Insights into CO₂ Fixation Pathway of Clostridium autoethanogenum by Targeted Mutagenesis

Fungmin Liew,a,b Anne M. Henstra,a Klaus Winzer,a Michael Köpke,b Sean D. Simpson,b Nigel P. Mintona

BBSRC/EPSRC Synthetic Biology Research Centre (SBRC), School of Life Sciences, University Park, The University of Nottingham, Nottingham, United Kingdom; LanzaTech Inc., Skokie, Illinois, USA

ABSTRACT The future sustainable production of chemicals and fuels from nonpetrochemical resources and reduction of greenhouse gas emissions are two of the greatest societal challenges. Gas fermentation, which utilizes the ability of acetogenic bacteria such as Clostridium autoethanogenum to grow and convert CO₂ and CO into low-carbon fuels and chemicals, could potentially provide solutions to both. Acetogens fix these single-carbon gases via the Wood-Ljungdahl pathway. Two enzyme activities are predicted to be essential to the pathway: carbon monoxide dehydrogenase (CODH), which catalyzes the reversible oxidation of CO to CO₂, and acetyl coenzyme A (acetyl-CoA) synthase (ACS), which combines with CODH to form a CODH/ACS complex for acetyl-CoA fixation. Despite their pivotal role in carbon fixation, their functions have not been confirmed in vivo. By genetically manipulating all three CODH isogenes (acsA, cooS1, and cooS2) of C. autoethanogenum, we highlighted the functional redundancies of CODH by demonstrating that cooS1 and cooS2 are dispensable for autotrophy. Unexpectedly, the cooS1 inactivation strain showed a significantly reduced lag phase and a higher growth rate than the wild type on H₂ and CO₂. During heterotrophic growth on fructose, the aca inactivation strain exhibited 61% reduced biomass and the abolishment of acetate production (a hallmark of acetogens), in favor of ethanol, lactate, and 2,3-butanediol production. A translational readthrough event was discovered in the uniquely truncated (compared to those of other acetogens) C. autoethanogenum aca gene. Insights gained from studying the function of CODH enhance the overall understanding of autotrophy and can be used for optimization of biotechnological production of ethanol and other commodities via gas fermentation.

IMPORTANCE Gas fermentation is an emerging technology that converts the greenhouse gases CO₂ and CO in industrial waste gases and gasified biomass into fuels and chemical commodities. Acetogenic bacteria such as Clostridium autoethanogenum are central to this bioprocess, but the molecular and genetic characterization of this microorganism is currently lacking. By targeting all three of the isogenes encoding carbon monoxide dehydrogenase (CODH) in C. autoethanogenum, we identified the most important CODH isogene for carbon fixation and demonstrated that genetic inactivation of CODH could improve autotrophic growth. This study shows that disabling of the Wood-Ljungdahl pathway via the inactivation of aca (encodes CODH) significantly impairs heterotrophic growth and alters the product profile by abolishing acetate production. Moreover, we discovered a previously undescribed mechanism for controlling the production of this enzyme. This study provides valuable insights into the acetogenic pathway and can be used for the development of more efficient and productive strains for gas fermentation.
level (9–11), but in vivo and genetic studies of CODH in acetogens are lacking.

Clostridium autoethanogenum, a model acetogen, is able to grow on CO as a sole carbon and energy source and produce ethanol, acetate, 2,3-butanediol, and lactate (12, 13). Whole-genome sequencing of this acetogen revealed the presence of three putative CODHs: CAETHG_1620-1621 (acsA), CAETHG_3005 (cooS1), and CAETHG_3899 (cooS2) (14). acsA is in an 18-kbp cluster with genes of the WLP and is predicted to encode the CODH component of the CODH/ACS complex, while cooS1 is localized upstream of a gene encoding a putative 4Fe-4S ferredoxin binding domain-containing protein and ferredoxin oxidoreductase. cooS2 appears to be an orphan. Transcriptomic studies have shown that all three genes are expressed, with acsA and cooS1 being among the most highly expressed genes within the genome (15, 16).

Given the number of CODHs within C. autoethanogenum, it is unknown whether all of them are essential for autotrophic growth and are true isozymes or have distinct functions. Here, we addressed this question by independently inactivating all three CODH-encoding genes by ClosTron mutagenesis (17–19) and addressed this question by independently inactivating all three CODH-encoding genes by ClosTron mutagenesis (17–19) and then examined the impact on autotrophy and product formation. The mutant strain in which acsA was inactivated is particularly interesting because it essentially has a disabled WLP and, as a consequence, displays a radically different metabolite distribution, including the complete abolition of acetate formation.

Intriguingly, compared to the acsA genes of other acetogens, that of C. autoethanogenum uniquely contains an in-frame TGA stop codon. The encoded enzyme is therefore predicted to be truncated by some 231 amino acids and the gene effectively split in two: CAETHG_1621 and CAETHG_1620. By using FLAG-tagged acsA variants, the apparent acsA truncation event was investigated.

