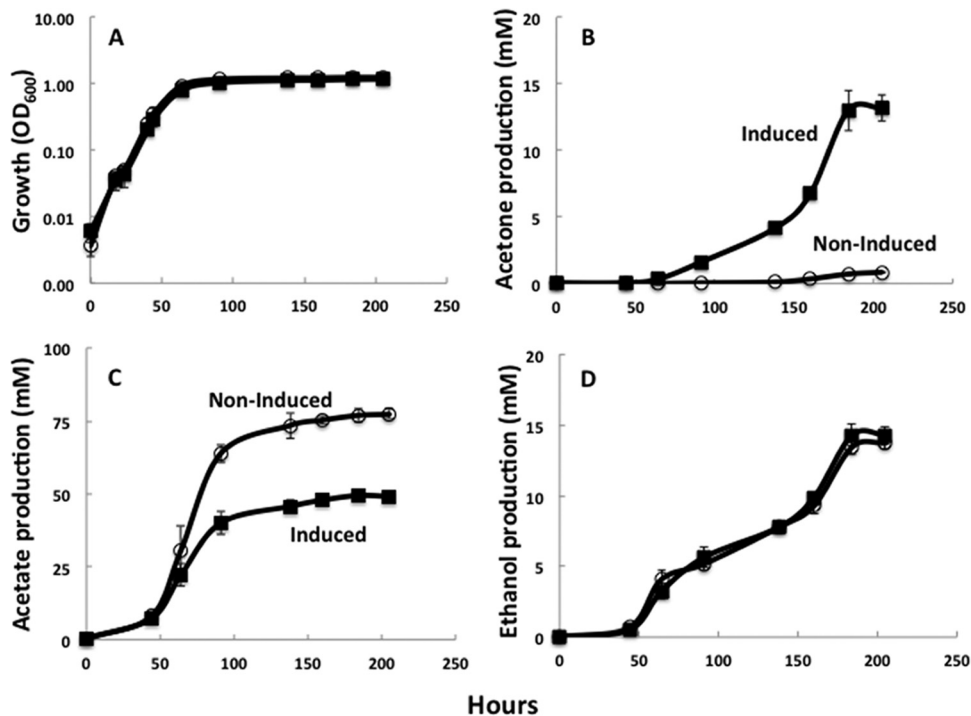


FIG 7 Cell growth and production of acetone, acetate, and ethanol by the *C. ljungdahlii* wild-type strain containing plasmid pB3 under autotrophic growth conditions with CO as the electron donor. (A) Cell growth; (B) acetone production; (C) acetate production; (D) ethanol production. Data are the means and standard deviations for triplicate cultures. Where no error bar is seen, the bar is smaller than the size of the symbol.

with the pB3 plasmid produced some acetone, with a corresponding decrease in the production of acetate compared with a control containing the pKRAH1 plasmid lacking the acetone production genes under fructose fermentation conditions (Fig. 6). No acetone production was detected in the strain containing the control plasmid pKRAH1, suggesting that the acetone production was solely due to the induction of the synthetic acetone pathway.

Acetone production in the strain with pB3 was substantially increased and acetate production proportionally decreased with lactose induction during fructose fermentation (Fig. 6). All strains produced small (ca. 1 to 2 mM) quantities of ethanol, but the lactose-induced pB3-containing strain produced slightly less ethanol (1.1 mM) than the strain containing the control pKRAH1 plasmid (1.6 mM). When the total carbon appearing in the potential end products acetate, ethanol, and acetone are considered, ca. 60% of the carbon appeared in acetone in the induced pB3-containing strain. Acetone-producing strains grew slightly slower but reached a final cell density comparable to that of the control. *C. ljungdahlii* gains less ATP when it produces acetone than when acetate is the end product (Fig. 1).

