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CRISPR/Cas9-based efficient genome editing in \textit{Clostridium ljungdahlii}, an autotrophic gas-fermenting bacterium

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Author Contributions

$^†$H.H. and C.C. contributed equally to this work. H.H, C.C and N.L. performed the experiments. Y.G, H.H and N.P.M. designed the experiments and wrote the manuscript. P.R., S.Y. and W.J. assisted in experimental design and data analysis.
ABSTRACT:

Acetogenic bacteria have the potential to convert single carbon gases (CO and CO$_2$) into a range of bulk chemicals and fuels. Realization of their full potential is being impeded by the absence of effective genetic tools for high throughput genome modification. Here we report the development of a highly efficient CRISPR/Cas9 system for rapid genome editing of *Clostridium ljungdahlii*, a paradigm for the commercial production of ethanol from synthesis gas. Following the experimental selection of two promoters (P$_{thl}$ and P$_{araE}$) for expression of *cas9* and the requisite single guide RNA (sgRNA), the efficiency of system was tested by making precise deletions of four genes, *pta*, *adhE1*, *ctf* and *pyrE*. Deletion efficiencies were 100%, >75%, 100% and >50%, respectively. The system overcomes the deficiencies of currently available tools (more rapid, no added antibiotic resistance gene, scar-less and minimal polar effects) and will find utility in other acetogens, including pathogen *Clostridium difficile*.

KEYWORDS: CRISPR/Cas9, rapid genome editing, strong promoters, *Clostridium ljungdahlii*, autotrophic bacterium, gas fermentation
The use of fossil fuels is no longer tenable. A finite resource, their use is damaging the environment through pollution and global warming. Alternative, environmentally friendly sources of chemicals and fuels are required. To date the focus has been on using lignocellulose as a feedstock for microbial fermentation. However, its recalcitrance to deconstruction is making the development of economic processes extremely challenging. One alternative is to directly capture carbon before incorporation into lignocellulosic biomass. Acetogenic bacteria, typified by *Clostridium ljungdahlii*, are able to capture carbon (CO or CO$_2$) through the Wood-ljungdahlii pathway, allowing them to grow on a spectrum of waste gases from industry (e.g., steel manufacture and oil refining, coal and natural gas) to produce ethanol (1-3). They can also consume ‘synthesis gas’ (mixtures of CO, CO$_2$ and H$_2$) made from the gasification of renewable resources, such as biomass in the form of domestic or agricultural waste. Acetogenic gas fermentation can, therefore, produce ethanol in any geographic region without competing for food or land resources (1-4).

The real potential of acetogens, however, resides in their capacity to produce chemicals and fuels other than ethanol (5). This will require the redesign and implementation of more efficient metabolic pathways, adapting them to high performing manufacturing processes. The first tentative steps towards such goals have been made in recent years through the development and implementation of classical tools for the modification of acetogens such as *Clostridium ljungdahlii* and the closely related *Clostridium autoethanogenum* (5-9). The tools used to date, however, have been limited to either the introduction of autonomous plasmids encoding selected
enzymes involved in product formation (5), the generation of insertional mutants
using ClosTron mutagenesis (6) or the insertion of antibiotic resistance genes by
homologous recombination (8) and the single crossover integration of suicide
plasmids (9). In other work, a butyrate production from C. acetobutylicum was
integrated into the C. ljungdahlii genome via homologous recombination, and
thereafter modified by excision of the catP antibiotic resistance marker used for
selection of the integrated DNA. The latter was achieved through the action of an
introduced heterologous cre recombinase on loxP target sites that flanked the catP
gene (7). Despite this progress, the developed methods and tools are in the main
cumbersome and time consuming, being reliant on a number of labor intensive
screening steps to identified the desired cell lines. Moreover, their deployment results
in mutants with properties that are less than ideal. Thus, those mutants generate by
single crossover integration of plasmids can revert through plasmid excision as a
consequence of recombination between the duplicated regions of homology that
mediated plasmid integration (9). On the other hand, the presence of inserted DNA in
those mutants made using the ClosTron (6) or via double crossover integration of
mutant alleles carrying antibiotic resistance genes (8), generate mutants that are not
immune from polar effects on downstream genes. Moreover, even in those instances
where the selectable marker is removed using Cre-LoxP technology, the final strain
inevitably carries a scar in the chromosome (7).

