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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Gene replacement and elimination using λRed- and FLP-based tool to re-direct carbon flux in acetogen biocatalyst during continuous CO_2/H_2 blend fermentation

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Abstract A time- and cost-efficient two-step gene elimination procedure was used for acetogen *Clostridium* sp. MT1834 capable of fermenting CO_2/H_2 blend to 245 mM acetate ($p < 0.005$). The first step rendered the targeted gene replacement without affecting the total genome size. We replaced the acetate *pta-ack* cluster with synthetic bifunctional acetaldehyde-alcohol dehydrogenase (*al-adh*). Replacement of *pta-ack* with *al-adh* rendered initiation of 243 mM ethanol accumulation at the expense of acetate production during CO_2/H_2 blend continuous fermentation ($p < 0.005$). At the second step, *al-adh* was eliminated to reduce the genome size. Resulting recombinants accumulated 25 mM mevalonate in fermentation broth ($p < 0.005$). Cell duplication time for recombinants with reduced genome size decreased by 9.5 % compared to *Clostridium* sp. MT1834 strain under the same fermentation conditions suggesting better cell energy pool management in the absence of the *ack-pta* gene cluster in the engineered biocatalyst. If the first gene elimination step was used alone for *spo0A* gene replacement with two copies of synthetic formate dehydrogenase in recombinants with a shortened genome, mevalonate production was replaced with 76.5 mM formate production in a single step continuous CO_2/H_2 blend fermentation ($p < 0.005$) with cell duplication time almost nearing that of the wild strain.

Keywords Acetogens · Continuous fermentation · Gene elimination · Gene replacement · Mevalonate · Formate

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Introduction

Energy production from fossil fuels and manufacture of food and chemicals are associated with multi-step oxidation of organic carbon of carbohydrates to inorganic carbon of carbon dioxide (CO_2) with oxygen as the end electron acceptor. An economic approach to use vent gas of >100 MW power plants utilizing integrated coal gasification combined cycle (IGCC) (waste gas 100 % CO_2) for biocatalysis to carbon of carbohydrates gains strong interest. The CO_2 may be directly biocatalyzed to chemicals and food components without employing the photosynthesis part of the global carbon cycle. Bacteria are known for the highest ratio of cell surface area-to-cell volume [8] with cell duplication time of a few dozen minutes under optimal growth conditions, compared to longer cell division times for higher plants and algae. Also, bacteria-based biocatalysis does not require excessive land reserve, watering, and process-associated manpower and fuel use, thus making clear the benefits of complete commercial biotransformation of CO_2 using bacterial biocatalysts [5, 15, 27].

Inorganic carbon of industrial waste gases has become an attractive raw material for microbial biocatalysis to fuels, chemicals and food components [1, 11–13, 16]. Naturally occurring acetogens reduce inorganic carbon of CO_2 and carbon monoxide (CO) to organic compounds with two or more carbons [1, 12, 13, 16]. Carbonaceous materials for production of synthesis gas (CO, CO_2 and H_2) are coal, acid-hydrolyzed lignocellulosic biomass and natural gas (steam reforming) available at ~$2.50 per 1 GJ in the US and some other countries. The CO and CO_2 can be selectively fermented to specialty, commodity chemicals, food components or fuels using metabolically engineered biocatalysts with high product selectivity [5, 27] as

opposed to chemical syngas catalysis offering low product selectivity. The CO_2-rich syngas or captured CO_2 with added hydrogen can be completely fermented by an engineered biocatalyst with zero CO_2 process emissions to the target product if an inexpensive H_2 source is available (water hydrolysis using electricity produced by the DOE approved high efficiency solar panels in situ) [5, 27].

Mevalonic acid is a key intermediate organic product of the mevalonate pathway in living organisms. The mevalonate market is still small with no added commercial mevalonate production as a chemical or nutrient. However, other components of the mevalonate pathway have an annual market with more than 10 million metric tons annually for isobutene produced from petroleum at a target cost $\sim$0.9 € kg^{-1} [29]. There are reports on engineering of biocatalysts producing terpenoids via synthetic mevalonate pathway from glucose as the source of carbon [31]. Mevalonate is a valuable nutrient for growing microorganisms [22, 28]. Mevalonic lactone was recited as a combustion fuel additive [20]. The importance of this and similar reports on direct inorganic carbon reduction to organic carbon of carbohydrates [3–5, 7, 27] is obvious *in lieu* of the increasing commercial activity associated with proposed manned remote space travel and the potential such technology renders to mitigation of global warming.

Formic acid is used in the manufacture of leather, textiles, pharmaceuticals, rubber, and for preservation of silage as well as along with the use in the processing of agricultural produce with the annual global market of 573 thousand metric tons [14] at the bulk price $\sim$\$750.00–850.00 metric ton^{-1}. Petroleum cracking is the main source for formic acid precursors [8, 19]. The CO_2 as petroleum cracking waste heavily adds to the global warming and climate change.

