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Development of a High-Efficiency Transformation Method and Implementation of Rational Metabolic Engineering for the Industrial Butanol Hyperproducer *Clostridium saccharoperbutylacetonicum* Strain N1-4

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ABSTRACT While a majority of academic studies concerning acetone, butanol, and ethanol (ABE) production by *Clostridium* have focused on *Clostridium acetobutylicum*, other members of this genus have proven to be effective industrial workhorses despite the inability to perform genetic manipulations on many of these strains. To further improve the industrial performance of these strains in areas such as substrate usage, solvent production, and end product versatility, transformation methods and genetic tools are needed to overcome the genetic intractability displayed by these species. In this study, we present the development of a high-efficiency transformation method for the industrial butanol hyperproducer *Clostridium saccharoperbutylacetonicum* strain N1-4 (HMT) ATCC 27021. Following initial failures, we found that the key to creating a successful transformation method was the identification of three distinct colony morphologies (types S, R, and I), which displayed significant differences in transformability. Working with the readily transformable type I cells (transformation efficiency, 1.1×10^6 CFU/ μ g DNA), we performed targeted gene deletions in *C. saccharoperbutylacetonicum* N1-4 using a homologous recombination-mediated allelic exchange method. Using plasmid-based gene overexpression and targeted knockouts of key genes in the native acetone-butanol-ethanol (ABE) metabolic pathway, we successfully implemented rational metabolic engineering strategies, yielding in the best case an engineered strain (*Clostridium saccharoperbutylacetonicum* strain N1-4/pWIS13) displaying an 18% increase in butanol titers and 30% increase in total ABE titer (0.35 g ABE/g sucrose) in batch fermentations. Additionally, two engineered strains overexpressing aldehyde/alcohol dehydrogenases (encoded by *adh11* and *adh5*) displayed 8.5- and 11.8-fold increases (respectively) in batch ethanol production.

IMPORTANCE This paper presents the first steps toward advanced genetic engineering of the industrial butanol producer *Clostridium saccharoperbutylacetonicum* strain N1-4 (HMT). In addition to providing an efficient method for introducing foreign DNA into this species, we demonstrate successful rational engineering for increasing solvent production. Examples of future applications of this work include metabolic engineering for improving desirable industrial traits of this species and heterologous gene expression for expanding the end product profile to include high-value fuels and chemicals.

KEYWORDS ABE fermentation, *Clostridium*, biofuel, genetics

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Given the pressing need for alternative sources of liquid transportation fuels that are both renewable and economically feasible, much attention has surrounded microorganisms capable of converting biomass-derived sugars into suitable replacement fuels (1). Acetone-butanol-ethanol (ABE) fermentation by certain members of the anaerobic bacterial genus *Clostridium* serves as a promising solution to the need for renewable liquid fuels, either through the direct use of butanol as a “drop-in” fuel (2) or by using all three products as precursors for catalytic conversion to hydrocarbons with molecular weights similar to those found in gasoline, diesel, or aviation fuel (3). In addition to the utility of their end products, solvent-producing *Clostridium* organisms are valued for their ability to metabolize a variety of carbon sources such as pentoses, hexoses, oligosaccharides, and lignocellulose hydrolysates, permitting significant flexibility in the selection of biological feedstocks (4, 5).

Since the first large-scale implementation of ABE fermentation with *Clostridium acetobutylicum* during World War I (6), a number of other solventogenic *Clostridium* species have been discovered and subsequently employed for industrial operation, including *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (7). Compared to the other major industrial ABE producers, *C. saccharoperbutylacetonicum* is characterized by its high selectivity toward butanol (as high as 85% of the total solvents produced), low sporulation frequency (a desirable trait for industrial operation), and wide range of metabolizable carbohydrates (8–10). First detailed in 1960 (8), *C. saccharoperbutylacetonicum* has been the subject of numerous academic studies highlighting the diverse renewable feedstocks that can be utilized by this species, including molasses (11), palm oil (12), cassava (13), sago starch (14), rice bran (15), agricultural waste (16), and lignocellulosic hydrolysate (17–19). Evaluating the species from an engineering perspective, other studies have demonstrated that *C. saccharoperbutylacetonicum* is amenable to operating in a continuous mode (20–22) as well as incorporating *in situ* separation strategies such as liquid-liquid extraction (23) and membrane pervaporation (24). Thus, the demonstrated industrial scalability, feedstock flexibility, and downstream processability associated with *C. saccharoperbutylacetonicum* indicate that this species is highly attractive for use in industrial biofuel production.

Despite the favorable fermentative characteristics of *C. saccharoperbutylacetonicum*, a major drawback associated with this species is the distinct lack of tools and techniques available for performing genetic manipulations. This is in stark contrast to *C. acetobutylicum* and *C. beijerinckii*, for which transformation methods have existed for more than 20 years (25, 26). Building on these methods, rational metabolic engineering of these two species has enabled significant progress in expanding substrate utilization, improving oxygen tolerance, eliminating sporulation, increasing solvent titers/productivities, and enabling the generation of valuable end products beyond ABE (27–31). Without an established transformation method, stable host/vector system, and efficient gene disruption strategy, the types of advances made using rational metabolic engineering in *C. acetobutylicum* and *C. beijerinckii* are not possible for *C. saccharoperbutylacetonicum* without cumbersome and tedious screening of traditional mutagenesis libraries. To our knowledge, only one report from 2007 has detailed a transformation method for any *C. saccharoperbutylacetonicum* strain (strain N1-4 ATCC 13564) (32). Other than this report and a single follow-up study by the same group in 2008 (33), we were unable to find any reports demonstrating transformation methods or heterologous gene expression for any of the *C. saccharoperbutylacetonicum* strains [which include strain N1-4 ATCC 13564 and its two derivatives, strain N1-4 (HMT) ATCC 27021 and strain N1-504 ATCC 27022 (9)]. As strain N1-4 ATCC 13564 has long been deaccessioned, we attempted the published transformation method (32) using the publicly available strain N1-4 (HMT) ATCC 27021 (proposed to be the current type strain [9]) and were unable to obtain any transformants. Therefore, we sought to develop a genetic transformation method for *C. saccharoperbutylacetonicum* strain N1-4 (HMT) ATCC 27021 (hereafter referred to as *C. saccharoperbutylacetonicum* N1-4), determine if plasmid-based gene overexpression and targeted gene deletion would be possible, and

importantly, demonstrate improvements in its fermentation performance using rational metabolic engineering.

