Physiology, genomics, and pathway engineering of an ethanol-tolerant strain of Clostridium phytofermentans

Andrew C. Tolonen¹*, Trevor R. Zuroff²‡, Ramya Mohandass³, Magali Boutard¹, Tristan Cerisy¹, Wayne R. Curtis²

¹Genoscope-CEA, CNRS-UMR8030, Université d’Évry, Évry 91057, France
²Department of Chemical Engineering, The Pennsylvania State University, University Park, PA 16802
³Department of Genetic Engineering, SRM University, Kattankulathur 603203, India
‡ Current address: Biodomain, Shell Technology Center, 3333 Highway 6 South, Houston TX 77082

*These authors contributed equally to this work.

#Corresponding author: atolonen@genoscope.cns.fr

Running Title: C. phytofermentans ethanol tolerance and production
Abstract

Novel processing strategies for hydrolysis and fermentation of lignocellulosic biomass in a single reactor offer large potential cost savings for production of biocommodities and biofuels. One critical challenge is retaining high enzyme production in the presence of elevated product titers. Toward this goal, the cellulolytic, ethanol-producing bacterium Clostridium phytofermentans was adapted to increased ethanol concentrations. The resulting ethanol-tolerant strain (ET strain) has nearly doubled ethanol tolerance relative to wild-type, but also reduced ethanol yield and growth at low ethanol concentrations. The genome of the ET strain has coding changes in proteins involved in membrane biosynthesis, the Rnf complex, cation homeostasis, gene regulation, and ethanol production. In particular, purification of the mutant bi-functional acetaldehyde CoA/alcohol dehydrogenase showed that a G609D variant abolished its activities, including ethanol formation. Heterologous expression of Zymomonas mobilis pyruvate decarboxylase and alcohol dehydrogenase in the ET strain increased cellulose consumption and restored ethanol production, demonstrating how metabolic engineering can be used to overcome disadvantageous mutations incurred during adaptation to ethanol. We discuss how genetic changes in the ET strain reveal novel potential strategies for improving microbial solvent tolerance.

Introduction

The conversion of lignocellulosic biomass to fuels and commodities represents a large-scale, renewable alternative to petroleum. This multi-step bioconversion is traditionally performed in a series of independent processes, but consolidated bioprocessing (CBP) is an alternative paradigm with potential economic advantages (1). In CBP, enzyme production, hydrolysis, and fermentation occur in a single reactor, leading to savings in capital and operating costs as well as increased efficiencies due to system synergies (2). Here we study Clostridium phytofermentans, a promising CBP candidate that ferments plant biomass primarily to ethanol (3, 4). C. phytofermentans hydrolyzes pre-treated corn stover (both glucans and xylans) with similar efficiencies to simultaneous saccharification and co-fermentation (SSCF) using commercial enzymes and xylose-fermenting yeast (5). Fermentation of pre-treated corn stover by C.
phytofermentans reaches a titer of 7 g L⁻¹ ethanol (6) and stable co-cultures of C. phytofermentans and Saccharomyces cerevisiae cdt-1 ferment ~70 g L⁻¹ cellulose to 22 g L⁻¹ ethanol (7), which is an ethanol concentration that reduces C. phytofermentans growth. Thus, application of CBP bacteria such as C. phytofermentans will likely require improving their solvent tolerances, but without compromising enzyme production or fermentation of soluble carbohydrates to ethanol.

Considerable effort has focused on adapting Clostridia to increased solvent levels and investigating the genetic and physiological changes associated with adaptation to solvents (8-13). Other studies have increased ethanol production in Clostridia that primarily ferment to products other than ethanol. C. cellulolyticum expressing pyruvate decarboxylase and alcohol dehydrogenase overcame pyruvate accumulation and shifted fermentation products from lactate to acetate and ethanol (14). In C. thermocellum, redirection of carbon flow through pyruvate kinase (15), inactivation of lactate dehydrogenase and phosphotransacetylase (16), and deletion of hydrogenases (17) all improve ethanol production. These results demonstrate that, although the genetic tools are only now being developed, engineering improved ethanol production is possible in cellulolytic Clostridia. However, development of strains that are both ethanol-tolerant and produce ethanol in high titers remains a significant challenge.

Here we seek to develop a strain of C. phytofermentans with both improved resistance and production of ethanol, particularly from cellulose. We isolated an ethanol-tolerant C. phytofermentans strain (ET strain) by serial transfer into increasing ethanol and characterized its growth and fermentation properties. We sequenced the
ET strain genome to reveal genomic mutations that arose during adaptation and
overcame reduced ethanol yield in the ET strain by heterologous expression of an
alternative ethanol formation pathway. We discuss how this study improves our
understanding of how microbes adapt to elevated concentrations of solvents such as
ethanol.