For some engineered acetogen biocatalyst strains, elimination of the acetate pathway and certain early sporulation genes did not negatively affect performance of the engineered biocatalysts but rather had a positive impact on their performance [3–5, 7, 27]. We optimized a Cre-*lox66*/*lox71*-based gene removal system for use in metabolic engineering of an acetogen-biocatalyst [4]. The system requires custom-synthesized enzyme Cre-recombinase, not always available. It is useful to have an additional gene elimination tool comprising a gene replacement step based on the exact match in sizes of the DNA sequences to exchange. Therefore, we felt that there was a need for a simple time- and cost-efficient gene elimination method, similar to that developed for enterobacteria [10]. As applied to acetogen engineering, such a gene elimination system also requires high efficiency electrotransformation of strict anaerobes [3–5, 7, 27].

A simple two-step λRed and FLP-based gene elimination procedure is reported for acetogen to terminate acetate production during continuous CO_2/H_2 blend fermentation. The construct rendering gene elimination effect comprised a gene suitable for selection of recombinants flanked with FRT sequences and also with fragments of genes just outside of the genes targeted for elimination, long enough to render homologous recombination based on a double crossover of the two DNA strands with matching lengths. Using this approach, the phosphotransacetylase-acetate kinase (*pta-ack*) cluster was replaced in the acetogen strain *Clostridium* sp. MT1834 with synthetic bi-functional acetaldehyde-alcohol dehydrogenase (*al-adh*). Recombinants had stable expression of the synthetic *al-adh* establishing 243 mM ethanol production by the fourth day of continuous CO_2/H_2 blend fermentation. If the *al-adh* was further eliminated via transforming recombinant cells with FLP-recombinase, the duplication time for the recombinant cells decreased by 6 min compared to 63 min for the wild type *Clostridium* sp. MT1834 strain under the same fermentation conditions suggesting improved cell energy balance. Elimination of the acetate-related Acetyl-CoA sink resulted in accumulation of 25 mM mevalonate in the fermentation broth. If early sporulation gene *spo0A* with surrounding sequences was replaced with an equal in size construct comprising two copies of synthetic formate dehydrogenase, the recombinant strain started producing 79.3 mM formate and increased cell duplication time to 62 min.

This is the first report on optimization of λRed and FLP-based gene elimination procedure for engineering acetogen biocatalyst capable of continuous CO_2/H_2 blend fermentation along with decreased by 9.5 % recombinant cell duplication time compared to the wild type acetogen strain suggesting better cell energy pool management in the absence of the *ack-pta* cluster and *spo0A*.

Materials and methods

Bacterial strain and antibiotics

All manipulations with cells, media/solutions, glass- and plastic labware for microbial cultivation were performed under syngas blend (60 % CO + 40 % H_2 v/v) in a customized Anaerobe Chamber AS-580 (Anaerobe Systems, CA) with added section. Neoprene sleeves and gloves were used to maintain anaerobic conditions with <1 ppm of oxygen. The syngas blend composition was elaborated based on reported earlier empirical stoichiometry of the syngas fermentation process using various engineered biocatalysts to prevent CO starving causing quick initiation of a sporulation event [3–5, 7, 27]. A gas blend composed of 20 % CO_2 + 80 % H_2 (v/v) was used in continuous fermentations.

Strain *Clostridium* sp. MT1834 was isolated from oil well flooding water in the Saratov region (Russian Federation) in 2007 using the standard CO-enrichment procedure [23]. The Gram (+) strictly anaerobic catalase (−) spore-forming rod utilizes CO/CO_2 as the carbon source to make acetate only. The purified culture was grown in Syngas Fermentation Broth (SFB) or solidified with 1.2 % Syngas Fermentation Agar (SFA) [23]. Wheaton serum bottles with capped full size butyl rubber stoppers were used for liquid cultures and 100 mm Petri dishes were used for plating. Plates were incubated in vented anaerobic Vacu-Quick Jars (*Almore* International, Beaverton, OR). Syngas in bottles (1.76 kg cm^{-2}) and in jars (0.42 kg cm^{-2}) was exchanged every 6 h to ensure proper microbial feed. Those inoculated in anaerobic chamber bottles were incubated in a IncuShaker 10 l (Southwest Science, Bordentown, NJ, USA). The incubation temperature used was 36 °C.

Recombinant acetogens were grown in SFB or on SFA containing Chloramphenicol (Cm) (Sigma, St. Louis, MO, USA) 35 g ml^{-1} or without the antibiotic. Accumulation of certain recombinant DNA was performed in *E. coli* JM109 (New England BioLabs, Ipswich, MA) using Brain Heart Infusion (Becton–Dickinson, Laurel, MD, USA) in the presence of 40 g ml^{-1} Cm. For detection of acetogen colonies formed by recombinants producing formate only, SFA additionally contained 0.2 mg ml^{-1} bromocresol purple, the pH indicator rendering SFA purple color at neutral pH and turning color of the respective colonies and agar underneath to yellow at pH below 6.5.

Spo (-) phenotype detection

Exposure to 50 % ethanol for 1 h at 37 °C under anaerobic conditions kills spo^- recombinants [2–7, 25–27] in contrast to 100 % survival of the spo^+ parental strain. Validation of the spo^- genotype was performed using RT-PCR [3–5, 7, 27] with respective primers (Table 1).