Here we report the development of an efficient, robust, and repeatable genetic transformation method for *C. saccharoperbutylacetonicum* N1-4, along with the first reported targeted gene deletions of any *C. saccharoperbutylacetonicum* strain. A key finding was the discovery of multiple phenotypic subtypes of *C. saccharoperbutylacetonicum* N1-4 that displayed dramatic differences in transformability via electroporation. After establishing a repeatable transformation method, we selected 12 genes across the ABE metabolic network for overexpression studies, report batch fermentation data for five strains that displayed altered solvent titers, and report the fermentation performance of three gene deletion strains. These metabolic engineering efforts resulted in several engineered strains with improved ABE production. This study opens the door to future metabolic engineering efforts in *C. saccharoperbutylacetonicum* N1-4 through gene overexpression and targeted gene deletions, as well as newly developed genome-editing tools recently adapted for use in *Clostridium*. Moreover, we believe that these advances make *C. saccharoperbutylacetonicum* N1-4 a competitive candidate for industrial biofuel production.

RESULTS

Initial transformation attempts and identification of morphological subtypes of *C. saccharoperbutylacetonicum* N1-4. Many clostridia are notorious for their genetic intractability, often due to their unwillingness to accept foreign DNA. Thus, our first challenge was to develop a protocol capable of overcoming the DNA restriction-modification systems, membrane-associated DNase activity, morphological heterogeneity, and overall low transformation efficiencies displayed by most clostridia (34). Transformation via electroporation was our natural first choice given the high transformation efficiencies compared to conjugation or other DNA transfer methods. Recently, an excellent review (34) of successful DNA transfer procedures for clostridia was published, providing a starting point for establishing appropriate electroporation parameters.

After determining an appropriate liquid and solid growth medium (2× YTG [pH 6.5], consisting of 16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, 10 g/liter glucose, with an additional 15 g/liter agar for solid medium), and antibiotic for selection (erythromycin [Ery], with a measured MIC of ~10 μg/ml), we attempted electroporation of mid-log-phase cells (optical density at 600 nm [OD₆₀₀], ~ 0.6) using a *dam dcm* methylated *Escherichia coli-Clostridium* shuttle vector deemed pWIS_empty, a derivative of the pIMP vector backbone commonly employed for transformations in *C. acetobutylicum* ATCC 824 (26, 35). Following liquid recovery and incubation on 2× YTG + Ery (20 μg/ml) plates, we observed no transformant colonies. To overcome potential type II DNA endonuclease activity, we performed *in vivo* methylation of pWIS_empty prior to electroporation using the *Bacillus subtilis* phage φ3T I methyltransferase (expressed on *E. coli* plasmid pAN1). This procedure protects plasmid DNA from targeted degradation in *C. acetobutylicum* ATCC 824 through methylation of 5'-GGCC-3' and 5'-GCNCG-3' DNA sequences and was the key to achieving efficient transformations in *C. acetobutylicum* (25). Still, following DNA methylation and electroporation, no transformant colonies were observed. As electroporation parameters have dramatic effects on transformation efficiency, we tested a wide range of parameters (electroporation voltages, resistances, and buffers) as well as competent cell preparation methods (log-phase versus stationary-phase cells, membrane solubilization, and washing procedures). Again, we did not observe transformants under any of these conditions.

While exploring additional transformation strategies, we happened to make a seemingly unrelated discovery; upon plating on solid 2× YTG medium, three distinct colony morphologies of *C. saccharoperbutylacetonicum* N1-4 could be observed, which we called regular (R), irregular (I), and sticky (S) (Fig. 1A). While all three morphological subtypes originated from stocks purchased from ATCC (ATCC 27021), one ATCC stock