Induction of the genes for acetone production also yielded acetone with a proportional decrease in acetate production when carbon monoxide was the electron donor (Fig. 7), demonstrating that the lactose-inducible system also functioned under autotrophic growth conditions. Acetone accounted for a lower proportion of carbon flow (25%) than during growth on fructose. Further investigation to account for these differences is warranted.

Conclusions. The results demonstrate that the *bgaR*- P_{bgaL} lactose-inducible system developed for *C. perfringens* (23) functions well in *C. ljungdahlii* and provides an effective strategy for the

inducible expression of genes for enzymes that can divert carbon and electron flow away from acetate and toward the production of more desirable products. Inducible systems for novel product formation may be particularly helpful when carbon dioxide is the carbon feedstock for commodity production. Conversion of acetyl-CoA to acetate, the predominant terminal pathway for carbon dioxide reduction in wild-type *C. ljungdahlii*, yields more ATP than readily envisioned pathways for potential alternative products. Therefore, a strategy for effectively producing the catalytic biomass required for generating desired products may be to first grow *C. ljungdahlii* via carbon dioxide respiration with acetate as the end product and then, once sufficient biomass is available, shift metabolism to produce desired products with an inducible system, such as the one described here. Genetic manipulation to increase expression of components for extracellular electron transfer has proven to be an effective strategy for enhancing microbe-to-electrode electron transfer (34), and thus inducing expression of proteins that promote electrode-to-cell electron transfer for microbial electrosynthesis at the proper stages of biofilm development is a likely strategy for further optimizing this process. Furthermore, the inducible system described here should aid in further basic studies of the physiology of *C. ljungdahlii*, which has become an important model organism for the study of acetogenesis due to the increasing availability of genetic tools for this organism.

ACKNOWLEDGMENTS

This research was supported by the Advanced Research Projects Agency-Energy (ARPA-E), U.S. Department of Energy, under award no. DE-AR0000159.

We thank Stephen B. Melville for providing plasmids pAH2 and

pKRAH1. We are grateful for the technical support provided by Joy Ward, Trevor Woodard, and Cory Gillis.