Table 1 Primers for RT-PCR

Gene	Product size, bp	Forward and reverse primers
ack	385	5′-tgggaactagatgcggtgat-3′; 5′-agtgcctctccccttttctt-3′
pta	355	5′-gggaagtgcaaaacacgaat-3′; 5′-agcttgtgcttgaactgctg-3′
al-adh	394	5′-ataaagccttgacattcttt-3′; 5′-tacgtcaggattattaccaa-3′
fdhλ	921	5′-ctccttctgtagccggtctg-3′; 5′-cccaagacgtggaaggtaaa-3′

Determination of cell duplication time

In an anaerobic chamber, a single 36–48 h old colony with approximately 2×10^5 CFU, was inoculated in 20 ml of SFB in a 100 ml serum bottle, the bottle was capped and pressurized with CO_2/H_2 blend, then incubated in a shaker at 300 r min^{-1} for 17 h and 36 °C outside the anaerobic chamber. The bottle was then placed in an anaerobic chamber, and 1 ml of the culture was taken to prepare and plate decimal dilutions as described [2]. The cell duplication times were calculated for *Clostridium* sp. MT1834 and its recombinants according to the Eq. 1:

$$N = N_0 2^n, \text{ where } N_0 \text{ is the number of cells in the sample at time 0, and } n \text{ is the cell duplication time, min} \quad (1)$$

This equation is based on assumptions that (1) the studied acetogen strains do have simple binary cell division cycle (2) are not triggered to sporulate due to the test performance [2–7, 25–27], and (3) a single cell forms a colony.

N_0 was determined using the sample dilution scheme presented in Fig. 1.

Continuous syngas fermentation

BioFlo 2000 (2.5 l) vertical bioreactors (New Brunswick Scientific, Edison, NJ, USA) with two Rushton impellers 3.5 cm apart and 2.5 cm from the bottom of the vessel were used for single stage continuous syngas fermentation. Ten cm flat disk Pall spargers with pore size 0.5 m (Pall Corporation, Jersey Village, TX, USA) covered the bottoms of each vessel. Sterile filtered syngas blend as above was used in all experiments. The impeller speed was set at 200 RPM. The fermentation broth volume was maintained at 1.8 l at pH 6.4 ± 0.1 adjusting it with 0.1 m filtered pre-reduced 5 M NH_4OH. Each bioreactor with 1.5 l of pre-reduced sterile SFB was purged with syngas for 24 h with the pH and temperature controls set P-I-D until resazurin became colorless indicating anaerobiosis. Each bioreactor was inoculated with 250 ml of overnight seed cultures with OD_{600} 3.65 ± 0.15 either of *Clostridium* sp. MT1834, or its recombinants: *Clostridium* sp. MT EtOH124 $pta^-ack^-al\text{-}adh^+$, *Clostridium* sp. MT563 $pta^-ack^-al\text{-}adh^-$, and *Clostridium* sp. MTFCOOH (fdh^+).

Seed batch cultures were obtained in BioFlo 2000 bioreactors. Inoculated bioreactors for continuous syngas fermentations were kept running with no liquid flow and syngas flow 8 ml min^{-1} until the OD_{600} reached 6.60 ± 0.15. At that point the liquid flow was gradually increased from 0 to 1.5 ml min^{-1} at maintaining the OD_{600} 6.60 ± 0.16 and the gas flow constant for the 25 day long

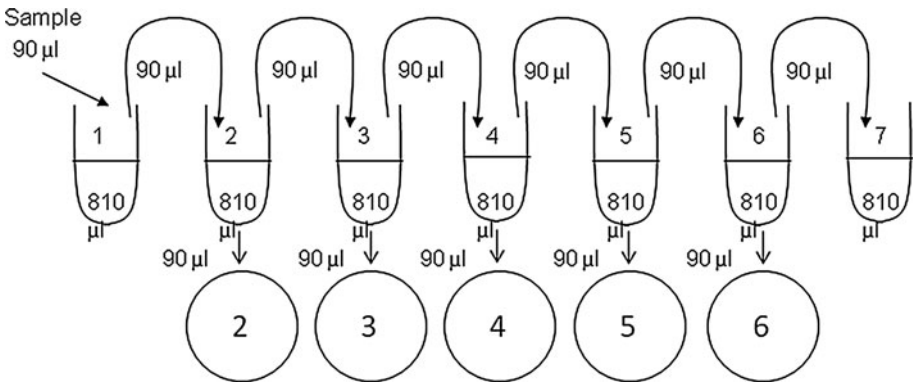

Fig. 1 Decimal dilutions used to determine CFU in the sample (N_0)

runs. Each bioreactor was peristaltic pump fed with sterile pre-reduced SFB from two connected in parallel 38 l vented bottles per each bioreactor. The waste culture broths were gravity flow to two similar sterile bottles kept under strict anaerobiosis. Vents of all bottles were connected to the reservoir with oxygen-free sterile nitrogen connected to nitrogen bottles via an Ultra Precision/Low Flow Regulator QPV (Equilibar, Fletcher, NC, USA) to equilibrate the changes in liquid volumes. Waste culture broths passing to waste culture 38 l bottles through sterile flasks with extra ports were sampled aseptically under anaerobiosis every 15 min (96 data points daily) to monitor the OD_{600}, detection of ethanol, and acetate using HPLC and also for collecting fresh cells for DNA extraction and electrotransformation experiments.