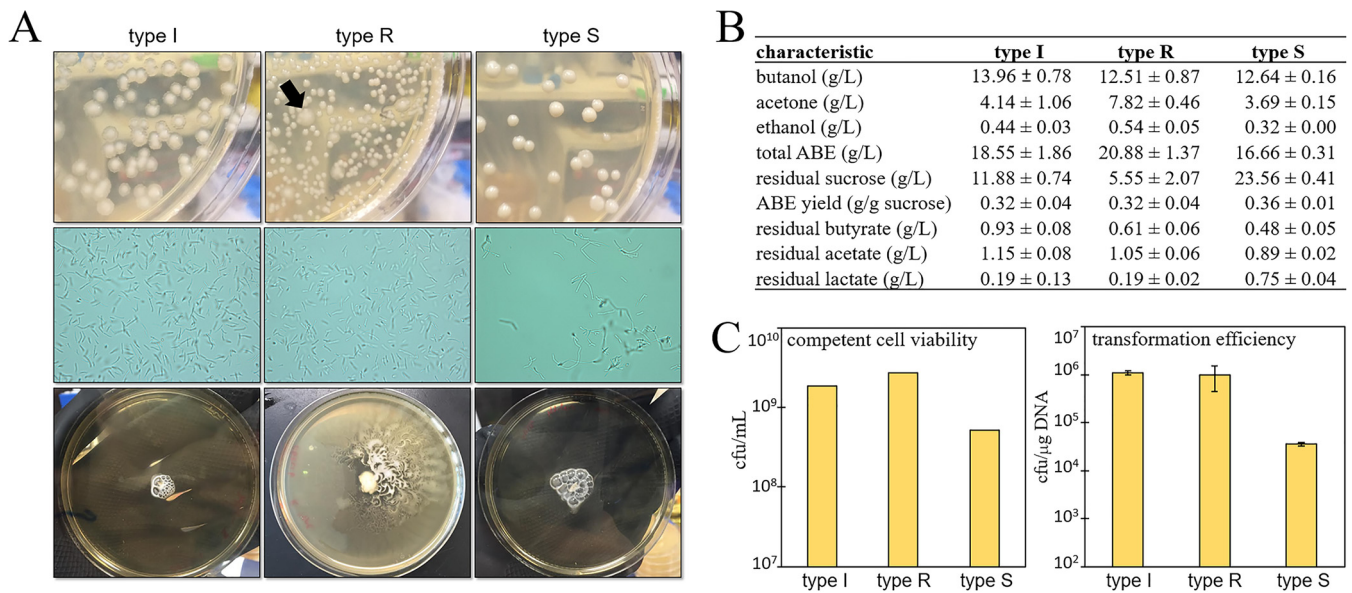


FIG 1 Comparison of three morphological subtypes of *C. saccharoperbutylacetonicum* N1-4. (A) Top row, colonies of each subtype cultured on TYA plates. The black arrow on the type R plate indicates an example of the spontaneous appearance of a type I colony. Middle row, bright-field microscopy images of the three subtypes, with samples diluted from liquid $2\times$ YTG cultures ($OD_{600} \sim 1.0$). Bottom row, motility assays on solid TYA plates. (B) Batch flask fermentation comparison of the three subtypes in PL7S media with samples taken 66 h postinoculation. Fermentations were performed in biological triplicates, for which we report the average. (C) Competent cell viability and transformation efficiency of the three subtypes using the optimized transformation protocol. Electroporations were performed in triplicate.

yielded mixtures of R and I variants (no S variants), while an independently purchased ATCC stock yielded only the S morphology, the latter being the subtype used for our initial transformation attempts. The three subtypes were sequenced for 16S rRNA and showed 100% identity agreement with that of the published sequence (NCBI accession number NC_020291.1). Additionally, we confirmed that all three variants harbor the expected 136-kb megaplasmid (NCBI accession number NC_020292.1), which features a number of seemingly unrelated genes as well as many hypothetical proteins (see Fig. S2 in the supplemental material). Thus, the phenotype differences could not be explained by megaplasmid loss, a phenomenon known to lead to strain degeneration (the loss of ability to produce solvents) in *C. acetobutylicum* ATCC 824 (4).

While type R colonies featured a circular/round form and smooth margin, type I colonies had an irregular form and undulate margin (Fig. 1A). Type S colonies (named for their sticky texture) appeared to have characteristics of both type I and type R, with a slightly irregular form and entire margin, but were convex in elevation (compared to a slightly raised elevation for R and I). Interestingly, the type R phenotype appeared to be unstable; spontaneous conversion to the type I phenotype was observed upon restreaking (Fig. 1A). To probe this phenomenon further, three type R colonies were cultured in separate liquid cultures for 120 h, dilutions were plated onto solid media, and the resulting colony phenotypes (either type R or type I) were enumerated after 48 h of incubation. The results varied widely for the three original cultures; culture A yielded 39% type R colonies, culture B yielded 89% type R colonies, and culture C yielded 100% type R colonies. Thus, the R phenotype was unstable, and the conversion of colonies from type R to type I occurred spontaneously at variable rates. The reverse phenotype conversion (type I to type R) was not detected under any conditions, indicating that the type I morphology was likely stable. Similarly, we did not observe any morphology conversions in the offspring of type S colonies, which appeared to be another stable phenotype.

Additional assays showed high motility for type R cells, low motility for type I and S cells, visible spore formation for all three subtypes, and visibly different cellular morphologies according to light microscopy (Fig. 1A). Slight differences in batch solvent production were observed for the three subtypes, with the highest butanol

production by type I (14.0 g/liter) and the highest acetone production by type R (7.8 g/liter) (Fig. 1B). Type S showed lower sucrose utilization, with 23.6 g/liter remaining at the end of the fermentation, potentially related to the lower cell viability observed for this strain, although this subtype displayed a higher ABE yield (0.36 g/g sucrose) than did the other subtypes.