CRISPRs (clustered regularly interspaced short palindromic repeats) function as a
prokaryotic acquired immune system, conferring resistance to exogenous genetic
elements such as plasmids and phages \((10)\). In type I and III systems, the pre-crRNA transcript is cleaved within the repeats by CRISPR-associated ribonucleases, releasing multiple small crRNAs. In type II systems, crRNA-tracrRNA hybrids complex with Cas9 to mediate interference. The CRISPR nuclease Cas9 is targeted by a short guide RNA that recognizes the target DNA via Watson-Crick base pairing. The guide sequence within these CRISPR RNAs can be easily replaced by a sequence of interest to retarget the Cas9 nuclease to a gene of choice. In recent years, the CRISPR-Cas9 system has been developed into a powerful tool for high-efficiency genome editing \((10, 11)\), by using a Cas9 nuclease, CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), which work together to bind and cut at any target DNA site \((12-14)\), or a simplified system, in which crRNA and tracrRNA were reprogrammed to yield a chimeric single guide RNA (sgRNA) that was more easily designed and manipulated \((12, 15, 16)\). Recently, this powerful genetic tool has been harnessed for genome editing in two industrially relevant clostridial species, *Clostridium cellulolyticum* and *Clostridium beijerinckii* \((17, 18)\). The former is a cellulolytic bacterium that is capable of directly converting lignocellulosic biomass into chemical commodities \((19, 20)\), while the latter is highly effective at fermenting biomass-derived hydrolysates \((21-23)\). Here we sought to establish a similar system in *C. ljungdahlii*, a paradigm for clostridial acetogens, based on a CRISPR/Cas9-based editing system developed in our laboratory for use in *E.coli* \((24)\).

As shown in Figures 1A and B, we constructed a plasmid that contained a sgRNA expression cassette and the *Streptococcus pyogenes* cas9, together with the requisite *C.*
ljungdahlii-derived DNA required to mediate repair of the CRISPR/Cas9 catalyzed double strand break. For effective operation of the envisaged system, it is crucial that the incorporated CRISPR/Cas9 elements are well expressed in C. ljungdahlii under the growth conditions to be used. Accordingly, a number of heterologous promoters from C. acetobutylicum were cloned upstream of a promoter-less lacZ reporter gene, introduced into C. ljungdahlii on an autonomous plasmid, and the relative production of β-galactosidase was assayed at 24, 48 and 72 h time points during heterotrophic growth on fructose as the carbon source (Figure 2). Promoters from C. ljungdahlii were deliberately avoided to rule out unintended homologous recombination between the final plasmid and the chromosome. The promoters tested were the P araE promoter of the C. acetobutylicum gene CA_C1339, together with the promoters of several solventogenic genes that had previously been characterized, namely, P thi (25, 26), P pthl (25, 26), and P adc (26). The data obtained demonstrated that P thi and P araE promoters exhibited much greater activity at all three time points than P pthl and P adc. The P thi and P araE promoters were, therefore, selected to mediate expression of Cas9 and sgRNA, respectively, in C. ljungdahlii.