RESULTS

acsA is essential for autotrophy in C. autoethanogenum. To determine the roles of acsA in supporting autotrophy in C. autoethanogenum, we first inactivated the gene by ClosTron mutagenesis (17–19), which resulted in an acsA knockout (KO) strain (see Fig. S1 in the supplemental material). Both the KO and wild-type (WT) strains were subjected to autotrophic batch growth on either CO or H₂-CO₂ (2:1) to assess the role of acsA in catalyzing CO oxidation and/or fixation of CO₂ (using H₂ as a reductant). As shown in Fig. 1A, the acsA KO strain displayed no sign of growth on CO following 48 days of incubation, whereas the WT reached an optical density at 600 nm (OD⁶⁰⁰) of 1.98 after day 8. Under H₂-CO₂ conditions, the acsA KO strain was unable to grow within 27 days, whereas the WT achieved stationary phase (OD⁶⁰⁰ of 0.17) after ~day 15 (Fig. 1B). In an attempt to restore autotrophy, plasmid pMTL83151-PacsA-acsAfull containing acsA was conjugated into the acsA KO strain to generate a complementation strain. The complementation strain was able to restore growth and acetate formation on CO, albeit growing to an OD⁶⁰⁰ of 1.10 after a growth lag phase of ~21 days (see Fig. S2 in the supplemental material) and generating 15% less acetate (P = 0.009). The growth characteristics of the plasmid control strain (harporing pMTL83151-PacsA) were generally similar to those of the WT, except that the lag phase was longer by 5 days (Fig. 1A; see also Fig. S2 in the supplemental material).

Inactivation of acsA abolishes acetate formation during heterotrophic growth. In order to gain an insight into the role of acsA during heterotrophic growth, we next investigated the growth and product profile of the acsA KO strain on fructose. The acsA KO strain fully exhausted the supplemented 10 g/liter fructose but reached a 61% lower OD⁶⁰⁰ (P < 0.0001) and exhibited a longer growth lag phase than the WT (Fig. 2A). The 3.8-fold higher head-space pressure recorded at the end of the growth experiment with the acsA KO strain (Fig. 2B) indicates that more CO₂ was being released from fructose metabolism as a result of the organism’s inability to reassimilate the released CO₂ as a consequence of its dysfunctional WLP.

In terms of metabolite production, the acsA KO strain only transiently produced a trace amount of acetate (2.6 mM on day 2.8) while growing on fructose (Fig. 2C). In contrast, the WT strain generated 86.0 mM acetate under the same conditions (Fig. 2C). In the acsA KO strain, most of the carbon from fructose was diverted from acetate toward reduced products ethanol and 2,3-butanediol and toward lactate, as evident in increases of 113, 138, and 125%, respectively, relative to the WT (Fig. 2D to F). Similar to autotrophic growth on CO (mentioned earlier), the plasmid expression of acsAfull in the acsA KO strain partially restored the phenotypes of heterotrophic growth by reducing the growth lag phase to WT levels and increased the OD⁶⁰⁰ from 1.77 (acsA KO) to 3.11, which is 69% of the WT level (Fig. 2A). The complementation strain also synthesized 30.78 mM acetate (up from the 2.5 mM of the acsA KO), 65.35 mM ethanol (down from the 102.74 mM of the acsA KO), and 6.66 mM 2,3-butanediol (down from 10.95 mM) (Fig. 2C to E). Plasmid expression of acsA (pMTL83151-PacsA-acsAfull) in the WT had only minimal effects

![FIG 1](image-url) The acsA KO strain is unable to grow autotrophically on 200 kPa CO (A) or 130 kPa H₂ plus 70 kPa CO₂ (B). Symbols: black circles, WT (n = 4 for CO; n = 3 for H₂-CO₂); red squares, acsA KO strain (n = 3). Error bars show the standard error of the mean.
on growth and metabolite production during growth on CO (see Fig. S3 in the supplemental material) or under heterotrophic growth conditions (see Fig. S4 in the supplemental material).

Translational readthrough of acsA. Genome sequencing of C. autoethanogenum (14, 20, 21) revealed the presence of an internal TGA stop codon within the acsA gene, splitting the gene into coding sequences (CDSs) CAETHG_1621 (1,203 bp) and CAETHG_1620 (684 bp) (see Fig. S5 in the supplemental material). Sanger sequencing confirmed the presence of the TGA stop codon (data not shown).