/The vent of each bioreactor was connected via a sterile filter to the carousel mechanism distributing samples from various bioreactors to the Portable NDIR Syngas Analyzer Gasboard 3100P (Wuhan Cubic Optoelectronics CO., Ltd, China) (http://www.gassensor.com.cn/English/Product/108155822.html) via a 6.3 cm copper line. The line and the carousel mechanism were automatically purged with sterile oxygen-free nitrogen after each sampling to prevent cross-contamination during sampling. Vent gas components CO, CO_2 and H_2 were analyzed in real-time every 15 min (96 data points daily) for each bioreactor. The data were collected in five independent 25 day-long continuous syngas or CO_2/H_2 blend fermentation runs for five independent clones of the above strains each and presented based on significance of differences between means.

Electrotransformation hardware, cuvettes, electrodes and electrotransformation

A Benchtop Centrifuge EBA^{TM} 21 (Cardinal Health, OH) was placed inside an anaerobic chamber to concentrate cells under anaerobic conditions from the waste culture broth (see continuous fermentation section) collected aseptically and to preserve anoxic conditions for the samples and the fermentation setups. Samples (10 ml each) were collected to sterile sealed purged with nitrogen, and chilled on ice serum bottles and transferred to an anaerobic chamber. There the cells were transferred to 15 ml disposable polypropylene centrifuge tubes, chilled on ice and concentrated via centrifugation. The resulting pellets were washed once with sterile cold 0.1 M pre-reduced sucrose and resuspended in the same solution to make 0.1 ml from each originally 10 ml sample of the waste culture broth. Syngas Biofuels Energy, Inc. electrotransformation generator [24] (Fig. 2), disposable cuvettes (sterile 0.5 ml PCR tubes imbedded into ice block) and their proprietary nickel-titanium alloy flat parallel electrodes were used for electric treatment with single 6 ms square pulses at 6,250 V with a digital recording of the pulse current as described [2–7, 24–27]. Cell viability was monitored by counting colonies grown on SFA inoculated using aliquots of decimal sample dilutions [25].

DNA isolation

The DNA isolation was performed under anoxic conditions through the clear lysate stage to preserve DNA preps from damage with the products of membrane oxidation [2–7, 25–27] with modifications. Cell collection, cold wash with sterile 0.1 M Sucrose in HPLC-grade water, treatment with 5 mg ml^{-1} lysozyme and SDS lysis were performed in an anaerobic chamber to preserve the DNA preps from the damage with the products of membrane oxidation. All subsequent steps were performed on the bench: the RNA digestion, Proteinase K treatment to decrease the viscosity of the aqueous phase and deactivate endo- and exo-nuclease activities, de-proteinization by Tris-equilibrated (pH 8.0) Phenol/Chloroform/Isoamyl Alcohol mixture (25:24:1 vol/vol/vol) in the presence of 1 M NaCl, and precipitation of DNA with three volumes of ethanol in 0.1 M Sodium Acetate (pH 5.2). All enzymes and chemicals were purchased from Sigma, St. Luis, MO. Upon the extraction, the DNA from *Clostridium* sp. MT1834 and its recombinants was additionally purified with a Qiagen Gel Extraction Kit

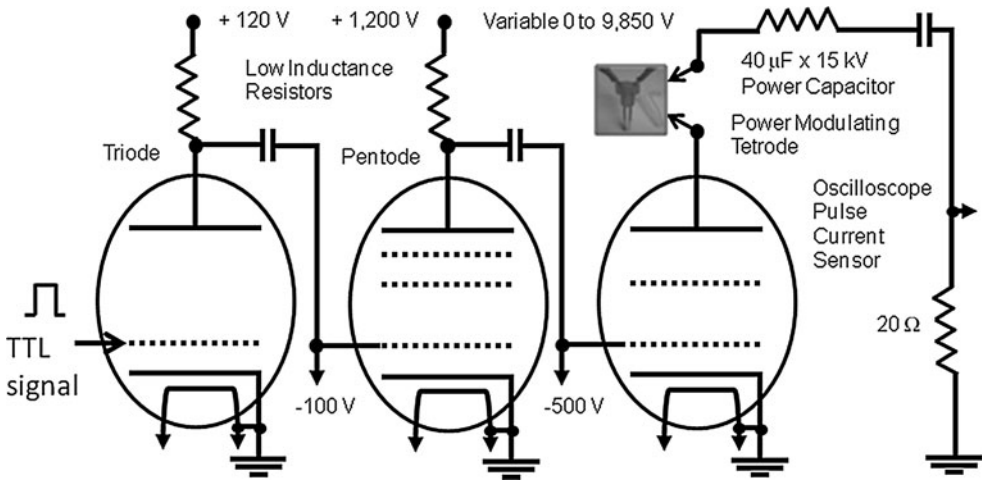

Fig. 2 Hardware used to elecrotransform acetogens (http://www.syngasbiofuelsenergy.com/Patents/RUpatent2005776.pdf)

(Valencia, CA) to decrease degradation with endogenous endo- and exo-nucleases.