Materials and Methods

Culturing: *C. phytofermentans* ISDg ATCC 700394 was grown anaerobically by
preparing cultures in a Coy anaerobic chamber with a 1.5% H₂/98.5% N₂ atmosphere.
Cultures were incubated without shaking at 30°C in GS2 medium (18) adjusted to pH=7
supplemented with carbon sources as described in the text. Growth kinetics were
monitored by optical density (OD₆₀₀) in sealed 100-well microtiter plates ( Bioscreen
9502550) as previously described (19); cultures were briefly shaken to resuspend cells
before each optical density measurement. Cellulose, cellobiose, and glucose cultures
for substrate consumption and fermentation product analysis were grown in 100 mL
serum bottles and sealed with butyl rubber stoppers after degassing.

The *C. phytofermentans* ethanol tolerant (ET) strain was selected by serial
transfer (1:50 dilution) into culture tubes containing 10 ml of GS2 medium supplemented
with increasing ethanol concentrations. Starting with cultures in 4% v/v (31.5 g L⁻¹)
ethanol, cultures were transferred weekly to fresh medium containing the same ethanol
concentration and 1% higher ethanol. If no growth was observed at the higher
concentration after 1 week, cultures were re-transferred to the same ethanol
concentrations. If a culture grew at a higher ethanol concentration, this culture was transferred again to that ethanol concentration and 1% greater ethanol. Growth was observed at 5% ethanol after 7 weekly transfers, 6% after 13 transfers, and 7% after 19 transfers. Each time ethanol tolerance was improved, cells were plated, individual colonies were picked, and liquid cultures were re-inoculated to ensure that selection was based on a specific strain with increased ethanol tolerance, not a consortium of strains that collectively survive in increased ethanol. The ET strain is thus a colony-purified isolate from a mother culture that grew in GS2 medium supplemented with 7% (v/v) ethanol. After colony-purification, it was confirmed the ET strain has a similar ethanol resistance to the mother culture.

**Cellulose and fermentation analysis:** Cellulose remaining in culture was measured by taking a 1 mL sample from a 10 mL culture tube with a sterile syringe, placing it in a pre-weighed 1.7 mL micro-centrifuge tube, and centrifuging at 13,000 x g for 10 minutes. The supernatant was removed and the cellulose pellet was washed and centrifuged again at 13,000 x g for 10 minutes. The rinsed pellet was placed at 70˚C to dry until reaching a constant mass. The contribution of cellular biomass to total cellulose weight was not accounted for and assumed to be minimal due to low anaerobic biomass yields.

Fermentation product concentrations were measured in 0.22 μm filtered culture supernatant using an Agilent 1100 HPLC with a Jasco RI-1531 refractive index detector (RID) and an Aminex HPX-87H cation exchange column (BioRad). The HPLC was run using a 0.01 M sulfuric acid (0.01 M) mobile phase, 65˚C column temperature, 30˚C RID.
temperature, 25 µL sample volume, and 0.6 mL/min operating flow rate. Product formation is reported relative to the concentration in the medium at the point of inoculation. Gas phase measurements were made by removing 1 mL of headspace and injecting 100 µL into a gas chromatograph (SRI Instruments, multiple gas analyzer, Model # 8610C). Argon was used as a carrier gas and the pressure was set at 30 psig. A stainless steel molecular sieve 13x and silica gel packed columns were used for sample separation and the components were detected using a TCD detector. The column compartment temperature was held initially at 40°C for 3.5 minutes, then ramped to 160°C for 2 minutes and to 300°C for 10 minutes, after which the column was allowed to cool to 40°C for the remainder of the sample run.