Having established the basic components of the C. ljungdahlii CRISPR/Cas9 system, a plasmid (pMTLcas-pta, Figure 1A) capable of targeting the pta (CLJU_c12770) gene encoding phosphotransacetylase was assembled and transferred into C. ljungdahlii by electroporation. In parallel, two control plasmids, pMTLcase (lacking the pta homology arms) and pMTLHa (carrying the pta homology arms, but lacking Cas9 and sgRNA), were also electroporated into C. ljungdahlii (Table S2).
Transformed cells were plated on YTF (27) solidified media supplemented with 5 µg/mL thiamphenicol and those colonies visible after 3 days of incubation subjected to PCR screening in order to detect the desired double-crossover genotype. The primers were designed such that a 3,313-bp PCR amplified fragment would be generated from the wild-type pta allele, whereas the mutant pta allele would generate a 2,300-bp DNA fragment. Of the 8 and 10 colonies obtained in two independent experiments (Table 1) evidence for the presence of the desired pta deletion was obtained in all cases. In contrast, no pta-deletion events were observed in the electro-transformants that carried the control plasmids, indicating that the generation of mutants was specific to the presence of a fully functional CRISPR/Cas9 system. In a significant number of cases, however, the PCR screening suggested that the colonies were composed of both mutant and wild-type populations, as evidenced by the presence of both the 2,300-bp and 3,313-bp PCR-amplified DNA fragments (Table 1 and Figure 1C). These data may suggest that, at this stage in the development of the system and protocol, that complete cleavage of the target site by the Cas9/sgRNA complex is not occurring. More importantly, however, several colonies represented pure, clonal populations of the desired mutant, as evidenced by the generation of only a 2,300-bp PCR-amplified DNA fragment, and the complete absence of the 3,313-bp DNA fragment associated with the wild-type genotype (Figure 1C). The 2,300-bp PCR-amplified DNA fragment was also extracted and sequenced to further confirm the desired deletion (Fig. S1 in supplementary data). These data show the desired deletion mutant can be isolated in a single step, requiring no further restreaking and
Phenotypic characterization of a \textit{pta} mutant revealed that the production of acetic acid had been substantially reduced compared to the wild-type strain (Figure 3). This is consistent with the central role of phosphotransacetylase in the acetate acid-forming pathway (28). It was also notable that the growth rate of the mutants was significantly impaired in comparison to the wild-type (Figure 3). The \textit{pta}-dependent acetic acid-forming pathway represents a major ATP donor in \textit{C. ljungdahlii} during syngas fermentation (5). Impairment of growth is, therefore, most likely a consequence of the reduction in the cells ability to generate ATP.

To further exemplify the system, three further \textit{C. ljungdahlii} genes were targeted. These were, \textit{adhE1} (CLJU\_c16510, encoding a bifunctional aldehyde/alcohol dehydrogenase), \textit{ctf} (CLJU\_c39430, which encodes acyl-CoA transferase) and \textit{pyrE} (CLJU\_c35680, which encodes orotate phosphoribosyltransferase). In every instance, evidence for the presence of mutant populations in some, or all, of the transformant colonies was obtained by PCR screening. In contrast to the result with \textit{pta}, some of the colonies, although a minority, appeared to be composed of entirely wild-type cells. Nevertheless, the majority of the colonies were composed of either mutant or a mixture of mutant and wild-type populations. In the case of \textit{ctf}, all (21 in total) of the colonies screened were composed of mutant and wild-type cells. In the case of \textit{pyrE}, the majority (15 out of 22) comprised mutant and wild-type cells, with one pure mutant and 6 wild-type colonies. In contrast, in the case of \textit{adhE1}, no mixed colonies were evident. Rather the transformant colonies comprised 14 deletion mutants and 4
wild-type cell lines.

To address the issue of mixed populations, colonies carrying both mutant and wild-type cells from the ctf and pyrE deletion experiments were resuspended in 20 µL liquid YTF medium (supplemented with 5 µg/mL thiamphenicol) and re-streaked onto YTF agar plates (supplemented with 5 µg/mL thiamphenicol). Following two days of incubation, 15 well isolated individual colonies from either the ctf or pyrE mixed mutant cell lines, respectively, were selected for PCR screening. In all cases, a single PCR-amplified DNA fragment was obtained consistent with the mutant allele (Table 2). These data indicate that, at least in these instances, pure mutant populations can easily be isolated from primary colonies composed of mixed mutant and wild-type alleles by one-step subculturing. This may suggest that the prolonged incubation of cells in the continued presence of the active, targeted Cas9/sgRNA plasmid leads to total conversion of cells to mutants. Alternatively, ctf and pyrE mutants may possess a growth advantage over the wild-type cells, leading to their predominance as well isolated colonies. All these three deletion events were further confirmed by sequencing (Fig. S1 in supplementary data).