By fusing a FLAG tag to the N or C terminus of AcsA, the acsA translation pattern in C. autoethanogenum and Escherichia coli was investigated by Western blot analysis. In addition, the TGA stop codon was replaced with a TCA or TAA codon by splicing by overhang extension PCR (SOE-PCR). Modified genes were expressed from plasmids. The truncation of the 69-kDa full-length AcsA protein can result in proteins of 44 and/or 25 kDa (Fig. 3B). With a C-terminal FLAG tag, a 69-kDa protein was detected in C. autoethanogenum crude lysates (Fig. 3A), while a 25-kDa protein band was absent. With the N-terminal FLAG tag variant, the 44- and 69-kDa proteins were both detected, with the intensity of the 44-kDa protein band being higher. When the TGA codon was replaced with a TCA codon, only the 69-kDa protein was detected. These results indicate that translational readthrough of the acsA TGA codon occurs with low frequency in C. autoethanogenum and there is no independent translation of the downstream CDS.

E. coli is a model microorganism for the study of selenocysteine synthesis and is capable of selenocysteine incorporation (22, 23).

FIG 2 Growth, headspace pressure, and metabolite profiles of the C. autoethanogenum WT, acsA KO, and complementation strains on 10 g/liter fructose. Panels: A, growth profile; B, headspace pressure profile; C, acetate profile; D, ethanol profile; E, 2,3-butanediol profile; F, lactate profile. Black, WT; red, acsA KO strain; blue, complementation strain. n = 3. Error bars show the standard error of the mean.
Lyase of E. coli cells that expressed acsA variants encoding either N- or C-terminally FLAG-tagged proteins failed to produce the 69-kDa full-size AcsA product (Fig. 3C). Instead, the 44-kDa truncated protein was detected. However, an internal stop codon was also introduced into the acsA sequence, which is predicted to generate a full-size AcsA product (Fig. 3C). Replacement of the internal stop codon with a TCA serine codon successfully generated the full-size ~69-kDa truncated protein. However, the 44-kDa N- or C-terminally FLAG-tagged proteins failed to produce the expected protein sizes.

cooS1 and cooS2 are dispensable for autotrophy. Besides acsA, the genome of C. autoethanogenum contains two additional putative CODHs: cooS1 and cooS2. These are unable to compensate for acsA and are therefore predicted to be monofunctional, as shown above. Accordingly, the acsA KO strain would be expected to grow on fructose (10 g/liter) and oxidize CO (22.4 mmol) into CO2 by using the unperturbed cooS1- and/or cooS2-encoded CODHs. In experiments that compared the acsA KO strain to the WT grown mixotrophically on CO plus fructose, the WT strain generated 2.03 mmol of CO, while the WT control consumed 8.52 mmol of CO (Fig. 4B). Both strains completely exhausted the fructose at the end of the experiment (data not shown).

In the absence of a WLP, the consumption of 10 g/liter fructose (or an absolute amount of 2.8 mmol) is expected to produce a maximum CO2 level of 5.6 mmol (1 mol of fructose yields 2 mol of pyruvate, which in turn is decarboxylated into acetyl-CoA with the concomitant release of 2 mol of CO2) (Fig. 5). In acetogens, a large proportion of the released CO2 is fixed into acetyl-CoA. Since the WT produced 8.3 mmol of CO2 during mixotrophic growth on 2.8 mmol fructose and 8.52 mmol of CO (Fig. 4B), which is greater than the theoretical maximum of 5.6 mmol of CO2, CO oxidation must have occurred, as opposed to the direct use of CO in the carbonyl branch of the WLP. These results indicate that while the WT strain is able to oxidize CO during mixotrophic growth, neither cooS1 nor cooS2 in the acsA KO strain is able to catalyze CO oxidation. Instead, the physiological roles of these monofunctional CODHs under mixotrophic growth conditions may lie in the direction of CO2 reduction since additional CO was produced by the acsA KO strain.

By Clostron mutagenesis, cooS1 and cooS2 of C. autoethanogenum were independently inactivated to assess their roles in autotrophy (see Fig. S1 in the supplemental material). When grown on pure CO, the cooS1 KO strain displayed some growth deficiencies, including an ~2.9-day-longer lag phase, and achieved a 42% lower OD600 (P = 0.0002) than the WT (Fig. 6A). It produced 25% less acetate (P = 0.0004) and a similar amount of 2,3-butanediol (data not shown) but 64% more ethanol (not statistically significant) than the WT (Fig. 6B). The reduced growth of the cooS1 KO strain in the presence of CO may result in the accumulation of excess reducing equivalents that are consumed in ethanol biosynthetic pathways. Remarkably, under H2-CO2 conditions, the cooS1 KO strain is able to grow without an apparent lag phase and also reaches twice the OD600 of the WT (Fig. 6C).