Test for ethanol production

Each Petri dish with single colonies was replica-plated using Scienceware® velveteen squares for replica plating (Sigma, St. Louis, MO, USA) to a sterile dish. Dishes with plated replicas were marked to trace the original colonies for subsequent testing using RT-PCR and in fermentation vessels. After replica-plating, each original Petri dish was sprayed with the commercially available reagent for colorimetric ethanol detection ALCO-SCREEN (Chematics, Inc., North Webster, IN, USA). Colonies and agar spots with the colonies producing ethanol stained darker as described [27].

Sequencing

Sequencing of both DNA strands and DNA synthesis including primers, and DNA inserts for the pUC19 backbone were performed by Integrated DNA Technologies, Inc. (San Diego, CA, USA).

Promoter and terminator sequences

Promoter and terminator sequences used in both vectors were identified using Softberry Bacterial Promoter, Operon and Gene Finding tool (http://linux1.softberry.com/). Primers for the synthetic genes used in this project are listed in Table 1.

RT-PCR

Qiagen OneStep RT-PCR Kit and mRNAs isolated from clones of *Clostridium* sp. MT1834 and its recombinants were used as described [3–5, 7, 27]. Forward and reverse primers (Table 1) were used where appropriate.

Integration vector for *pta-ack* elimination in *Clostridium* sp. MT1834

Integration vector pFRT*al-adh* for elimination of *pta-ack* in *Clostridium* sp. MT1834 comprised a cassette inside a multiple cloning site of pUC19 (NEB, Ipschvich, MA, USA) serving as a backbone for DNA accumulation in *E. coli*. The first component of the cassette was the homology region H_1 (region 1375741…1376022 of NC_014328) followed by 34 bp FRT 5′-gaagttcctattctctagaaagtataggaacttc-3′ just upstream of terminator (NC_014328, region 8965…9061), *al-adh* (NC_014328, region 1791269…1793881) and the promoter sequence (region 2156 to 2233 of NC_014328), separated with 5′aaa3′ spacers, followed by 34 bp FRT 5′-gaagttcctattctctagaaagtataggaacttc-3′and then flanked with the homology region H_2 (region 1378844…1379125 of NC_014328).

Replacement of *pta-ack* with *al-adh* for ethanol production and subsequent excision

Cells of *Clostridium* sp. MT1834 were electrotransformed with the mixture of 25 U of λRed recombinase (NEB, Ipschvich, MA) and 10 g of pFRT*al-adh*. Recombinant colonies were of the same color as the selective agar while the wild type (non-transformed) colonies stained yellow due to acetate production causing change of color created by a pH-indicator. In addition, ethanol-producing colonies were confirmed using a rapid test for ethanol production. The *al-adh*$^+$ recombinants, named *Clostridium* sp. MT EtOH124, were confirmed via RT-PCR in addition to

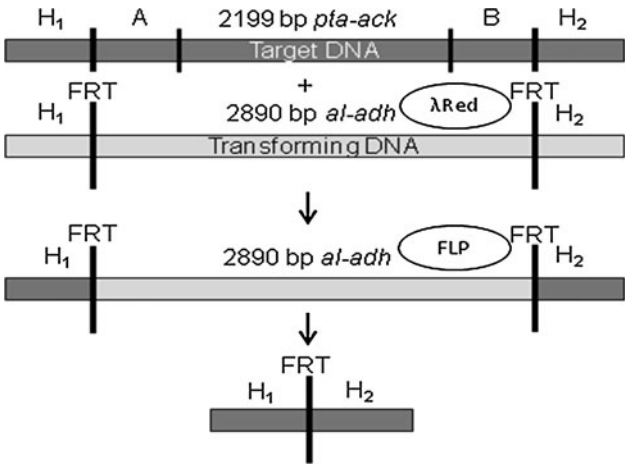

Fig. 3 The algorithm of *pta-ack* replacement with *al-adh* followed then by *al-adh* elimination

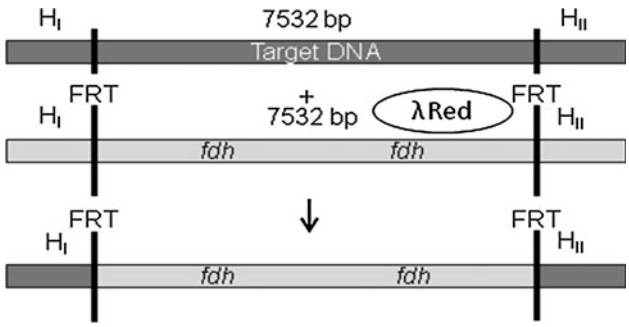

Fig. 4 The algorithm of *spo0A* replacement with two copies of synthetic *fdh*

ethanol detection using primers indicated in the Table 1, and then checked for cell duplication time and in continuous CO_2/H_2 fermentation experiments.