REFERENCES

- Cotter JL, Chinn MS, Grunden AM. 2009. Influence of process parameters on growth of *Clostridium ljungdahlii* and *Clostridium autoethanogenum* on synthesis gas. *Enzyme Microb. Technol.* 44:281–288. <http://dx.doi.org/10.1016/j.enzmictec.2008.11.002>.
- Cotter JL, Chinn MS, Grunden AM. 2009. Ethanol and acetate production by *Clostridium ljungdahlii* and *Clostridium autoethanogenum* using resting cells. *Bioprocess Biosyst. Eng.* 32:369–380. <http://dx.doi.org/10.1007/s00449-008-0256-y>.
- Tirado-Acevedo O, Cotter JL, Chinn MS, Grunden AM. 2011. Influence of carbon source pre-adaptation on *Clostridium ljungdahlii* growth and product formation. *J. Bioprocess. Biotech.* S2:001.
- Kopke M, Held C, Hujer S, Liesegang H, Wiezer A, Wollherr A, Ehrenreich A, Liebl W, Gottschalk G, Durre P. 2010. *Clostridium ljungdahlii* represents a microbial production platform based on syngas. *Proc. Natl. Acad. Sci. U. S. A.* 107:13087–13092. <http://dx.doi.org/10.1073/pnas.1004716107>.
- Kopke M, Mihalcea C, Bromley JC, Simpson SD. 2011. Fermentative production of ethanol from carbon monoxide. *Curr. Opin. Biotechnol.* 22:320–325. <http://dx.doi.org/10.1016/j.copbio.2011.01.005>.
- Lovley DR, Nevin KP. 2011. A shift in the current: new applications and concepts for microbe-electrode electron exchange. *Curr. Opin. Biotechnol.* 22:441–448. <http://dx.doi.org/10.1016/j.copbio.2011.01.009>.
- Lovley DR, Nevin KP. 2013. Electrobiocommodities: powering microbial production of fuels and commodity chemicals from carbon dioxide with electricity. *Curr. Opin. Biotechnol.* 24:385–390. <http://dx.doi.org/10.1016/j.copbio.2013.02.012>.
- Nevin KP, Hensley SA, Franks AE, Summers ZM, Ou J, Woodard TL, Snoeyenbos-West OL, Lovley DR. 2011. Electrosynthesis of organic compounds from carbon dioxide is catalyzed by a diversity of acetogenic microorganisms. *Appl. Environ. Microbiol.* 77:2882–2886. <http://dx.doi.org/10.1128/AEM.02642-10>.
- Nevin KP, Woodard TL, Franks AE, Summers ZM, Lovley DR. 2010. Microbial electrosynthesis: feeding microbes electricity to convert carbon dioxide and water to multicarbon extracellular organic compounds. *mBio* 1(2):e00103-10. <http://dx.doi.org/10.1128/mBio.00103-10>.
- Drake HL, Daniel SL, Kusel K, Matthies C, Kuhner C, Braus-Stromeyer S. 1997. Acetogenic bacteria: what are the in situ consequences of their diverse metabolic versatility? *Biofactors* 6:13–24. <http://dx.doi.org/10.1002/biof.5520060103>.
- Ragsdale SW. 2008. Enzymology of the wood-Ljungdahl pathway of acetogenesis. *Ann. N. Y. Acad. Sci.* 1125:129–136. <http://dx.doi.org/10.1196/annals.1419.015>.
- Ragsdale SW, Pierce E. 2008. Acetogenesis and the Wood-Ljungdahl pathway of CO₂ fixation. *Biochim. Biophys. Acta* 1784:1873–1898. <http://dx.doi.org/10.1016/j.bbapap.2008.08.012>.
- Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET. 2012. Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. *Curr. Opin. Biotechnol.* 23:364–381. <http://dx.doi.org/10.1016/j.copbio.2011.10.008>.
- Fast AG, Papoutsakis ET. 2012. Stoichiometric and energetic analyses of non-photosynthetic CO₂-fixation pathways to support synthetic biology strategies for production of fuels and chemicals. *Curr. Opin. Chem. Eng.* 1:380–395. <http://dx.doi.org/10.1016/j.coche.2012.07.005>.
- Leang C, Ueki T, Nevin KP, Lovley DR. 2013. A genetic system for *Clostridium ljungdahlii*: a chassis for autotrophic production of biocommodities and a model homoacetogen. *Appl. Environ. Microbiol.* 79:1102–1109. <http://dx.doi.org/10.1128/AEM.02891-12>.
- Tremblay PL, Zhang T, Dar SA, Leang C, Lovley DR. 2012. The Rnf complex of *Clostridium ljungdahlii* is a proton-translocating ferredoxin: NAD⁺ oxidoreductase essential for autotrophic growth. *mBio* 4(1):e00406-12. <http://dx.doi.org/10.1128/mBio.00406-12>.
- Kopke M, Mihalcea C, Liew F, Tizard JH, Ali MS, Conolly JJ, Al-Sinawi B, Simpson SD. 2011. 2,3-Butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. *Appl. Environ. Microbiol.* 77:5467–5475. <http://dx.doi.org/10.1128/AEM.00355-11>.