Unlike the autotrophic growth of the cooS1 KO strain with CO, that of the cooS2 KO strain is not significantly affected. The cooS2 KO strain even showed a mild decrease in the growth lag phase but a lower final OD600 in the presence of CO than the WT (Fig. 6A). This result, combined with a lack of impact on H2-CO2 autotrophic growth (Fig. 6C), suggests that cooS2 is not heavily involved in autotrophy of C. autoethanogenum.
DISCUSSION
Understanding the fundamentals of C1 metabolism in acetogens is a prerequisite for their further development as a chassis for the sustainable production of chemicals and fuels from waste gases.

By independently disrupting all three CODH isogenes in \textit{C. autoethanogenum}, we investigated their roles in supporting autotrophy. The complete absence of growth of the \textit{acsA} KO strain on CO or H\textsubscript{2}-CO\textsubscript{2} demonstrated that \textit{acsA} is absolutely essential for aut-
otrophy under both CO and H$_2$-CO$_2$ conditions and that unperturbed cooS1 and cooS2 are unable to compensate for the loss of acsA function. In Methanosarcina acetivorans, which has two CODH/ACS paralogs, deletion studies showed that the microorganism can grow autotrophically in CO when one of these genes was deleted but not when both were deleted (24).

During glycolysis, 1 mol of hexose sugar is metabolized to 2 mol of acetyl-CoA, 2 mol of CO$_2$ (generated during the pyruvate:ferredoxin oxidoreductase reaction), and 8 reducing equivalents (Fig. 5). Acetogens such as C. autoethanogenum utilize the 8 reducing equivalents to reassimilate the released 2 mol of CO$_2$ into an additional acetyl-CoA, resulting in complete carbon conversion (3). Based on this, a concept called acetogenic mixotrophy was recently proposed to improve biofuel and biochemical yields (25). The acsA KO strain constructed in this study provides a unique opportunity to examine the effect of a disabled WLP on a microbe that normally performs acetogenic mixotrophy. The growth of the acsA KO strain on fructose was significantly impaired, highlighting the role of CODH/ACS in biomass formation during heterotrophic growth.

Acetate production, which generates an ATP per molecule of acetyl-CoA via substrate level phosphorylation, is one of the hallmarks of acetogens (3). Hence, it is surprising that the acsA KO strain produced significantly more ethanol, 2,3-butanediol, and lactate than the WT while growing on fructose. Production of these products serves as an alternative sink to the disabled WLP (due to acsA inactivation) for reducing equivalents generated by glycolysis (Fig. 5), resulting in a redistribution of electron and carbon fluxes. By a similar approach, acetate production could be significantly reduced and carbon redistributed to ethanol when inactivating hydrogenase maturation proteins (and, therefore, hydrogenase activity) in Clostridium thermocellum (29). Certain acetogens, including C. autoethanogenum, harbor the enzyme aldehyde:ferredoxin oxidoreductase (14), which could reduce acetic acids into acetaldehyde using reduced ferredoxin, followed by the formation of ethanol via ethanol dehydrogenase. Accordingly, the lack of acetate production in the acsA KO strain could be due to an increase in the rate of acetic acid reduction. Collectively, our results demonstrated that although a functional WLP allows higher carbon utilization efficiency during heterotrophic growth by fixing CO$_2$ into biomass and acetate, the biosynthesis of ethanol, 2,3-butanediol, and lactate is significantly reduced in the absence of an additional reductant (e.g., H$_2$ or CO).

The acsA gene of C. autoethanogenum is uniquely truncated because of an internal TGA stop codon. The acsA genes of five other clostridial acetogens, Clostridium ljungdahlii (30), “Clostridium ragdalet” (12), Clostridium carboxidivorans P7 (31), Clostridium aceticum (32), and Clostridium difficile 630 (33), with identical

![FIG 6 Growth and metabolite profiles of the C. autoethanogenum WT and cooS1 and cooS2 KO strains on CO or H$_2$-CO$_2$. Panels: A, growth profile on CO; B, metabolite profile on CO; C, growth profile on H$_2$-CO$_2$; D, metabolite profile on H$_2$-CO$_2$. Columns: black, WT (n = 4 for CO, n = 3 for H$_2$-CO$_2$); green, cooS1 KO strain (n = 3); purple, cooS2 KO strain (n = 3). Error bars show the standard error of the mean.](mbio.asm.org)
targeted mutagenesis of codh in c. autoethanogenum

operon topologies showed no gene-splitting event (see Fig. S5A in the supplemental material). Instead, a TCA serine codon is present in these acetogens. Given the essential role of acsA in acetogenesis, it was of interest to determine whether posttranscriptional mechanisms allowed translational readthrough beyond the internal stop codon in c. autoethanogenum. One such mechanism is the incorporation of selenocysteine at the TGA codon, which is generally reliant on a characteristic bacterial selenocysteine insertion sequence (bSECIS) immediately downstream of the stop codon. A bSECIS element was not detected in the c. autoethanogenum acsA gene by using an algorithm (34), but such an element was uncovered via manual examination (see Fig. S5B in the supplemental material). The catalytic activities of many selenoproteins are often superior to those of their cysteine-dependent counterparts (35).