Elimination of *al-adh* and measurement of the cell duplication time

Cells of *Clostridium* sp. MT EtOH124 were electrotransformed with the mixture of 25 U FLP-recombinase (NEB, Ipschvich, MA, USA). Recombinant colonies not producing ethanol were identified using ALCO-SCREEN test combined with replica plating for subsequent clone analysis. Colonies with RT-PCR confirming the absence of *al-adh* were named *Clostridium* sp. MT563 (*al-adh*$^-$), and checked for cell duplication time and in continuous CO_2/H_2 fermentation experiments. The algorithm of *pta-ack* replacement with *al-adh* followed then by *al-adh* elimination is shown in Fig. 3.

Size-matching replacement of cluster comprising sequences flanking *spo0A* via double crossover homologous recombination using two copies of synthetic *fdh* in the presence of λRed recombinase.

Rationale

Recombinant colonies expressing synthetic *fdh* to make formate will change the pH indicator color producing yellow colonies. The algorithm of *spo0A* replacement with two copies of synthetic *fdh* is shown in Fig. 4.

Integration vector pMT*fdh*.2 for elimination of *spo0A* in *Clostridium* sp. MT563 (*al-adh*$^-$) and establishing formate production.

Integration vector pMT*fdh*.2 comprised a cassette inside a multiple cloning site of pUC19 (NEB, Ipschvich, MA, USA) serving as a backbone for DNA accumulation in *E. coli*. The first component of the cassette was the homology region H_I (region 1221894…1222144 of NC_014328) followed by 34 bp FRT 5′-gaagttcctattctctagaaagtataggaacttc-3′ just upstream of the synthetic *fdh*: terminator (NC_014328, region 8965…9061), *fdhλ* (region 777256…779385 of NC_014328), *fdh* subunit D (region 781536…781967 of NC_014328), NADH dehydrogenase I subunit E (*dhIE*) (region 782263…782748 of NC_014328), NADH dehydrogenase I subunit F (*dhIF*) (region 782741…784540 of NC_014328), formylmethanofuran dehydrogenase subunit E (*fmdhE*) (region 996986…997510 of NC_014328) under the promoter sequence (region 2156 to 2233 of NC_014328) (all in duplicate), followed by 34 bp FRT 5′-gaagttcctattctctagaaagtataggaacttc-3′and then flanked with the homology region H_{II} (region 1224525..1224695 of NC_014328), all separated with 5′-aaa-3′ spacers.

The upstream region of the substituted fraction of the *Clostridium* sp. MT563 chromosome comprised a downstream portion of the ORF for the gene sharing 98 % homology with the gene encoding 1-deoxy-D-xylulose-5-phosphate synthase in NC_014328, region 1220447…1222144.

Cells of *Clostridium* sp. MT563 were electrotransformed with the mixture of 25 U of λRed recombinase (custom-made by Fermentas, Vilnius, Lithuania) and 10 g of pMT*fdh*2 using the electrotransformation procedure [2–7, 24–27]. Decimal dilutions of samples were plated on non-selective SFA with pH indicator to obtain single colonies. Colonies with RT-PCR that confirmed the presence of *fdhλ* were named *Clostridium* sp. MTFCOOH (*fdh*$^+$), checked for cell doubling time and in continuous CO_2/H_2 blend fermentation.

HPLC analysis

For HPLC analysis a standard mixture containing formate, acetate, ethanol, acetaldehyde, acetone, acetoacetate, ethyl acetoacetate, butyraldehyde, butyrate, *n*-butanol and

mevalonate was used on an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 55 °C. The mobile phase consisted of the 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1}. Detections were performed via refractive index using a Waters 2414 Infra Red Detector (Milford, MA, USA). The minimal detection level was 0.3 M. The samples were prepared by mixing of 0.5 ml 0.1 m filtered fermentation broth with 0.5 ml 1.76 M H_3PO_3 and filtering again the resulted solution through a 0.1 M filter to HPLC vial immediately prior the HPLC detection procedure.

Statistical analysis

The significance of differences between means for all fermentation experiments was evaluated based on a one-sided *t* test as described by [21]. Daily sampling comprised 96 points for the listed liquid and gas components of the process for each vessel in operation for 25 days of continuous fermentation runs in BioFlo2000 in five independent replicas using syngas or CO_2/H_2 blends.

Results

Wild type *Clostridium* sp. MT1834 produced 245 mM acetate in the continuous CO_2/H_2 fermentation ($p < 0.005$) (Fig. 5) and had cell duplication time 63 min.

Express-detection of eliminated acetate production

Indicator pH added to agar for selection of recombinants helped easy distinguishing between non-transformed and recombinant colonies. There was 100 % correlation between the absence of yellow color and expression of *al-adh* by the recombinant colonies and vice versa for yellow colonies still retaining acetate production similar to that of the wild type strain *Clostridium* sp. MT1834.

Express-detection of ethanol production

Ethanol-containing areas of agar underneath recombinant colonies and ethanol-producing colonies themselves stained darker compared to the agar background and in the areas with colonies with no ethanol production after the plates were sprayed with the reagent for colorimetric ethanol detection ALCO-SCREEN.