Genome Sequencing and variant analysis of the ET strain: Twelve micrograms of genomic DNA were extracted from a 4 mL ET strain culture using the Sigma GenElute Bacterial Genomic DNA kit (NA2110). DNA was sequenced on an Illumina MiSeq instrument with an insert size of 795 bp and 300 bp paired-end reads. A total of 5,077,282 reads passed quality filtering and mapping by Picard-Tools (https://github.com/broadinstitute/picard), yielding a genome coverage of about 250-fold. Sequence variants (SNPs, indels) in the ET genome relative to the NCBI NC_010001.1 reference strain were identified using the GATK (20) (see supplementary information for detailed filtering and variant calling methods). FASTQ-formatted DNA sequencing files for the ET genome were submitted to the European Nucleotide Archive under Primary Accession PRJEB7255.
ADH purification and activity measurements: The cphy3925 genes from the WT and ET strains were cloned by Ligation-Independent Cloning (21) into pET-22B(+) as previously described (19). Genes were cloned with C-terminal His-tags using primers cphy3925F/R (Table 1) and confirmed by sequencing. Plasmids were transformed into E. coli BL21(DE3) (Novagen 70235), grown in 50 mL TB medium to OD600=1, and expression was induced by adding 500 µM IPTG and incubating overnight at 20°C. Cells were pelleted, resuspended in lysis buffer (50 mM phosphate buffer pH 8, 0.5M NaCl, 10 mM imidazole, 15% glycerol, 1 mM pefabloc (Sigma 76307)), and lysed by sonication (Cole-Parmer Vibracell CV33) with lysozyme (Novagen 71230). His-tagged proteins were purified from 50 mL culture on Ni-NTA spin columns (Qiagen 31014) and visualized on 12% SDS-PAGE gels (Novex 12% Bis-Tris Gel NP0342BOX). Enzyme activities were measured as described in (22) in 100 µL of 100 mM Tris HCl pH 8 containing cofactor (0.2 mM NADH(P)H or 2 mM NAD(P)+) and substrate (300 µM acetyl-CoA or CoA, 18 mM acetaldehyde, 2M ethanol) as appropriate. NAD(P)H was measured as 340 nm absorbance (extinction coefficient 6.22 mM⁻¹·cm⁻¹) using a SAFAS UVmc2 Spectrophotometer at room temperature.

Plasmid construction and transformations: pQexpE is derived from pQexp (23), a plasmid that replicates stably with erythromycin selection in E. coli (200 µg mL⁻¹ erythromycin) and C. phytofermentans (40 µg mL⁻¹ erythromycin on plates and 200 µg mL⁻¹ erythromycin in liquid culture). To construct pQexpE, the Z. mobilis pdc and adhB genes were PCR amplified from pES120 (24) using primers pdcAdhB_F/R (Table 1) and cloned into the unique BamHI and Pvul sites of pQexp. The insert was confirmed
by sequencing using primers pQexp F/R. pQexpE was conjugally transferred to C. phytofermentans using the donor strain E. coli S17-1. Conjugation were performed as described in (23, 25) except polyethersulfone membranes were used to support 50 µL culture mixtures, and only nalidixic acid without trimethoprim was used to select against E. coli following mating. Positive C. phytofermentans transconjugants containing pQexpE were confirmed by colony PCR using primers pQexp F/R to amplify the 2.9 kb pdcl/adhB operon.

Results and Discussion

Isolation and physiology of an ethanol tolerant C. phytofermentans strain: The growth of wild-type (WT) C. phytofermentans was monitored in cultures supplemented with 0, 2, 4, 6, or 7% (v/v) ethanol (Fig 1A) and generation time (hours) and maximum cell densities (OD₆₀₀) were calculated (Table S1). The WT strain grew similarly at 0% ethanol and 2% ethanol, but was significantly inhibited at 4%, and very little growth was observed at 6 and 7% ethanol. In contrast, the ET strain grows at 4, 6, and 7% with a maximum cell density (OD₆₀₀) at 7% ethanol similar to that of the WT strain at 4% ethanol (Fig 1B, Table S1).

While more ethanol resistant, the ET strain grows more slowly relative to WT and to reduced cell yield in cultures without added ethanol (Fig 1). Glucose consumption by ET was also slower than WT, reflecting the reduced growth rate. When grown on 30 g L⁻¹ glucose, the ET strain consumed only 20 g L⁻¹ substrate in 200 hours, while the WT strain completely exhausted the glucose in less than 150 hours (Fig S1). Thus, the
enhanced growth of the ET strain at high ethanol is accompanied by reduced growth under standard conditions, supporting the ET strain has altered physiology, similar to C. thermocellum adapted to 5% ethanol (26, 27). However, we observed no morphological differences between ET and WT colonies grown on GS2 agar plates or cells grown in liquid GS2 medium (Fig S2).

The ET strain also produces less ethanol than WT per unit of sugar consumption. For example, ethanol yield (mol product per mol glucose equivalent consumed) by the ET strain decreased 25-50% relative to WT when growing on cellulose, cellobiose, and glucose (Fig 2A), whereas acetate yield was similar between strains (Fig 2B). We also measured cellobiose consumption and formation of the primary fermentation products ethanol, acetate, and formate in WT and ET cultures grown in cellobiose medium supplemented with 0, 3.75, or 7% ethanol (Fig 2C). Ethanol production by WT was significantly lower at 3.75% ethanol and cellobiose consumption and fermentation ceased at 7% ethanol. In contrast, cellobiose consumption and ethanol production by the ET strain decreased only slightly with increased ethanol supplementation, demonstrating the robustness of the ethanol-resistance phenotype. In all, these results highlight the phenotypic advantages of the ET strain to metabolize and produce ethanol at elevated ethanol concentrations, but at the expense of lower ethanol yields and slower growth at low ambient ethanol.