- Tracy BP. 2012. Improving butanol fermentation to enter the advanced biofuel market. *mBio* 3(6):e00518-12. <http://dx.doi.org/10.1128/mBio.00518-12>.
- Wang T, Ma X, Du G, Chen J. 2012. Overview of regulatory strategies and molecular elements in metabolic engineering of bacteria. *Mol. Biotechnol.* 52:300–308. <http://dx.doi.org/10.1007/s12033-012-9514-y>.
- Dong HJ, Tao WW, Zhang YP, Li Y. 2012. Development of an anhydrotetracycline-inducible gene expression system for solvent-producing *Clostridium acetobutylicum*: a useful tool for strain engineering. *Metab. Eng.* 14:59–67. <http://dx.doi.org/10.1016/j.ymben.2011.10.004>.
- Feustel L, Nakotte S, Durre P. 2004. Characterization and development of two reporter gene systems for *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* 70:798–803. <http://dx.doi.org/10.1128/AEM.70.2.798-803.2004>.
- Girbal L, Mortier-Barriere I, Raynaud F, Rouanet C, Croux C, Soucaille P. 2003. Development of a sensitive gene expression reporter system and an inducible promoter-repressor system for *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* 69:4985–4988. <http://dx.doi.org/10.1128/AEM.69.8.4985-4988.2003>.
- Hartman AH, Liu HL, Melville SB. 2011. Construction and characterization of a lactose-inducible promoter system for controlled gene expression in *Clostridium perfringens*. *Appl. Environ. Microbiol.* 77:471–478. <http://dx.doi.org/10.1128/AEM.01536-10>.
- Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP. 2007. The Clostron: a universal gene knock-out system for the genus *Clostridium*. *J. Microbiol. Methods* 70:452–464. <http://dx.doi.org/10.1016/j.mimet.2007.05.021>.
- Nariya H, Miyata S, Kuwahara T, Okabe A. 2011. Development and characterization of a xylose-inducible gene expression system for *Clostridium perfringens*. *Appl. Environ. Microbiol.* 77:8439–8441. <http://dx.doi.org/10.1128/AEM.05668-11>.
- Nuyts S, Van Mellaert L, Theys J, Landuyt W, Lambin P, Anne J. 2001. The use of radiation-induced bacterial promoters in anaerobic conditions: a means to control gene expression in *Clostridium*-mediated therapy for cancer. *Radiat. Res.* 155:716–723. [http://dx.doi.org/10.1667/0033-7587\(2001\)155\[0716:TUORIB\]2.0.CO;2](http://dx.doi.org/10.1667/0033-7587(2001)155[0716:TUORIB]2.0.CO;2).
- Tummala SB, Welker NE, Papoutsakis ET. 1999. Development and characterization of a gene expression reporter system for *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* 65:3793–3799.
- Al-Hinai MA, Fast AG, Papoutsakis ET. 2012. Novel system for efficient isolation of *Clostridium* double-cross-over allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. *Appl. Environ. Microbiol.* 78:8112–8121. <http://dx.doi.org/10.1128/AEM.02214-12>.
- Jones DT, Woods DR. 1986. Acetone-butanol fermentation revisited. *Microbiol. Rev.* 50:484–524.
- Morita M, Malvankar NS, Franks AE, Summers ZM, Giloteaux L, Rotaru AE, Rotaru C, Lovley DR. 2011. Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. *mBio* 2(4):e00159-11. <http://dx.doi.org/10.1128/mBio.00159-11>.
- Melville SB, Labbe R, Sonenshein AL. 1994. Expression from the *Clostridium perfringens* cpe promoter in *C. perfringens* and *Bacillus subtilis*. *Infect. Immun.* 62:5550–5558.
- Tanner RS, Miller LM, Yang D. 1993. *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial ribosomal-RNA homology group-I. *Int. J. Syst. Bacteriol.* 43:232–236. <http://dx.doi.org/10.1099/00207713-43-2-232>.
- Zhang Y, Shang X, Lai S, Zhang G, Liang Y, Wen T. 2012. Development and application of an arabinose-inducible expression system by facilitating inducer uptake in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 78:5831–5838. <http://dx.doi.org/10.1128/AEM.01147-12>.
- Leang C, Malvankar NS, Franks AE, Nevin KP, Lovley DR. 2013. Engineering *Geobacter sulfurreducens* to produce a highly cohesive conductive matrix with enhanced capacity for current production. *Energy Environ. Sci.* 6:1901–1908. <http://dx.doi.org/10.1039/c3ee40441b>.