By investigating the translational pattern of FLAG-tagged AcS protein by Western blot analysis, we showed that a partial and inefficient translational readthrough event occurs in c. autoethanogenum as formation of the truncated AcS protein is the main product. It is not clear whether the ability of c. autoethanogenum to generate mature and truncated AcS is a novel regulatory mechanism for an as-yet-unknown physiological purpose or whether it poses a handicap that hinders acetogenesis. A search of the curated genome (21) revealed 52 CDSSs with TGA stop codons closely followed by an in-frame CDS (see Table S1 in supplemental material), including a selenocysteine formate dehydrogenase (36). Thus, acsA may not be the only c. autoethanogenum gene with an internally translated stop codon.

In contrast to c. autoethanogenum, plasmid expression of the FLAG-tagged AcS protein in E. coli (a model organism for selenocysteine formation and incorporation) did not result in the formation of a translational readthrough product. This result indicates that the translational readthrough event in acsA is c. autoethanogenum specific and is unlikely to involve selenocysteine incorporation. Stop codon readthrough is not uncommon and especially prevalent for the UGA codon, while UAA is a more efficient translational stop signal (37). When the TAA stop codon of acsA replaced the internal TGA stop codon, translation of the full-length AcS peptide in c. autoethanogenum and E. coli was completely eliminated. Stop codon readthrough depends on the competition between a release factor and a near-cognate tRNA (37). The genome of c. autoethanogenum encodes one tRNA with an anticodon 5'-CCA-3' (CAETHG_R0046) for tryptophan.

In this study, inactivation of either cooS1 or cooS2 moderately lowered biomass formation during growth on CO but did not negatively impact growth on H2-CO2, highlighting functional redundancies in acetogens that harbor multiple CODH isoforms. The orthologous cooS1 and cooS2 genes of c. ljungdahlii were reported to be expressed at lower levels when the bacterium was grown autotrophically on CO rather than heterotrophically on fructose, leading the authors to hypothesize that monofunctional CODHs do not significantly contribute to the oxidation of CO, but it is the bifunctional CODH/ACS complex that is mainly responsible (16, 38). In agreement with this hypothesis, during mixotrophic growth on fructose plus CO, c. autoethanogenum WT, but not the acsA KO strain (which still carries the functional cooS1 and cooS2 genes), was able to oxidize CO.

During growth on H2-CO2, the cooS1 KO strain, surprisingly, grew without an apparent lag phase and reached twice the OD600 of the WT strain. Since the reduced [CO] moiety is enclosed within the CODH/ACS complex to lower internal thermodynamic barriers during acetyl-CoA synthesis (39–41), the presence of another CODH may act as a competitor for CO2. The inactivation of cooS1 would, therefore, increase the efficiency of the CODH/ACS enzyme complex, leading to the observed enhanced growth on H2-CO2, and may represent a metabolic engineering strategy to improve gas utilization efficiency in acetogens that harbor multiple CODH-encoding isoforms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For the bacterial strains used in this study, see Table S2 in the supplemental material. The E. coli strains used for general plasmid propagation, cloning, and conjugation were cultivated at 37°C in LB medium in the presence of antibiotics (25 µg/ml chloramphenicol, 100 µg/ml spectinomycin). C. autoethanogenum DSM 10061 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and cultivated under strict anaerobic conditions in CaGM medium.

CaGM growth medium contains (per liter) 0.25 g of NH4Cl, 0.1 g of KCl, 0.2 g of KH2PO4, 0.2 g of MgSO4 · 7H2O, 0.02 g of CaCl2 · 2H2O, 1 g of yeast extract, 0.5 ml of 2-g/liter resazurin, 20 g of 2-(N-morpholino)ethanesulfonic acid (MES), 0.05 g of Fe(SO4)2 · 7H2O, 0.25 g of sodium acetate · 3H2O, 0.05 g of nitrolitriacetic acid (NTA), 10 g of fructose (only for heterotrophic growth), 10 ml of a trace element (TSE) solution, and 10 ml of Wolfe’s vitamin solution. The composition of the TSE solution (per liter) was 2 g of nitrolitriacetic acid, 1 g of MnSO4 · H2O, 0.8 g of FeSO4 · 7(NH4)2 · 6H2O, 0.2 g of CoCl2 · 6H2O, 0.2 mg of ZnSO4 · 7H2O, 0.02 g of CuCl2 · 2H2O, 0.02 g of NaMoO4 · 2H2O, 0.02 g of Na2SeO3 · 2H2O, 0.02 g of NiCl2 · 6H2O, and 0.02 g of Na2WO4 · 2H2O. The vitamin solution composition (per liter) was 2 mg of biotin, 2 mg of folic acid, 10 mg of pyridoxine hydrochloride, 5 mg of thiamine HCl, 5 mg of riboflavin, 5 mg of nicotinic acid, 5 mg of calcium pantothenate, 0.1 mg of vitamin B12, 5 mg of p-aminobenzoic acid, and 5 mg of thiotic acid. The medium was prepared anaerobically, and the pH of the medium was adjusted to 5.8 before sterilization. Prior to inoculation, 100 ml of CaGM medium was reduced with 1 ml of reducing agent (1 g of cysteine HCl/100 ml of water) and 1 ml of reducing agent 2 (7.64 g of NTA, 5.33 g of Na2CO3, and 8.5 ml of TICl/100 ml of water).