Phenotypic characterization of an adhE1 mutant revealed that the production of ethanol had been significantly reduced compared to the wild-type strain (Figure 4A). This is consistent with the essential role of bifunctional aldehyde/alcohol dehydrogenase in the ethanol-forming pathway (28). The ctf mutant M-ctf also showed impaired growth compared to the wild-type strain, leading to decreased production of acetic acid and ethanol (Figure 4B). The phenotypic effect of deleting
ctf is in agreement with the previously reported result, in which ctf was inactivate by single-crossover homologous recombination (7). In addition, the deletion of pyrE resulted in the desired FOA-resistant cells, which can grow on solidified medium supplemented with FOA (Figure 4C).

In summary, here we report the first demonstration of CRISPR/Cas9 genome editing in an industrially important clostridial acetogen, C. ljungdahlii. This genetic tool facilitates rapid and precise chromosomal manipulation in C. ljungdahlii and overcomes intrinsic limitations in those methods described to date for constructing mutant strains for industrial applications. Mutants made are scar-less and devoid of undesirable antibiotic resistance markers, an essential feature of chassis destined for process scale-up. The system should prove applicable to other autotrophic Clostridium species for efficient genome editing, both industrially important (e.g., C. autoethanogenum) and pathogens (e.g., Clostridium difficile). More importantly, our systems will form the basis of more elaborate implementations of Synthetic Biology approaches in acetogens, such as gene regulation, elucidating gene function, pathway engineering and more sophisticated cell factory designs.
METHODS

Strains, Media, and Reagents

The *E.coli* host strain DH5α *E. coli* strain was used for plasmid cloning and maintenance. It was grown in LB medium supplemented with chloramphenicol (12.5 µg/mL) when needed. The *C. ljungdahlii* strain used was DSM 13528. It was grown in YTF medium (27) or a modified ATCC medium 1754 (2-fold increased concentration of all the mineral elements; fructose was removed) with a headspace of CO\textsubscript{2}-H\textsubscript{2}-N\textsubscript{2} (56%/20%/9%/15%; pressurized to 0.2 MPa). In addition, 5 µg/mL of thiamphenicol (Wako Pure Chemical Industries, Osaka, Japan) was supplemented as needed for plasmid selection. KOD plus Neo and KOD FX DNA polymerase (Toyobo, Osaka, Japan) was used for high fidelity DNA amplification and PCR screening of the desired genotypes of *C. ljungdahlii*. The oligonucleotide primers used were synthesized by GenScript (Nanjing, China). Plasmids were constructed using conventional restriction digestion enzymes and ligase purchased from Thermo Fisher Scientific (USA) and Takara (Dalian, China), respectively, or assembled through the ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China). Plasmid isolation and DNA purification were performed with kits purchased from Axygen (Hangzhou, China).

Plasmid Construction

The CRISPR/Cas9 editing plasmid pMTLcas-pta was assembled from the following fragments: linear plasmid pMTL83151 (29) obtained by double digestion
with SauI and XhoI; the native cas9 of Streptococcus pyogenes was obtained by PCR using the primers cas9fw/cas9rw and pCas as the template; the P_{thl} and P_{aroE} promoters were obtained from Clostridium acetobutylicum using the primers pthlrw/pthlfw and paraEfw/paraErw, respectively; sgRNA (which targets the 20-nt target spacer of the pta gene) was obtained by PCR using the primers ptagRNAs/gRNAaster and pTargetF as template; the two homologous arms (HAs) that flank the open reading frame of pta were amplified from C. acetobutylicum genomic DNA using the primers ptauas/ptaus and ptads/ptadas.