Development of a high-efficiency transformation protocol for *C. saccharoperbutylacetonicum* N1-4 subtype I. Two observations indicated that the three *C. saccharoperbutylacetonicum* N1-4 variants might exhibit different transformation efficiencies. Qualitatively, we observed what appeared to be high extracellular polysaccharide (EPS) production in liquid cultures from type R colonies: after several days of incubation in PL7S medium, culture supernatant remained cloudy and somewhat viscous, and centrifugation resulted in loose pellets (see Fig. S1 in the supplemental material). This was unlike type I and S cultures, which demonstrated very little cloudiness in aged cultures and yielded compact pellets following centrifugation. Second, we observed significantly lower cell viability (in terms of CFU per milliliter) for type S cultures than for I and R types. As both EPS production and cell viability are factors known to impact transformation efficiencies, we expanded our electroporation efforts to all three *C. saccharoperbutylacetonicum* N1-4 subtypes. Using the same initial electroporation procedure (and *dam dcm* methylated plasmid) that failed to yield transformants for type S cells, we were able to obtain a substantial number of erythromycin-resistant transformants for type I and R subtypes (transformation efficiencies on the order of 10^4 CFU/ μ g DNA). Transformants were confirmed by plasmid purification from liquid cultures, followed by DNA restriction digest gels, which showed bands consistent with the transformed pWIS_empty vector. With improvements in electroporation parameters (by testing voltages between 1.0 and 2.0 kV and resistances between 200 and infinite Ω) and competent cell preparation, we achieved transformation efficiencies of 1.1×10^6 ($\pm 9.3 \times 10^4$) and 9.8×10^5 ($\pm 5.4 \times 10^5$) CFU/ μ g DNA for type I and R cells, respectively (Fig. 1C). Interestingly, we observed an increase of 1 order of magnitude in transformation efficiencies using *C. saccharoperbutylacetonicum* N1-4 type I by performing competent cell preparation at room temperature (versus 4°C, as is used for preparations of *C. acetobutylicum* competent cells [25]). Comparing the viabilities of competent cells stocks of the three subtypes, it appeared that the significantly lower transformability of the S subtype was largely due to its decreased viability. Despite this, the optimized transformation method developed using type I cells permitted successful transformations of type S cells, albeit at significantly lower efficiencies. Unexpectedly, the suggested difference in EPS production between type R and I variants did not appear to have a significant impact on transformability.

Development of targeted gene knockout (KO) method for *C. saccharoperbutylacetonicum* N1-4. Recent advances in the genetic manipulation of clostridia (primarily *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052) have afforded several methods for targeted gene disruption, including (i) heterologous expression of mobile group II introns with the CloStron system (36), (ii) allelic exchange via homologous recombination (37, 38), and very recently, (iii) clustered regularly interspaced short palindromic repeat(s) (CRISPR)-Cas9 genome editing (39–41). Given the observed instability and off-target effects associated with mobile group II introns (42) as well as the start-up time required to adapt an efficient CRISPR-Cas9 system for use in *C. saccharoperbutylacetonicum* N1-4, we chose here to pursue a double-crossover-based allelic exchange (DCAE) method. Given the instability of the subtype R phenotype and the relatively low transformation efficiencies of subtype S cells, we chose to work exclusively with *C. saccharoperbutylacetonicum* N1-4 subtype I for genetic manipulations.

To perform targeted gene KOs, we constructed the plasmid series pLOR, containing an erythromycin resistance-based allelic exchange cassette and the necessary genetic elements for plasmid maintenance in *E. coli* (ColE1 replicon and ampicillin resistance). Notably, we omitted the Gram-positive replicon RepL (present on the pWIS vector backbone) to avoid the need for plasmid curing. To test this system, we first targeted the *pyrF* gene from *C. saccharoperbutylacetonicum* N1-4 (CSPA_RS05335) for deletion, as