Cell growth on liquid medium was monitored spectrophotometrically by measuring optical density at 600 nm (OD600). Changes in headspace pressure were measured with a Mosecap pressure gauge (Omega Engineering). For growth of c. autoethanogenum on agar plates, YTF solid medium (10 g/liter fructose, 10 g/liter yeast extract, 16 g/liter tryptone, 0.2 g/liter sodium chloride, 15 g/liter bacteriological agar [Oxoid], pH 5.8), with antibiotics (7.5 µg/ml thiamphenicol, 6 µg/ml clari-thromycin) where appropriate, was used. All mutagenesis work was performed inside an anaerobic workstation at 37°C (Don Whitley Scientific Ltd.). For strain comparisons, three or four biological replicates of WT or recombinant c. autoethanogenum strains were grown in 250-ml serum bottles containing 50 ml of CaGM medium with 10 g/liter fructose, 200 g/liter CO, 10 g/liter fructose plus 200 g/liter CO2, or 130 g/liter K2HPO4 plus 70 K2PO4 as the growth substrate. Incubation at 37°C was done with agitation (225 rpm) inside New Brunswick Innova shakers (Eppendorf). A standardized 0.5-OD600 equivalent of exponentially growing cultures was used as the inoculum.

DNA manipulation. DNA manipulation and cloning were carried out according to standard techniques described by Sambrook and Russell (42). Genomic DNA from c. autoethanogenum was isolated with a DNeasy Blood and Tissue kit (Qiagen) for PCR diagnostics. For Southern blot analysis, genomic DNA of c. autoethanogenum was extracted as described by Bertram and Dürre (43). Plasmid DNA from c. autoethanogenum was isolated with a QIAprep Spin Miniprep kit (Qiagen) with the DNeasy Blood and Tissue kit (Qiagen) for PCR diagnostics. For Southern analysis, genomic DNA from c. autoethanogenum was isolated with a QIAprep Spin Miniprep kit (Qiagen) with the DNeasy Blood and Tissue kit (Qiagen) for PCR diagnostics. For Southern analysis, genomic DNA from c. autoethanogenum was isolated with a QIAprep Spin Miniprep kit (Qiagen) with the DNeasy Blood and Tissue kit (Qiagen) for PCR diagnostics.
DNA polymerase (NEB). For the primers used in this study, see Table S4 in the supplemental material. Primers were designed with Geneious (Biomatters) and synthesized by Sigma-Aldrich or Eurofins. Sanger sequencing of plasmids and amplicons was carried out by Source Bioscience (United Kingdom).

**Plasmid vectors.** All of the plasmids used in this study were derived from the pMTL80000 series of modular *E. coli*–Clostridium shuttle vectors (44) (see Table S4 in the supplemental material). For the construction of plasmid pMTL83151-P_acsa, the promoter region of *C. autoethanogenum* acsa (CAETHG_1621) was amplified with oligonucleotides P_acsa-NotI-F and P_acsa-Ndel-R and then cloned into plasmid pMTL83151 (44) by using the NotI and Ndel restriction sites. To construct the acsa overexpression/complementation plasmid, acsa<sup>full</sup> (CAETHG1620-1621) was first subjected to SOE-PCR (45) with oligonucleotides listed in Table S3 in the supplemental material to remove an internal NdeI site at nucleotide position 342 of CAETHG_1620, resulting in a change in the nucleotide sequence from CATATG to CACATG while retaining the same encoded amino acid sequence. Following cleavage with Ndel and SacI, this ampiclon was cloned into plasmid pMTL83151-P_acsa to generate plasmid pMTL83151-P<sub>acsa</sub>-acsA<sub>full</sub>.