Here, every 20-nt target sequence (5'-N_{20}NGG-3', where N represents any nucleotide) chosen for sgRNA targeting was examined in advance by alignment search (NCBI BLAST), aiming to ensure no sequence with high nucleotide acid sequence similarity in the genome and thus avoid the potential “off-target” in gene deletion. Then, the sgRNA scaffold and P_{aroE} promoter were assembled by an overlap extension PCR, which resulted in the P_{aroE}-sgRNA fragment. This fragment was then assembled with the above HAs fragments by a second round of overlap extension PCR, which yielded the fragment P_{aroE}-sgRNA-HA. Thereafter, the linear pMTL83151 vector, and DNA fragments encompassing P_{thl}, cas9, and P_{aroE}-sgRNA-HA were assembled to give the CRISPR/Cas9 editing plasmid pMTLcas-pta using the ClonExpress MultiS One Step Cloning Kit (Fig. 1A). When preparing the other three plasmids for the adhE1, ctf and pyrE deletion, the pMTLcas-pta plasmid was used as the template, and the sgRNA and homologous arms cassette were changed accordingly.
To construct the pMTLcasc control plasmid ($\text{cas9} + \text{sgRNA}$, without HAs), the pMTLcas-pta plasmid was digested with $XbaI$ and $XhoI$ to remove the original sgRNA and HAs. The resulting linear vector was then assembled (using the ClonExpress MultiS One Step Cloning Kit) with a single $\text{pta}$-specific sgRNA fragment that was PCR-amplified from the pTargetF template using the primers $\text{ptagRNAs/gRNAasre}$. Similarly, the other control plasmid pMTLHa (HAs, without $\text{cas9}$ and sgRNA) was obtained by removing $\text{cas9}$ and sgRNA from pMTLcas-pta by $\text{NotI-SalI}$ double digestion, followed by blunt-ending and self-ligation.

The reporter plasmids were constructed as follows: the chloramphenicol acetyltransferase gene ($\text{catP}$) was amplified from the pMTL83151 plasmid with the $\text{catP-Sacl-For/catP-Clai-Rev}$ primers and digested with $\text{SacI/Clai}$. The cleaved fragment was then ligated with pXY1 plasmid digested with the same restriction enzymes, to yield the plasmid pLXY1. Next, the $\text{lacZ}$ reporter gene was excised from pIMP1-Pthl-LacZ by $\text{BamHI-SmaI}$ digestion and ligated with the pLXY1 plasmid digested with the same restriction enzymes, to give the plasmid pLXY1-Pthl-LacZ.

Subsequently, the $\text{P}_{\text{ptb}}$, $\text{P}_{\text{adc}}$, $\text{P}_{\text{araE}}$ promoters were PCR-amplified using genomic DNA of $C. \text{acetobutylicum}$ ATCC 824 as template and $\text{Pptb-Pstl-For/Pptb-BamHI-Rev}$, $\text{Padc-Pstl-For/Padc-BamHI-Rev}$, and $\text{ParaE-Pstl-For/ParaE-BamHI-Rev}$ as primers. This reaction was digested with $\text{Pstl/BamHI}$ and ligated into the pLXY1-Pthl-LacZ plasmid, which was also digested with the same restriction enzymes to replace the original $\text{P}_{\text{thl}}$ promoter. The outcomes of this procedure were three new reporter plasmids, namely pLXY1-$\text{P}_{\text{ptb}}$-LacZ, pLXY1-$\text{P}_{\text{adc}}$-LacZ and pLXY1-$\text{P}_{\text{araE}}$-LacZ.
Gene Deletion by CRISPR/Cas9 Plasmids