Integration of pFRT*al-adh* and expression of *al-adh*

Syngas Biofuels Energy, Inc. generator rendered the frequency of pFRT*al-adh* integration into the chromosome of *Clostridium* sp. MT1834 $8.2 \pm 0.3 \times 10^{-3}$ per the number

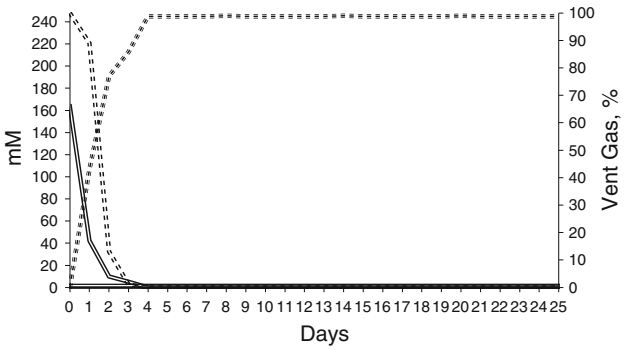

Fig. 5 Continuous CO_2/H_2 fermentation by *Clostridium* sp. MT1834:acetate (*triple dotted line*); vent gases: H_2 (*double dotted line*); CO (*double broken line*) and CO_2 (*double solid line*) ($p < 0.005$)

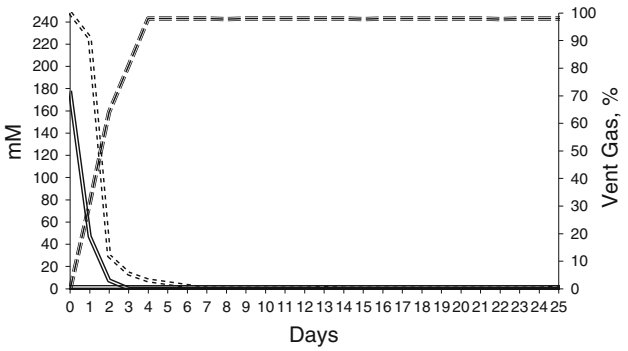

Fig. 6 Continuous CO_2/H_2 fermentation by *Clostridium* sp. MT EtOH124: ethanol (*triple solid line*); went gases: H_2 (*double dotted line*), CO (*double solid line*), CO_2 (*triple solid line*)

of recipient cells. Up to several ethanol-producing colonies were detected on each Petri dish inoculated with the third decimal dilution of the electrotransformed samples. The obtained ethanol-producing recombinants were confirmed for the expression of *pta, ack* and *al-adh* using RT-PCR and primers indicated in Table 1. Clones of *Clostridium* sp. MT EtOH124 $pta^{-}ack^{-}al\text{-}adh^{+}$ checked in continuous CO_2/H_2 blend fermentation established steady production of 243 mM ethanol at the expense of acetic acid on the fourth day ($p < 0.005$) (Fig. 6), and had cell duplication time of the wild type strain *Clostridium* sp. MT1834.

Elimination of *al-adh* and its effect on the cell duplication time

Clones of *Clostridium* sp. MT563 $pta^{-}ack^{-}al\text{-}adh^{-}$ that resulted from electrotransformation experiments with 25 U FLP-recombinase were detected using the ALCO-SCREEN test as not producing ethanol. Such clones were found at a frequency $1.2 \pm 0.06 \times 10^{-3}$ per the number of recipient cells. The RT-PCR confirmed the absence of expression of *pta*, *ack* and *al-adh* in clones of *Clostridium*

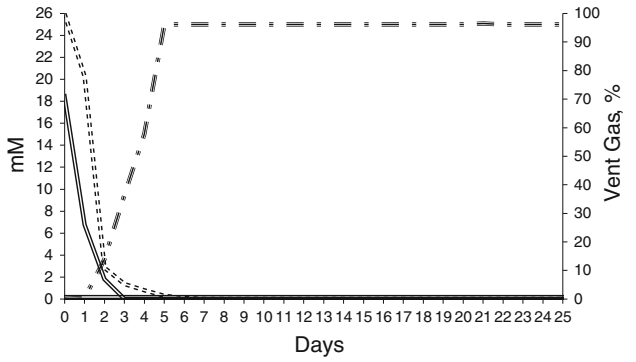

Fig. 7 Continuous fermentation of CO_2/H_2 blend by *Clostridium* sp. MT563 (*al-adh*$^-$): Mevalonate (*triple dash-dotted line*); went gases: H_2 (*double dotted line*), CO (*double solid line*), CO_2 (*triple solid line*)

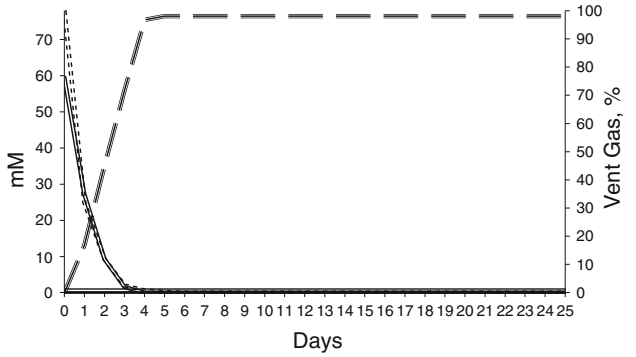

Fig. 8 Continuous fermentation of CO_2/H_2 blend by *Clostridium* sp. MTFCOOH (*fdh*$^+$): formate (*long dashed triple line*); went gases: H_2 (*double dotted line*), CO (*double solid line*), CO_2 (*triple solid line*)

sp. MT563. Continuous CO_2/H_2 fermentation experiments revealed establishing of the stable gas blend fermentation profile after 83 h past inoculation thus confirming shortening of the cell duplication time by 6 min (57 min) as compared to the wild type strain *Clostridium* sp. MT1834. *Clostridium* sp. MT563 *pta*$^-$*ack*$^-$*al-adh*$^-$ started accumulating 25 mM mevalonic acid in fermentation broth ($p < 0.005$) (Fig. 7).