A FLAG tag sequence (encoding the amino acid sequence DYKD-DDDK) was fused to the N or the C terminus of acsa, which was then cloned into plasmid pMTL83151 to generate four plasmid variants to examine the *C. autoethanogenum* acsa translation pattern. The first plasmid, pMTL83151-P_acsa-FLAG-acsa(TGA), has an N-terminally FLAG-tagged acsa gene. It was constructed by initial PCR amplification of native acsa with oligonucleotides Ncol-FLAG-acsa-F and acsa-HindIII-R and cloning of the fragment generated into plasmid pMTL83151 by using the Ncol and HindIII restriction sites. This was followed by PCR amplification of a DNA fragment encompassing the native promoter with primers P_acsa-SacI-F and P_acsa-Ncol-R and its cloning between the SacI and Ncol sites of plasmid pMTL83151. The second plasmid, pMTL83151-P<sub>acsa</sub>-acsA(TGA)-FLAG, was generated by the amplification of acsa and its native promoter with oligonucleotides P_acsa-SacI-F and acsa-FLAG-BamHI-R and cloning of the product into plasmid pMTL83151 by using the SacI and BamHI restriction sites.

The third plasmid, pMTL83151-P<sub>acsa</sub>-acsA(TCA)-FLAG, has the internal TGA stop codon of *C. autoethanogenum* acsa mutated to a TCA serine codon. To assemble this plasmid, SOE-PCR was performed with oligonucleotides P_acsa-SacI-F, acsa(TCA)-SOE-B, acsa(TCA)-SOE-C, and acsa-FLAG-BamHI-R and then the product was cloned into plasmid pMTL83151 by using the SacI and BamHI restriction sites. Similarly, the fourth plasmid, pMTL83151-P<sub>acsa</sub>-acsA(TAA)-FLAG, consists of an acsa variant that has the internal TGA codon mutated to another stop codon, TAA. This plasmid was constructed by first performing SOE-PCR with primers P_acsa-SacI-F, acsa(TAA)-SOE-B, acsa(TAA)-SOE-C, and acsa-FLAG-BamHI-R and then cloning the product into plasmid pMTL83151 by using the SacI and BamHI restriction sites. The cloned promoter and CDS insert in all of the above-described plasmids were verified by Sanger sequencing.

For the construction of ClosTron retargeting plasmids, the appropriate intron–targeting regions within codon1, coo2, and acsa were generated in silico as previously described (18) using a web-based Perutka algorithm (46). DNA 2.0, Inc., then synthesized the 344-bp intron–targeting region and cloned it into ClosTron vector pMTL007C-E2 (18) by using restriction sites HindIII and BsrGI, resulting in plasmids pMTL007C-E2::coo2<sub>1</sub>_601<sub>1</sub>, pMTL007C-E2::coo2<sub>529</sub>, and pMTL007C-E2::acsA<sub>143</sub> (see Table S4 in the supplemental material).

**Plasmid transfer into *C. autoethanogenum*.** Plasmids were transformed into *E. coli* donor strain CA434 and then conjugated into *C. autoethanogenum* by previously established methods (15, 47, 48). Thiamphenicol (7.5 μg/ml) was used to select for cat<sup>T</sup>-based plasmids. Trimethoprim (10 μg/ml) was used to counterselect against *E. coli* strain CA434 after conjugation. For the validation of plasmid overexpression and plasmid complementation strains, plasmids were isolated from *C. autoetha-

The ClosTron KO strain. Clones with multiple ClosTron insertions were omitted from downstream studies. For the complementation of the acsa KO strain, plasmid pMTL83151-P<sub>acsa</sub>-acsA<sub>full</sub> was conjugated into this strain and verified by restriction digestion analysis of rescued plasmids from the transconjugants (data not shown).

**Preparation of crude lysates.** Transformed *E. coli* strain XL1-Blue MR<sup>F</sup> and *C. autoethanogenum* were cultured in 50-ml Falcon tubes inside an anaerobic cabinet. Transformed *E. coli* was cultivated in 50 ml of LB medium supplemented with 20 μM glucose, 10 μM Na<sub>2</sub>SO<sub>4</sub>, and 25 μg/ml chloramphenicol for 23 to 28 h. *C. autoethanogenum* transconjugants were cultivated in 10 ml of YTF medium supplemented with 15 μg/ml thiamphenicol for 44 to 51 h.

Cell pellets were harvested by centrifugation at 4°C for 7,197 × g for 10 min and then resuspended in 300 μl of lysis buffer (30 mM Tris-HCl [pH 7.4], 100 mM NaCl) containing fresh 20 mg/ml chicken lysozyme. Following incubation at 37°C for 45 min, the cell suspensions were sonicated with a Bioruptor Plus (Diagenode) for 60 cycles of 30 s of sonication and 30 s of rest per cycle at 4°C. Following ultracentrifugation at 20,238 × g for 5 min at 4°C, the supernatant was harvested as the soluble fraction, whereas the pellet represented the insoluble fraction and was resuspended in 300 μl of lysis buffer without chicken lysozyme. Protein contents were quantified with Bradford reagent (Sigma-Aldrich) with bovine serum albumin as the standard. Both the soluble and insoluble fractions of crude lysates were stored at −20°C for further analysis.