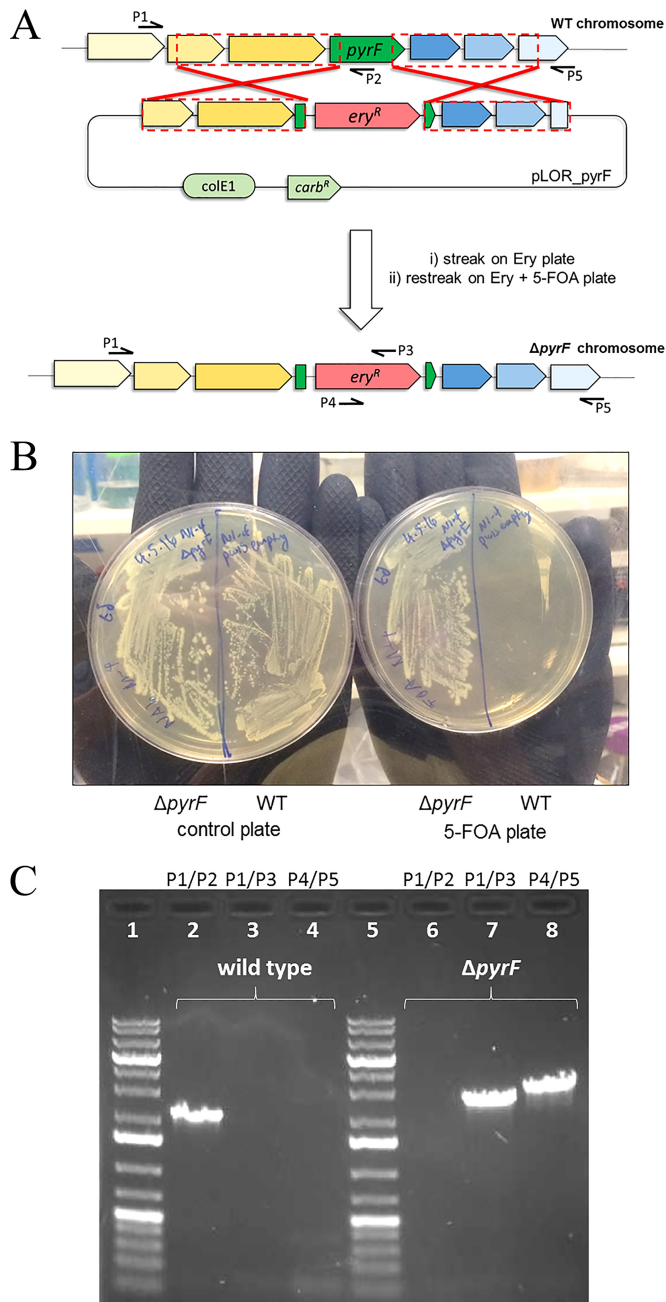


FIG 2 (A) Diagram of the DCAE method applied to targeted KO of the *C. saccharoperbutylacetonicum* N1-4 *pyrF* gene. Dashed red boxes indicate the ~2-kb regions of homology present in pLOR_*pyrF*. Red X's represent homologous recombination events taking place between the *C. saccharoperbutylacetonicum* N1-4 chromosome and pLOR_*pyrF*. P1 to P5 indicate annealing regions for primers used in colony PCR confirmation of successful mutants. (B) Demonstration of a successful *pyrF* mutant. Liquid 2× YTG cultures (OD₆₀₀ ~1.0) of Δ *pyrF* and wild-type (WT) control strains were streaked and incubated (72 h) on a 2× YTG plate (left) and a 2× YTG–500 mg/liter 5-FOA plate (right). (C) DNA electrophoresis gel displaying results of colony PCRs for the isolated *pyrF* mutant as well as a wild-type control. PCR bands from lanes 7 and 8 were gel extracted and analyzed by Sanger sequencing to further confirm the desired allelic exchange event.

mutants of *pyrF* are known to exhibit resistance to the toxic antimetabolite 5-fluoroorotic acid (5-FOA) (37). Additionally, as *pyrF* is necessary for the biosynthesis of uracil, *pyrF* mutants should display uracil auxotrophy. For performing this targeted gene KO, we constructed plasmid pLOR_*pyrF*, containing ~2-kb regions of homology that flank a majority of the *pyrF* coding region (Fig. 2A). Following electroporation of

type I cells, recovery, and a 72-h incubation on erythromycin-containing plates, we obtained about a dozen transformants. Since *pyrF* mutants should exhibit resistance to 5-FOA, we cultured one transformant in liquid medium until it reached the late log phase (OD_{600} of approximately 1.0) and plated dilutions on $2\times$ YTG plates containing 500 mg/liter 5-FOA. Consistent with the expected $\Delta pyrF$ phenotype, the transformant was able to grow on the 5-FOA plate, while a wild-type control strain did not display any growth (Fig. 2B). The transformant also displayed the expected uracil auxotrophy as evidenced by its need for uracil supplementation for growth on defined solid media (see Fig. S5 in the supplemental material). This transformant was further evaluated by colony PCR, which confirmed the predicted allelic exchange event (Fig. 2C). Thus, we demonstrated a successful DCAE method for gene KO in *C. saccharoperbutylacetonicum* N1-4 using a nonreplicating vector.

Manipulation of N1-4 fermentative pathways using rational metabolic engineering. With a viable transformation method, host/vector system for gene expression, and targeted gene KO method, we sought to apply these techniques for rational metabolic engineering of *C. saccharoperbutylacetonicum* N1-4 fermentative pathways (Fig. 3A). We began by performing a bioinformatic analysis of the putative ABE-related genes present in the recently published *C. saccharoperbutylacetonicum* N1-4 genome (43, 44) and comparing these to the confirmed ABE pathway genes present in the well-studied *C. beijerinckii* NCIMB 8052 (a close genetic relative of *C. saccharoperbutylacetonicum* N1-4 [45]) and the model ABE producer *C. acetobutylicum* ATCC 824 (Fig. 3C). Interestingly, we found many redundancies in the putative ABE pathway genes present in *C. saccharoperbutylacetonicum* N1-4 compared to the other two species (Fig. 3B). Despite these differences, there are still clear similarities in the clustering of certain ABE-related genes (for instance, the so-called *sol* operon of *adh-ctfA-ctfB-adc* genes necessary for solvent production in *C. acetobutylicum*) for the three species.

Without any prior knowledge on the function of these 49 predicted ABE pathway genes, we selected 12 genes/gene pairs for targeted gene expression studies (Table 2). Highlighted in Fig. 3A, we selected genes that spanned most of the primary ABE fermentative pathways to probe which of these genes had major bearings on the end product profile of batch fermentations. We purposefully included genes from clusters homologous to those found in *C. beijerinckii* and *C. acetobutylicum* (including *ptb2*, *buk1*, and *adh11*), in addition to genes outside these conserved clusters (including *thl4*, *adh5*, and *ctfAB2*) to investigate whether these redundant homologs might also be important for ABE fermentation in *C. saccharoperbutylacetonicum* N1-4. We cloned these 12 genes/gene pairs into the pWIS vector backbone under the control of the *C. acetobutylicum* crotonase (*crt*) promoter, which we found to be transcribed constitutively in *C. saccharoperbutylacetonicum* N1-4. Of these 12 starting vectors, 10 yielded a high number of *C. saccharoperbutylacetonicum* N1-4 (subtype I) transformants, while two constructs (both expressing *bcd* genes) did not yield any transformants.

To evaluate the 10 generated *C. saccharoperbutylacetonicum* N1-4 overexpression strains, we performed preliminary batch fermentations in an anaerobic chamber with PL75 growth medium, a derivative of the commonly used clostridial growth medium (CGM; see Materials and Methods). We selected sucrose (70 g/liter) as the carbon source, given that the feedstock sugarcane juice (primarily sucrose) is an attractive low-cost industrial carbon source (46), in addition to the fact that *C. saccharoperbutylacetonicum* N1-4 has long been known to readily ferment sucrose-based feedstocks (11). Of the 10 strains, 5 appeared to show significant deviations in their end product profiles relative to a control strain and were used as the basis for detailed batch fermentation analysis (see Table 2). Fermentations of *C. saccharoperbutylacetonicum* N1-4/pWIS8, *C. saccharoperbutylacetonicum* N1-4/pWIS10, *C. saccharoperbutylacetonicum* N1-4/pWIS12, *C. saccharoperbutylacetonicum* N1-4/pWIS13, and *C. saccharoperbutylacetonicum* N1-4/pWIS14, and the control strain, *C. saccharoperbutylacetonicum* N1-4/pWIS_empty, revealed significant differences in both ABE titers and ratios among the six strains (Fig. 4). While *C. saccharoperbutylacetonicum* N1-4/pWIS10 (overexpressing the coenzyme A [CoA] transferase genes *ctfA2* and *ctfB2*) exhibited decreased ABE