Replacement of *spo0A* with synthetic *fdh* for formate production

Vector pMT*fdh*2 was integrated into the chromosome of *Clostridium* sp. MT MT563 at a frequency of $6.3 \pm 0.2 \times 10^{-3}$ per the number of recipient cells. The ease of recombinant clones detection was rendered by the same agar color change phenomenon provided by adding to the agar pH indicator. Formate producing colonies were yellow compared to colonies formed by non-transformed cells. All yellow colonies were confirmed for the expression of *fdh*λ using RT-PCR. Recombinant clones, *Clostridium* sp. MTFCOOH (*fdh*$^+$), revealed cell duplication time 62 min and demonstrated production of 76.5 mM formate ($p < 0.005$) in a single step continuous CO_2/H_2 blend fermentation established on the fifth day (Fig. 8).

Discussion

Design and integration of new pathways in microbial biocatalysts is a routine practice nowadays. However, not much had been done in the research on how to power new pathways. There are a few reports on gene elimination in acetogens which has rendered a fraction of cell energy pool to synthetic pathways [3–5, 7, 27]. It turned out that elimination of acetate production along with respective genes did not have much negative effect on the engineered biocatalyst performance. Elimination of the acetate pathway in the strains of acetogens we have studied, therefore, was most likely compensated by some other energy conservation mechanisms. This correlates well with the fundamental review highlighting energy conservation mechanisms in acetogenic bacteria [30]: ATP produced by oxidation of acetaldehyde to acetate is used by the methyl branch of the Acetyl-CoA pathway with resulting ATP balance zero. It appears that elimination of *pta-ack* in *Clostridium* sp. MT1834 did not lead to shortage of a cell energy pool to power ethanol production at the expense of eliminating acetate production.

There is another common misconception related to acetogens. It is associated with the so-called gas–water mass transfer barrier for syngas components believed as limiting the efficiency of syngas fermentation by acetogens [18]. If that was so, then how to explain that even in continuous CO_2/H_2 blend fermentation experiments, DCW was >2 g l^{-1} at H_2 and CO solubility 1 and 10 mg l^{-1}, respectively [6]? We suspect that gaseous substrates might be consumed directly by the biomass bypassing the water phase barrier when certain cell densities were reached at the proper bioreactor feed with microscopic gas blend bubbles. We believe that gas consumption by biocatalyst at its certain density when the process of continuous gas blend fermentation is stable, directly depends on CO/CO_2 and H_2 diffusion via lipid components of the cell surface under the right engineering design of the fermentation vessels.

What was surprising was that, when the acetate pathway (and then the synthetic ethanol pathway) was eliminated, the released Acetyl-CoA caused overproduction of mevalonic acid found in the fermentation broth (Fig. 5). The data suggests that the natural mevalonate pathway had a certain flexibility in performance and responded to the Acetyl-CoA fraction in excess, no longer used for acetate or ethanol production by the particular acetogen strain studied.

When synthetic formate dehydrogenase was integrated to replace *spo0A* and the surrounding portion of the genome, in addition to elimination of *pta-ack*, the fraction of the cell energy previously used to maintain replication of these genes was released. Such energy fraction was sufficient enough to power formate accumulation in fermentation broth (Fig. 6).

It was concluded that elimination of the *pta-ack* cluster did not negatively affect energy balance in the acetogen strain studied. In this report, elimination of acetate and acetaldehyde production via elimination of the *pta-ack* gene cluster shortened recombinant cell duplication time by 9.5 % suggesting improved cell energy pool balance. The data is in agreement with the earlier reported exact role of the acetate pathway in energy preservation in acetogens: each mol of ATP formed in the acetate pathway is then spent in the methyl branch [30]. This is the avenue to improve robustness of the acetogen-based biocatalysts for a number of applications. The approach of improving cell energy balance via elimination of unnecessary for the particular process genes is not new. Much has been done in shortening the genome of enterobacteria [9, 17]. This report is new as related to acetogen genome shortening and is associated with the powering of formate production. As in other reports on metabolic engineering of acetogens [2–7, 24–27], the only efficient electrotrasnformation was achieved with the use of the Syngas Biofuels Energy, Inc. electroporation generator, capable of driving cell samples with high internal electric capacitance to the state of cell membrane electric breakdown [24]. The importance of this report is also emphasized by the potential to use the acetogen route to make carbohydrates from CO_2 directly, thus mitigating global warming and climate change proportional to the commercial scale since bacteria render the best known surface to volume ratio having the path for gases (in) and the gas fermentation products (out) as short as possible [32].

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