**Western blot analysis.** Cell lysates and purified proteins were analyzed by sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE). NuPAGE LDS sample buffer (Invitrogen) and 83.3 mM (final concentration) dithiothreitol were added to each sample, and it was boiled at 100°C for 5 min to denature the proteins. The samples and Precision Plus Protein Kaleidoscope Standards (Bio-Rad Laboratories) were then loaded onto 4 to 12% NuPAGE Bis-Tris gels (Invitrogen) in the XCell SureLock Mini-Cell Electrophoresis System (Life Technologies), and NuPAGE MES running buffer (Invitrogen) was added to cover the electrodes. The samples were then subjected to electrophoresis at 150 V for 140 min to separate the proteins, after which the gel was removed from the cast, laid onto Amersham Hybond ECL nitrocellulose membrane (GE Healthcare), and then fitted into an XCell II Blot Module (Life Technologies). To transfer proteins to the membrane, Novex transfer buffer (10% [vol/vol] methanol) was added to the blot module and subjected to electrophoresis at 30 V for 2 h.

Following disassembly of the blot module, the membrane was blocked in 30 ml of TBS buffer (50 mM Tris-HCl, 150 mM NaCl [pH 7.5], 3%
Two-tailed, unpaired, parametric Student’s t tests were employed for comparisons with assistance from A.M.H., F.L., K.W., M.K., A.M.H., and N.P.M. analyzed the results. F.L., M.K., A.M.H., and N.P.M. wrote the manuscript. All of us discussed the results and commented on the manuscript.

REFERENCES


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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi=10.1128/mBio.00427-16/-/DCSupplemental.

Figure S1, TIF file, 1.7 MB.
Figure S2, TIF file, 0.2 MB.
Figure S3, TIF file, 0.1 MB.
Figure S4, TIF file, 0.1 MB.
Figure S5, TIF file, 1.5 MB.
Figure S6, TIF file, 0.9 MB.
Table S1, DOCX file, 0.02 MB.
Table S2, DOCX file, 0.02 MB.
Table S3, DOCX file, 0.02 MB.
Table S4, DOCX file, 0.02 MB.

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N.P.M., S.D.S., K.W., and F.L. conceived the project. F.L., K.W., A.M.H., and M.K. designed the experiments. F.L. performed the experiments with assistance from A.M.H., F.L., K.W., M.K., A.M.H., and N.P.M. analyzed the results. F.L., M.K., A.M.H., and N.P.M. wrote the manuscript. All of us discussed the results and commented on the manuscript.

S. D. S. and N. P. M. performed the targeted mutagenesis with mild agitation. Following three 5-min washes in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% [vol/vol] Tween [pH 7.5]), 4% of 3,3’,5,5'-tetrathoxybenzidine detection substrate (Sigma-Alrich) was added to the membrane, which was incubated at room temperature for 5 min before gentle rinsing with deionized H₂O. The membrane was air dried for 30 min before an image was captured with an EOS 60D DSLR camera (Canon).

Analytical chemistry. Analysis of metabolites was performed with the Varian ProStar HPLC (high-performance liquid chromatography) system equipped with a refractive index detector operated at 30°C and an Aminex HPX-87H column (1,300 by 7.8 mm; particle size, 9 μm; Bio-Rad Laboratories) kept at 30°C. Slightly acidified water was used (0.005 M H₂SO₄) as the mobile phase with a flow rate of 0.5 ml/min. To remove proteins and other cell residues, samples were centrifuged at 20,238 × g for 5 min and the supernatant was filtered with Spartan 13/0.2 filters. Ten microliters of the supernatant was then injected into the HPLC system for analysis. Measurements of headspace gas composition were carried out on a Varian CP-4900 micro gas chromatograph with two installed channels. Channel 1 was a 10-m Mol-sieve column running at 70°C with 200 kPa argon and a backflush time of 4.2 s, while channel 2 was a 10-m PPO column running at 90°C with 150 kPa helium and no backflush. The injector temperature for both channels was 70°C. The run time was set to 120 s, but all of the peaks of interest eluted before 100 s.

Alignment of acaA nucleotide sequences. The nucleotide sequences of acaA from C. autoethanogenum (GenBank accession no. NC_022592), C. ljungdahlii (CP001666), “C. ragsdalei” (HQ876032), C. carboxidivorans P7 (HM590563), C. acetum (CP009687), and C. difficile 630 (NC_000908) were obtained from NCBI. Multiple global sequence alignments with free-end gaps were performed with Geneious (Biomatters) version 6.1.7.

Data analysis and presentations. Statistical analysis was performed and graphically presented results were prepared with GraphPad Prism. Two-tailed, unpaired, parametric Student's t tests were employed for comparisons of means.