**Genome sequence of the ET strain:** The *C. phytofermentans* ET strain genome contains 12 variants relative to WT (Table 2), many fewer than a *C. thermocellum* ET genome with similar ethanol resistance that had 200-500 changes (9). While ethanol
214 resistance is likely a complex, multigenic trait, the small number of changes in the ET
215 strain genome shed light on DNA variants that could have functional roles in ethanol
216 tolerance. Two of the 12 mutations were in genes encoding transcriptional regulators
217 that could effectuate broad gene expression changes: PolB, the ß-subunit of DNA-
218 directed RNA polymerase, and a LysR regulator, Cphy3040. LysR-type regulators often
219 co-localize in the genome with their targets (28) and cphy3040 is adjacent to a gene
220 encoding a NAD-dependent aldehyde dehydrogenase, suggesting this regulator is
221 related to alcohol formation.
222 Ethanol increases the permeability of the cell membrane, resulting in toxic
223 leakage of metabolites out of the cell (29). Ethanol resistance thus often involves
224 membrane modifications such as altered protein content (8) or longer chain fatty acids
225 and more plasmalogen lipids (30) that increase membrane rigidity to mitigate the
226 fluidizing effect of ethanol. The ET strain has a D80N change in the putative acyl-
227 acceptor binding pocket (NCBI cd07989) of Cphy0233, a homolog of C. butyricum PlsD
228 that transfers a fatty acyl group to the sn-1 position of glycerol-3-phosphate in
229 phospholipid biosynthesis (31). The D80N Cphy0233 mutation may thus enable
230 synthesis of a more rigid, ethanol-resistant cell membrane by altering which fatty acids
231 are incorporated into phospholipids.
232 The ET strain has a C26S mutation in the RnfA subunit of the membrane-bound
233 Rnf complex that couples efflux of H⁺ (32) or Na⁺ (33) with electron transfer from
234 reduced ferredoxin to NAD⁺ (34). The resulting electrochemical gradient is harnessed
235 by an F0F1 ATPase for ATP synthesis. The C. phytofermentans Rnf complex

10
(Cphy0211-16) and F0F1 ATPase (Cphy3735-42) are highly expressed on all tested carbon sources (19) and may be important for energy conservation, similar to C. ljungdahlii (32). However, Rnf generates NADH, which may not be tolerated by the ET strain that cannot reoxidize NADH by AdhE-mediated ethanol formation (see below). The C26S RnfA variant may thus cripple the Rnf complex, which sacrifices ATP production, but benefit the ET strain by balancing cellular NADH/NAD⁺ ratios.

The ET strain also has mutations in two transporters putatively involved in cation homeostasis. Cphy0543 is homologous to MgtA, a P-type ATPase up-regulated at low ambient Mg²⁺ (35) to mediate Mg²⁺ uptake (36) or Ca²⁺/Mg²⁺ antiport (37). Cphy3778, the membrane component of an ABC transporter (PFAM PF06182), appears to be co-transcribed with Cphy3780, an ABC-type Na⁺ efflux protein (NCBI cd03267). In B. subtilis, this Na⁺ efflux system is induced by ethanol and is proposed to compensate for an influx of extracellular Na⁺ resulting from a weakened membrane barrier (38). The variants in these cation transporters may increase their activities to alleviate cation leakage due to ethanol stress.

**AdhE activities:** The ET strain has a G609D variant in Cphy3925 AdhE, a putative acetaldehyde-CoA dehydrogenase and alcohol dehydrogenase (ADH). The G609D mutation is in a conserved position in the active site of the C-terminal ADH domain (NCBI cd08178). A previous study reported an ethanol-tolerant C. thermocellum with AdhE mutations (P704L and H735R) that shifted cofactor specificity from NADH to NADPH, which was proposed to confer ethanol resistance by altering internal redox balance (9). To determine the effect of the G609D mutation on Cphy3925 enzyme
activity, we purified WT and ET versions of the enzyme (Fig 3A) and tested their in vitro catalysis of the two-step, bidirectional reactions converting acetyl-CoA to ethanol using either NAD or NADPH cofactors.