*C. ljungdahlii* was grown anaerobically at 37°C. The gene deletion was achieved by delivering the CRISPR/Cas9 editing plasmid into *C. ljungdahlii* by electroporation. Electrocompetent cells were prepared according to the previously described protocol (8). The electroporation procedure was slightly modified. Briefly, 200 µL of electrocompetent cells were thawed on ice and then mixed with 4 µg of plasmid DNA recovered from *E. coli* DH5α for approximately 10 min. The mixture of electrocompetent cells and plasmid DNA was then transferred into a 2 mm-gap electroporation cuvette (Harvard Apparatus, MA, USA) and pulsed with parameters set at 1.0 kV, 200 Ω and 50 µF using Gene Pulser Xcell microbial electroporation system (Bio-Rad). The cells were recovered in 2 mL YTF medium supplemented with 0.4% L-cysteine for 6 h. Then, they were plated onto YTF agar plates (supplemented with 5 µg/mL of thiamphenicol) at 37°C for approximately three days. When the colonies were visible on the agar plate, the primers listed in the Supplementary Table S2 were used for the PCR screening of the desired deletion events. Then, the PCR-amplified DNA fragments were extracted and sequenced to further confirm the deletions.

β-Galactosidase Assays

The plasmids pLXY1-P_{str}-LacZ, pLXY1-P_{psb}-LacZ, pLXY1-P_{adc}-LacZ, and pLXY1-P_{araE}-LacZ were transferred into *C. ljungdahlii* by electro-transformation. *C.
ljungdahlii cells that harbored the reporter plasmids were grown anaerobically at 37°C and harvested at 24, 48 and 72 h by centrifugation at 12,000 g for 5 min. The cell pellets were dissolved in the B-PER reagent (Thermo Scientific Pierce®, USA) and vortexed for 1 min for lysing the cells. The resulting cell lysate was heat-treated at 60°C for 30 min to remove heat-unstable proteins and, then, centrifuged at 12,000 g for 30 min. The supernatant was used for the β-galactosidase assays as previously reported (25).

Phenotypic Identification of Cells with pyrE Deletion

Cells with pyrE deletion can be identified on growth medium supplemented with 5-fluoroorotic acid (FOA), as FOA is highly toxic to cells harboring pyrE-based pyrimidine synthetic pathway. The assay was performed as previously described (30), excepted that solidified YTF medium supplemented with 2 mg/mL FOA was used as the assay medium.

Fermentation

All the C. ljungdahlii mutants were serially subcultured in YTF liquid medium (no antibiotic added) to eliminate the plasmid before fermentation. The fermentation was performed in 125 mL Wheaton serum bottles (Sigma-Aldrich, USA) with 30 mL working volume. Briefly, 100 µL of frozen stock was inoculated into 5 mL YTF liquid medium and incubated anaerobically at 37°C for 24 h. When the optical density (OD_{600}) of cells reached 0.5-1.0, approximately 5% (vol/vol) of the inoculum was transferred into 30 mL of a modified ATCC medium 1754 (2-fold increased
concentration of all the mineral elements; fructose was removed) with a headspace of
CO\textsubscript{2}-H\textsubscript{2}-N\textsubscript{2} (56%/20%/9%/15%; pressurized to 0.2 MPa) for fermentation. The
bottles were incubated horizontally in a shaking incubator at 37°C and 150 rpm. The
culture was sampled every 24 h and, then, the gases were added into the headspace
(again at 0.2 MPa).

**Analytical Methods**

Cell growth was followed by measuring the absorbance of the sample at $A_{600}$
(OD\textsubscript{600}) using a spectrophotometer (U-1800, Hitachi, Japan). The concentrations of
ethanol and acetate were measured by an internal standard method using gas
chromatography (7890 A, Agilent, Wilmington, DE, USA) equipped with a flame
ionization detector and a capillary column (Alltech ECTM-WAX). The analysis was
carried out under the following conditions: oven temperature, programmed from 85 to
150°C; injector temperature, 250°C; detector temperature, 300°C; nitrogen (carrier
gas) flow rate, 20 mL/min; hydrogen flow rate, 30 mL/min; air flow rate, 400 mL/min.
The internal standards were isobutanol, isobutyric acid and hydrochloric acid (for
acidification).
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SUPPORTING INFORMATION

Table S1 Strains and plasmids in this study

Table S2 Oligonucleotides used in this study

Fig. S1 Confirmation of the deletion of pta, adhE1, ctf and pyrE by sequencing

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Table 1. Results of the *pta* deletion in *C. ljungdahlii*