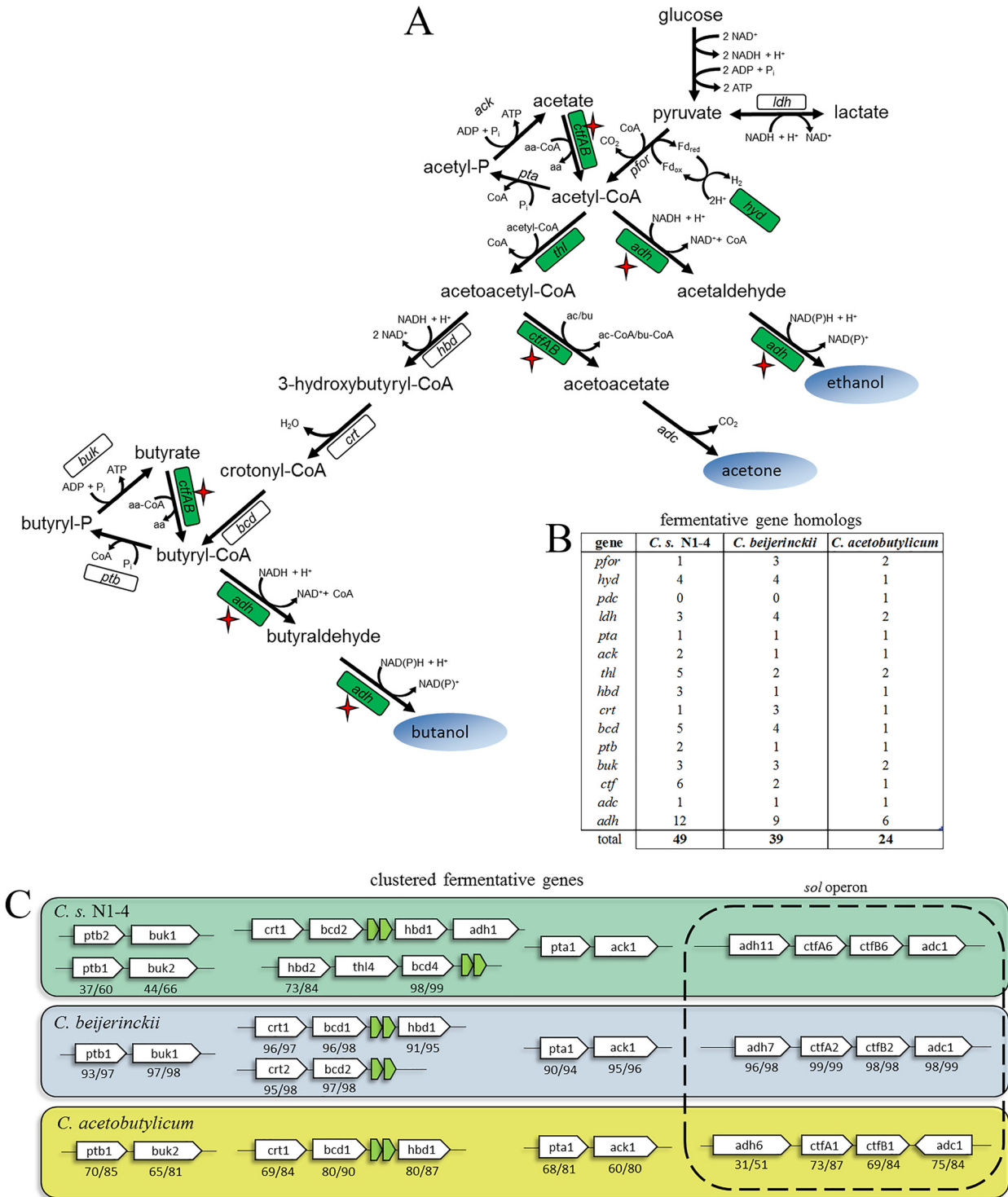


FIG 3 (A) Putative ABE fermentative pathways for *C. saccharoperbutylacetonicum* N1-4 based on *C. acetobutylicum* ABE metabolism. Genes with homologs selected for initial gene overexpression studies are boxed. The subset of these genes with reported batch fermentation data are highlighted in green. A red star indicates that a strain with a deletion in this gene was generated and evaluated by batch fermentation. Enzymes: *pfor*, pyruvate:ferredoxin oxidoreductase; *hyd*, hydrogenase; *pdc*, pyruvate decarboxylase; *ldh*, lactate dehydrogenase; *pta*, phosphotransacetylase; *ack*, acetate kinase; *thl*, thiolase; *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *crt*, crotonase; *bcd*, butyryl-CoA dehydrogenase complex; *ptb*, phosphotransbutyrylase; *buk*, butyrate kinase; *ctf*, CoA transferase; *adc*, acetoacetate decarboxylase; *adh*, alcohol and/or aldehyde dehydrogenase. Metabolites: aa-CoA, acetoacetyl-CoA; ac-CoA, acetyl-CoA; bu-CoA, butyryl-CoA; aa, acetoacetate; ac, acetate; bu, butyrate. (B) Numbers of fermentative gene homologs in three solventogenic clostridia based on bioinformatic analysis. Further details are presented in Table S2 in the supplemental material. (C) Diagram of clustered fermentative genes for *C. saccharoperbutylacetonicum* N1-4 (NCBI accession numbers NC_020291.1 and NC_020292.1), *C. beijerinckii* NCIMB 8052 (NCBI accession number NC_009617.1), and *C. acetobutylicum* ATCC 824 (NCBI accession numbers NC_003030.1 and NC_001988.2). Further details are presented in Table S2 in the supplemental material. Numbers below genes indicate the corresponding protein percent identity/similarity compared to the primary *C. saccharoperbutylacetonicum* N1-4 homolog.