The mutated Cphy3925 lost NAD(H)-dependent activities, but unlike the mutated AdhE in *C. thermocellum*, the G609D mutation did not acquire NADPH-dependent ADH activity (Fig 3B-E). Instead, our results support the ET strain arrested AdhE-mediated interconversion of acetyl-CoA, acetaldehyde, and ethanol, which helps explain why the *C. phytofermentans* ET strain had lower ethanol yield. AdhE loss-of-function could mitigate ethanol stress by reducing intracellular ethanol and its highly toxic precursor, acetaldehyde. *C. phytofermentans* encodes four Fe-dependent ADH in addition to Cphy3925 as well as a Zn-dependent ADH. All 6 ADH are expressed and Cphy3925 and Cphy1029 are among the most highly expressed proteins on all tested carbon sources (19, 39). *C. phytofermentans* thus likely produces ethanol by the concerted action of multiple ADH and these other ADH, especially Cphy1029, are responsible for ethanol produced by the ET strain.

**Ethanol pathway engineering:** To augment ethanol production by the ET strain, an alternative ethanol production pathway comprised of pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (AdhB) from *Zymomonas mobilis* (Fig 4A) was transferred into *C. phytofermentans* on the replicating plasmid pQexpE (Fig 4B). Together, these enzymes couple decarboxylation of pyruvate to ethanol with the oxidation of NADH, and thus represent an alternative ethanol formation pathway to AdhE. We chose to express
foreign enzymes rather than a WT copy of Cphy3925 because AdhE multimerizes (40) such that the mutant AdhE could have a dominant-negative effect in a merodiploid. Expression of pQexpE increased cellulolysis by ~30% in both WT and ET strains (Fig 5A) and boosted ethanol production by 70% relative to the ET strain (p < 0.01), thereby restoring ethanol yields to WT levels (Fig 5B). CO₂ production increased disproportionately relative to H₂ production in WT and ET strains expressing pQexpE (Table 3). Elevated CO₂ synthesis is likely due to increased pyruvate decarboxylation by the Pdc enzyme. Previous results showed that Pdc/AdhB expression enhanced cellulolysis and ethanol production in WT C. cellulolyticum, which was proposed to result from consumption of excess pyruvate that otherwise leads to metabolic arrest (14). Increased metabolism (cellulolysis and production of CO₂ and ethanol) by C. phytofermentans expressing Pdc/AdhB might be due to alleviation of inhibition by excess pyruvate. Alternatively, expression or activity of glycolytic enzymes might be regulated by NADH levels such that NADH reoxidation by Pdc/AdhB stimulates glycolysis, which results in increased substrate utilization.

Conclusions

In this study, we investigated the genetic basis and phenotypic consequences of microbial ethanol tolerance by isolating, characterizing, and engineering an ethanol resistant strain (ET) of Clostridium phytofermentans. The ET strain grows at higher ethanol concentrations than wild-type (Fig 1) and continues to produce ethanol at 7% ambient ethanol (Fig 2C), but has impaired growth (Fig 1) and ethanol yield (Fig 2A).
relative to wild-type. The genome sequence of the ET strain revealed 12 mutations in genes involved in diverse aspects of metabolism (Table 2) including a G609D variant in the bi-functional acetaldehyde CoA/alcohol dehydrogenase AdhE that abolishes its activity (Fig 3). We complemented the AdhE mutation in the ET strain by expressing pyruvate decarboxylase (Pdc) and alcohol dehydrogenase B (AdhB) from Zymomonas mobilis on the pQexpE plasmid (Fig 4), which boosted substrate conversion (Fig 5A) and restored ethanol production (Fig 5B).

Additional work is needed to enhance plant biomass degradation and ethanol formation rates and product titers by C. phytofermentans. Recently, improvement of C. phytofermentans growth on cellobiose, cellulose, and xylan by experimental evolution yielded strains that also produced ethanol more quickly (41). The genome sequence of the ET strain presented here suggests other novel approaches to potentially improve ethanol resistance and production. For example, our results suggest that further studies on ethanol resistance should focus on PIsD-mediated fatty acid incorporation into phospholipids, LysR-regulated gene expression patterns, overexpression of the Rnf complex to stimulate AdhE-mediated ethanol production, and preventing cation leakage.