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Elements</th>
<th>Experimental rounds</th>
<th>Results (M/P/W/T)*</th>
<th>Efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMTLcasc</td>
<td>Cas9+sgRNA</td>
<td>—</td>
<td>N</td>
<td>—</td>
</tr>
<tr>
<td>pMTLHa</td>
<td>Homologous arms</td>
<td>—</td>
<td>N</td>
<td>—</td>
</tr>
<tr>
<td>pMTLcas-pTa</td>
<td>Cas9+sgRNA+ Homologous arms</td>
<td>1</td>
<td>7/1/0/8</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4/6/0/10</td>
<td>100%</td>
</tr>
</tbody>
</table>

*M*: number of colonies that harbored mixed wild-type and gene-deleted cells; *P*: number of colonies that harbored pure gene-deleted cells; *W*: number of colonies that harbored pure wild-type cells; *T*: total number of colonies used for PCR screening.

*Efficiency*: probability of deletion events occurring, calculated as \((M+P)/T \times 100\%\).
Table 2. Results of the *adhE1*, *ctf* and *pyrE* deletion in *C. ljungdahlii*

<table>
<thead>
<tr>
<th>Gene targets</th>
<th>Deletion size (bp)</th>
<th>Experimental rounds</th>
<th>Before subculturing (M/P/W/T)*</th>
<th>Efficiency#</th>
<th>After subculturing (M/P/T)</th>
<th>Efficiency#</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>adhE1</em></td>
<td>2,600</td>
<td>1</td>
<td>0/3/1/4</td>
<td>75%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0/11/3/14</td>
<td>78%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>ctf</em></td>
<td>1,200</td>
<td>1</td>
<td>11/0/0/11</td>
<td>100%</td>
<td>0/5/5</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>10/0/0/10</td>
<td>100%</td>
<td>0/10/10</td>
<td>100%</td>
</tr>
<tr>
<td><em>pyrE</em></td>
<td>570</td>
<td>1</td>
<td>6/0/6/12</td>
<td>50%</td>
<td>0/7/7</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>9/1/0/10</td>
<td>100%</td>
<td>0/8/8</td>
<td>100%</td>
</tr>
</tbody>
</table>

*M*: number of colonies that harbored mixed wild-type and gene-deleted cells; *P*: number of colonies that harbored pure gene-deleted cells; *W*: number of colonies that harbored pure wild-type cells; *T*: total number of colonies used for PCR screening.

#Efficiency: probability of deletion events occurring, calculated as (M+P)/T×100%.
Fig. 1. Design of the CRISPR/Cas9 system for deletion of the *C. ljungdahlii* *pta* gene.

(A) Strategy for the construction of the plasmid pMTLcas-pta. (B) Strategy for *pta* deletion using pMTLcas-pta. The yellow star indicates the recognition site of the Cas9-sgRNA complex in the coding region of *pta*. (C) PCR screening of the *pta* deletion. The 3.3 and 2.3 kbp bands represent the wild-type and the *pta* deletion genotype, respectively. M: marker; WT: wild-type strain.
Fig. 2. Comparison of the strength of the four heterologous promoters ($P_{thl}$, $P_{araE}$, $P_{ptb}$ and $P_{adc}$ from *C. acetobutylicum* ATCC 824) in *C. ljungdahlii*. $P_{control}$: no promoter to drive *lacZ* expression. Data are representative of three replicates.
Fig. 3. Phenotypic effect after the *pta* deletion in *C. ljungdahlii*. WT: wild-type strain; M-pta: *pta*-deleted mutant. Data are representative of triplicate cultures.
Fig. 4. Phenotypic effect after deletion of *adhE1* (A), *ctf* (B) and *pyrE* (C) in *C. ljungdahlii*. WT: wild-type strain; M-adhE1: *adhE1*-deleted mutant; M-pyrE: *pyrE*-deleted mutant. Data from (A) and (B) are representative of triplicate cultures.

(A)

(B)


(C)

![Image](image.png)