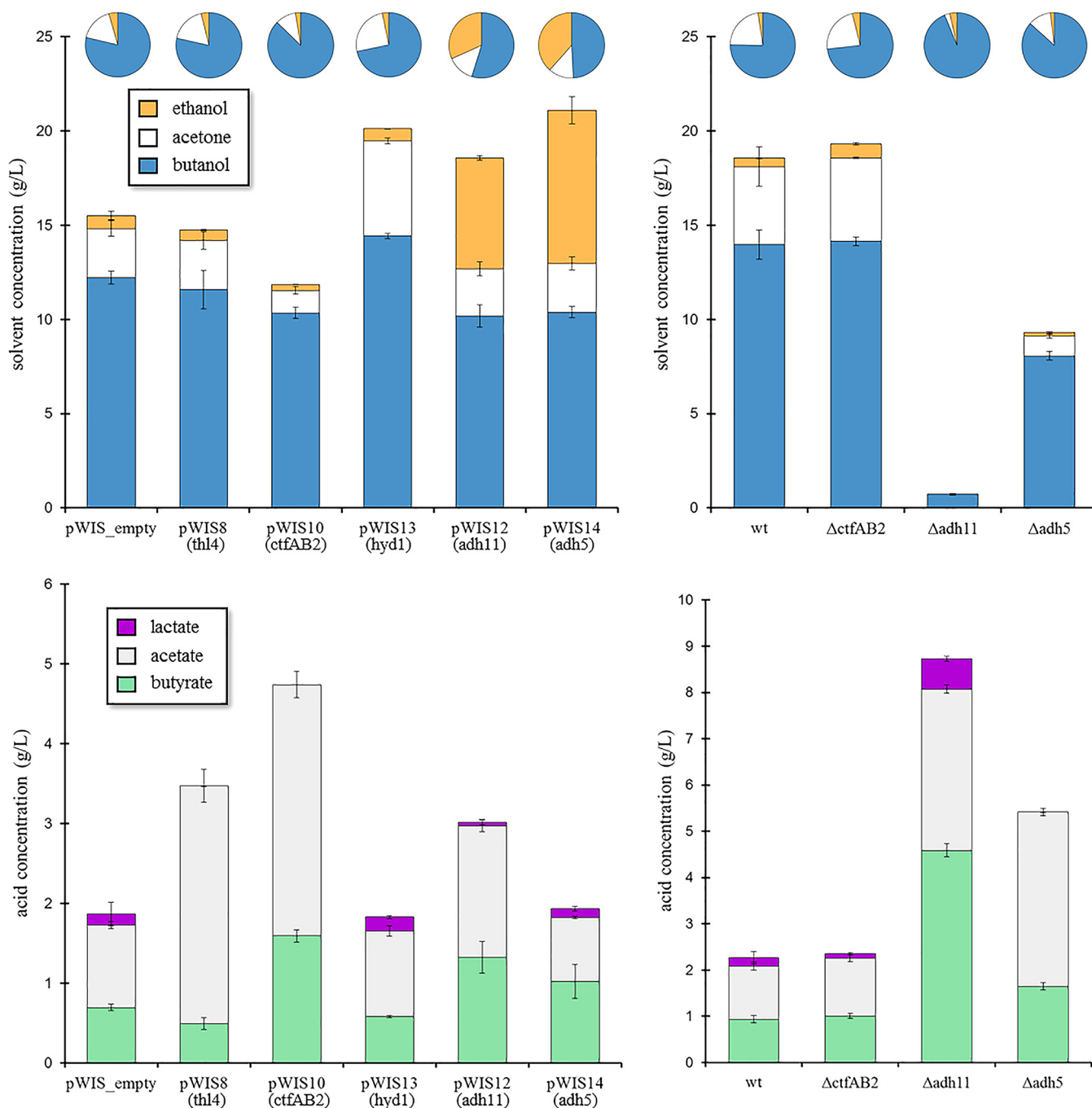


FIG 4 Batch solvent (top row) and acid (bottom row) production for engineered strains of *C. saccharoperbutylacetonicum* N1-4. Top left and bottom left plots display results for gene overexpression strains (with pWIS_empty as the control strain), while top right and bottom right plots display results for gene deletion strains (with vectorless wild-type [wt] type I as the control strain). Genes overexpressed in pWIS-harboring strains are identified in parentheses below their respective strain names. Fermentations were performed under anaerobic conditions in PL7S media (70 ml). Endpoint samples were taken 66 h after inoculation. Distribution of solvent end products are represented with pie charts for each strain. Further details are provided in Table S3 in the supplemental material.

production (with a 15% decrease in butanol and a >50% decrease in acetone and ethanol titers), *C. saccharoperbutylacetonicum* N1-4/pWIS13 (overexpressing the hydrogenase gene *hyd1*) showed significantly increased butanol and acetone titers (18% and 93% increases, respectively). Additionally, while *C. saccharoperbutylacetonicum* N1-4/pWIS12 and *C. saccharoperbutylacetonicum* N1-4/pWIS14 (overexpressing the alcohol/aldehyde dehydrogenase genes *adh11* and *adh5*, respectively) showed slight decreases in butanol titers, substantial increases in ethanol production for both of these strains