Acknowledgements

C. phytofermentans ISDg was kindly provided by Susan Leschine (University of Massachusetts, Amherst). ACT was supported by a CNRS Chaire d’Excellence and the Genoscope-CEA. TRZ was supported by a GRFP Fellowship from the National Science foundation (Grant No. DGE1255832) and a John and Jeannette McWhirter Fellowship.
from Pennsylvania State University. MR was supported by the Faculty Abroad Program, SRM University. Anaerobic growth capabilities for this work were financed by the Genoscope-CEA and a grant from the Department of Energy Advanced Research Project Agency - Energy (ARPA-e, #DE-AR0000092) to WRC. We thank Alex Rajangam for facilitating MR’s international exchange, Sergio Florez for useful feedback, Patrick Hillery and Sam Bjork for experimental assistance, and Nymul Khan for supporting GC analysis. We acknowledge the Huck Institute of the Life Sciences Shared Fermentation Facility for providing HPLC analytical capabilities and the Genoscope-CEA Sequencing Platform for sequencing the ET strain. Finally, we thank George Church and Noel Goddard for facilitating a sabbatical leave for WRC at Harvard where these studies were initiated.

References


Figure Legends
Fig 1 Growth of A wild-type (WT) and B ethanol tolerant (ET) C. phytofermentans strains in at 30ºC in GS2 medium with 3 g L⁻¹ glucose supplemented with ethanol (v/v): 0% (red square), 2% (green circle), 4% (blue triangle), 6% (yellow diamond), or 7% (black X). Data points of growth (OD₆₀₀) are means of triplicate 400 µl cultures in sealed microtiter plates; error bars are one standard deviation.

Fig 2 C. phytofermentans yields (mol product per mol glucose equivalent consumed) of A ethanol by WT (dark red) and ET (light red) strains and B acetate by WT (dark blue) and ET (light blue) strains. Statistical differences between WT and ET yield averages are indicated at p < 0.01 (*), p < 0.05 (**) and p < 0.1 (***) using a Student’s t-test. C C. phytofermentans WT and ET cellobiose consumption and fermentation products in GS2 cellobiose medium with 0, 3.75, or 7% (v/v) added ethanol. Bars show concentrations (mM) of cellobiose consumption (gray) and production of ethanol (red), acetate (blue), and formate (yellow) by WT (dark bars) and ET (light bars) strains. All cultures were grown in serum bottles containing GS2 medium at 30ºC with either 30 g L⁻¹ cellulose, 10 g L⁻¹ cellobiose, or 30 g L⁻¹ glucose and measured after 14 days. Bars are the average of 4 cultures; error bars are one standard deviation.

Fig 3 Comparison of purified Cphy3925 AdhE activities from wild-type and ET strains. A SDS-PAGE gel of purified Cphy3925 from wild-type (WT) and ET strain (G609D) showing single bands of the expected 95 kDa molecular weight. B-E Reactions for the two-step, bidirectional interconversion of acetyl-CoA acetaldehyde, and ethanol: B reduction of 300 µM acetyl-CoA to acetaldehyde (red), C reduction of 18 mM
acetaldehyde to ethanol (green), D oxidation of 2 M ethanol to acetaldehyde (purple),
and E oxidation of 18 mM acetaldehyde to acetyl-CoA (blue). Enzyme activities are mM
NAD(P)H µM enzyme$^{-1}$ second$^{-1}$ measured using NADH(P)H or NAD(P)+ cofactors. Bar
heights are average of duplicate activity measurements and error bars are one standard
deviation.

Fig 4 A Diagram of C. phytofermentans carbon metabolism showing insertion of the Z.
mobilis pdc/adhB alternative ethanol formation pathway. Enzymatic steps: {a} lactate
dehydrogenase (Cphy1117), {b} pyruvate formate lyase (Cphy1174), {c} pyruvate
ferredoxin oxidoreductase (Cphy3558), {d} Rnf ferredoxin:NAD+ oxidoreductase
complex (Cphy0211-6), {e} phosphate acetyl transferase (Cphy1326), {f} acetate kinase
(Cphy1327), {g} acetaldehyde dehydrogenase (Cphy1428 or Cphy3925), {h} alcohol
dercarboxylase (pdc) and {j} alcohol dehydrogenase (adhB). Dashed lines represent
multi-enzyme reactions where all enzymes are not listed. B Plasmid map of pQexpE for
pdc/adhB expression in C. phytofermentans. Plasmid features: gram-negative pUC
origin of replication (oriR pUC), C. phytofermentans pyruvate ferredoxin oxidoreductase
promoter (P3558) to express the Z. mobilis pdc and adhB genes, the RP4 conjugal
origin of transfer (oriT), the gram-negative/gram-positive erythromycin resistance gene
from TN1545 (ermR), the gram-positive pAMB1 origin (oriR pAMB1) and repE protein.

Fig 5 A Cellulose consumption and B ethanol production by non-plasmid bearing (-)
and pdc/adhB containing (+) WT and ET cultures at 30ºC in GS2 medium with 3 g L$^{-1}$ α-
cellulose after 30 days. Bars are the average of duplicate cultures in serum bottles from three independent experiments and error bars are one standard deviation. Statistical differences between treatments are indicated at p < 0.01 (*) and p < 0.05 (**) using a Student’s t-test.

Table legends

**Table 1** Bacterial strains, primers, and plasmids used in this study.

**Table 2** Genomic DNA variants in the ET strain including the gene name, encoded protein length, amino acid (if coding) or DNA variant, confidence value (Q) of the variant call, and annotation of the mutated protein. The probability a variant exists in the genome (p) is reported as a Phred-scaled probability Q=-10 * log_{10}(1-p)), meaning a Q value of a 100 indicates an error probability (1-p) of 10^{-10} (see GATK reports in the Supplementary Text for more information).

**Table 3** Carbon dioxide and hydrogen gas production (mM) by *C. phytofermentans* WT and ET cultures with and without the *Z. mobilis pdc/adhB* genes after 2 weeks growth at 30ºC in GS2 medium containing 100 g L^{-1} α-cellulose. Results are the average of four replicates.
### Bacterial Strains

<table>
<thead>
<tr>
<th>STRAIN NAME</th>
<th>DESCRIPTION</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli Top10</td>
<td>E. coli</td>
<td>Invitrogen Corporation</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>E. coli</td>
<td>Yale E. coli stock center</td>
</tr>
</tbody>
</table>

### Primers

<table>
<thead>
<tr>
<th>PRIMER NAME</th>
<th>DESCRIPTION</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdcAdhB_F</td>
<td>5' - TTTTTCG AATTCACCG G ATCCCTG CAG TAG G AG G AATTAACC- 3'</td>
<td></td>
</tr>
<tr>
<td>pdcAdhB_R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pQexp_F</td>
<td>Forward primer to confirm pdc,adhB insertion in pQexp</td>
<td>5' - AAACCTAGGTAATTGAGGAAAGTTACAATTA- 3'</td>
</tr>
<tr>
<td>pQexp_R</td>
<td>5' - GAATGGCGCCTGATGCG- 3'</td>
<td></td>
</tr>
<tr>
<td>cphy3925F</td>
<td>Forward primer to amplify cphy3925 coding sequence</td>
<td>5' - AAAGAAGGAGATAGGATCATGACGAAGAAAGTGGAATTA- 3'</td>
</tr>
<tr>
<td>cphy3925R</td>
<td>Reverse primer to amplify cphy3925 coding sequence</td>
<td>5' - G TG TAATG G ATAG TG ATCTTAATG G TG ATG G TG ATG TTTACCG TAG TACACTTTAAGATAG- 3'</td>
</tr>
</tbody>
</table>

### Plasmids

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>DESCRIPTION</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pES120</td>
<td>pES120</td>
<td>Jay Keasling Lab. University of California, Berkeley USA</td>
</tr>
<tr>
<td>pQexp</td>
<td>Replicating plasmid for C. phytofermentans</td>
<td>Andrew Tolonen Lab. Genoscope-CEA, Evry France</td>
</tr>
<tr>
<td>pQexpE</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

Source of Z. mobilis pdc,adhB genes

pQexp with Z. mobilis pdc,adhB cloned into the unique BamHI and PvuI sites
<table>
<thead>
<tr>
<th>Protein</th>
<th>Length (amino acids)</th>
<th>Amino Acid Variant</th>
<th>Confidence (G)</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cphy3925 (AdhE)</td>
<td>872</td>
<td>G609D</td>
<td>8929</td>
<td>Fe-dependent bifunctional acetaldehyde-CoA/alcohol dehydrogenase</td>
</tr>
<tr>
<td>Cphy0215 (RhoA)</td>
<td>191</td>
<td>C26S</td>
<td>5669</td>
<td>FMN-dependent nitroreductase</td>
</tr>
<tr>
<td>Cphy3255</td>
<td>229</td>
<td>20 bp insertion at Q91</td>
<td>19500</td>
<td>FMN-dependent nitroreductase</td>
</tr>
<tr>
<td>Cphy0543 (MgtA/B)</td>
<td>920</td>
<td>K417N</td>
<td>8661</td>
<td>P-type ATPase for Mg2+ uptake transporter or Ca2+/Mg2+ antiporter.</td>
</tr>
<tr>
<td>Cphy3778</td>
<td>258</td>
<td>Y229 transalu</td>
<td>1165</td>
<td>Na+ efflux transporter: ABC-2 transmembrane permease (PFAM PF06182)</td>
</tr>
<tr>
<td>Cphy0233 (Pld)</td>
<td>237</td>
<td>D80N</td>
<td>9245</td>
<td>Membrane synthesis: glycerol-3-phosphate acyltransferase. Transfers a fatty acid to the 1 position of glycerol-3-phosphate.</td>
</tr>
<tr>
<td>Cphy1077 (MurC)</td>
<td>489</td>
<td>G115D</td>
<td>8840</td>
<td>Peptidoglycan synthesis: ATP-dependent ligation of L-alanine and UDP-N-acetylmuramic acid to form UDP-N-acetylmuramyl-L-alanine</td>
</tr>
<tr>
<td>Cphy3404</td>
<td>351</td>
<td>D162N</td>
<td>8219</td>
<td>LysR transcriptional activator/repressor</td>
</tr>
<tr>
<td>Cphy3617 (Fub)</td>
<td>1278</td>
<td>D189Y</td>
<td>8624</td>
<td>5S ribosomal RNA-directed RNA polymerase</td>
</tr>
<tr>
<td>Cphy0267</td>
<td>524</td>
<td>G65Q (synonymous)</td>
<td>7470</td>
<td>Modification methyltransferase</td>
</tr>
<tr>
<td>Cphy3036 (ApdE)</td>
<td>581</td>
<td>L244L (synonymous)</td>
<td>9011</td>
<td>Thiamine biosynthesis: membrane-associated lipoprotein</td>
</tr>
</tbody>
</table>

**Energy and Metabolism**

- **Cphy3925 (AdhE) 872 G609D**: Fe-dependent bifunctional acetaldehyde-CoA/alcohol dehydrogenase.
- **Cphy0215 (RhoA) 191 C26S**: FMN-dependent nitroreductase.
- **Cphy3255 229**: 20 bp insertion at Q91.
- **Cphy0543 (MgtA/B) 920 K417N**: P-type ATPase for Mg2+ uptake transporter or Ca2+/Mg2+ antiporter.
- **Cphy3778 258 Y229 transalu**: Na+ efflux transporter: ABC-2 transmembrane permease (PFAM PF06182).

**Transport**

- **Cphy0233 (Pld) 237 D80N**: Membrane synthesis: glycerol-3-phosphate acyltransferase.
- **Cphy1077 (MurC) 489 G115D**: Peptidoglycan synthesis: ATP-dependent ligation of L-alanine and UDP-N-acetylmuramic acid to form UDP-N-acetylmuramyl-L-alanine.
- **Cphy3404 351 D162N**: LysR transcriptional activator/repressor.
- **Cphy3617 (Fub) 1278 D189Y**: 5S ribosomal RNA-directed RNA polymerase.
- **Cphy0267 524 G65Q (synonymous)**: Modification methyltransferase.
- **Cphy3036 (ApdE) 581 L244L (synonymous)**: Thiamine biosynthesis: membrane-associated lipoprotein.

**Membrane and Cell Wall**

- **Cphy0233 (Pld) 237 D80N**: Membrane synthesis: glycerol-3-phosphate acyltransferase.
- **Cphy1077 (MurC) 489 G115D**: Peptidoglycan synthesis: ATP-dependent ligation of L-alanine and UDP-N-acetylmuramic acid to form UDP-N-acetylmuramyl-L-alanine.

**Gene Regulation**

- **Cphy3404 351 D162N**: LysR transcriptional activator/repressor.
- **Cphy3617 (Fub) 1278 D189Y**: 5S ribosomal RNA-directed RNA polymerase.

**Non-Coding Changes**

- **Cphy0267 524 G65Q (synonymous)**: Modification methyltransferase.
- **Cphy3036 (ApdE) 581 L244L (synonymous)**: Thiamine biosynthesis: membrane-associated lipoprotein.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Gas produced (mL)</th>
<th>H₂ (mM)</th>
<th>CO₂ (mM)</th>
<th>H₂/CO₂ (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>26.3±0.4</td>
<td>4.21±0.11</td>
<td>6.26±0.08</td>
<td>0.67</td>
</tr>
<tr>
<td>WT (pdc, adhB)</td>
<td>40.0±5.4</td>
<td>4.62±0.13</td>
<td>10.02±0.07</td>
<td>0.46</td>
</tr>
<tr>
<td>ET</td>
<td>25.2±4.7</td>
<td>3.47±0.12</td>
<td>4.46±0.19</td>
<td>0.78</td>
</tr>
<tr>
<td>ET (pdc, adhB)</td>
<td>31.0±2.3</td>
<td>4.41±0.15</td>
<td>7.20±0.13</td>
<td>0.61</td>
</tr>
</tbody>
</table>