(8.5- and 11.8-fold-higher ethanol titers, respectively) made for higher overall ABE titers. In particular, *C. saccharoperbutylacetonicum* N1-4/pWIS14 produced the highest total ABE titer (21.1 g/liter) and ABE yield (0.39 g/g sucrose) observed in this study (see Table S3 in the supplemental material). Residual acid (butyrate and acetate) levels were consistent with the solvent production for the six strains, as relatively high overall acid accumulation was observed for the strains with decreased ABE production (*C. saccharoperbutylacetonicum* N1-4/pWIS8 and *C. saccharoperbutylacetonicum* N1-4/pWIS10).

Based on the above observations, we attempted to create *C. saccharoperbutylacetonicum* N1-4 mutants targeting the genes that hampered ABE production when overexpressed: *thl4* (expressed on *C. saccharoperbutylacetonicum* N1-4/pWIS8) and *ctfA2* and *ctfB2* (expressed on *C. saccharoperbutylacetonicum* N1-4/pWIS10), to explore if any beneficial effects could be observed by deleting these genes. Additionally, we wanted to determine if either *adh5* or *adh11* encoded an ethanol-specific dehydrogenase (rather than a bifunctional ethanol/butanol dehydrogenase); therefore, strains with deletions of these genes were also desired. Following the procedure that we employed for deleting the *pyrF* gene, we created KO vectors targeting the four genetic loci (pLOR_thl, pLOR_ctfAB2, pLOR_adh5, and pLOR_adh11) and performed transformations in *C. saccharoperbutylacetonicum* N1-4 (subtype I). After 72 h of incubation on 2× YTG solid medium, 4 to 12 colonies appeared on each of the transformation plates. The desired double-crossover mutants were isolated at a frequency of roughly one double-crossover KO for every eight screened colonies except for *thl4*, for which we were able to isolate only single-crossover mutants despite repeated efforts and extensive subcultures.

While batch fermentation of the Δ *ctfAB2* strain showed improvements relative to the *C. saccharoperbutylacetonicum* N1-4/pWIS_empty strain, comparison to the vectorless type I wild type showed comparable batch fermentation performance (Fig. 4). Thus, deletion of *ctfAB2* did not appear to significantly affect ABE production, while overexpression proved harmful to solvent production. Interestingly, deletion of either *adh5* or *adh11* resulted in significantly decreased ABE production. In particular, deletion of *adh5* led to decreases in titers of butanol, acetone, and ethanol of 42%, 75%, and 58%, respectively (compared to the vectorless wild type); while deletion of *adh11* abolished acetone and ethanol production and decreased the butanol titer to less than 1 g/liter. High acid accumulation in both Δ *adh5* and Δ *adh11* mutants confirmed the inability of these strains to effectively reduce CoA-bound intermediates to the typical alcohol products.

DISCUSSION

Since the discovery of an efficient gene transfer method over 20 years ago, *C. acetobutylicum* ATCC 824 has served as host for the vast majority of genetic engineering studies performed on solventogenic clostridia. This has been due largely to the well-studied physiology, fermentative metabolism, and industrial performance of this organism, dating back to the early 20th century. In the last 10 years, long-awaited progress in genetic tools for use in clostridia has afforded advanced genetic manipulations of *C. acetobutylicum*. Despite this, advanced genetic tools are of no use to genetically intractable organisms, as is the case for most known *Clostridium* species. Recent achievements in developing efficient transformation methods for historically intractable *Clostridium* species (e.g., *C. pasteurianum*, *C. cellulovorans*, and *C. ljungdahlii*) (47–49) have permitted novel genetic engineering efforts for these species. These accomplishments encourage the development of transformation methods and genetic tools for historically intractable solventogenic strains, particularly those with a history of strong industrial performance.

In this study, we present the development and application of a high-efficiency transformation method for the industrial butanol hyperproducer *C. saccharoperbutylacetonicum* strain N1-4 (HMT) ATCC 27021. Key to the development of this transformation method, we identified three distinct colony morphologies (types R, I, and S), which displayed significant differences in transformability. Presently, it is unclear whether

these distinct colony morphologies arise from genetic or epigenetic factors. The high frequency and unpredictability of type R to type I conversions suggest that epigenetic influences may be at play, although it is unclear which factors might stimulate this conversion. As type I and type S colonies were stable phenotypes under all conditions that we evaluated, mutations at one or more genetic loci could be responsible for the observed differences between these two subtypes. Comparing early descriptions of *C. saccharoperbutylacetonicum* colony morphologies (summarized in reference 9) to those that we observed, either type R or type S colonies seem to be consistent with these records, which reported domed, white, smooth colonies with entire/undulated margins. Although we did not find previous reports of plate motility assays being performed, our results demonstrated that type R and type S colonies could easily be distinguished using this assay (Fig. 1A). Differences in ABE production between the subtypes raised further questions as to the genetic or epigenetic factors which distinguish these subtypes. For the purposes of this study, we chose to perform all strain engineering using type I cells, given their stable phenotype and high transformation efficiencies, despite the higher total ABE production offered by subtype R (Fig. 1B). However, factors other than ABE titers are important in evaluating the industrial utility of different strains—certainly the production of EPS-like material from liquid cultures of type R colonies could present challenges for industrial applications.

Using a DCAE method for generating KOs permitted, to our knowledge, the first reported examples of targeted gene deletions in *C. saccharoperbutylacetonicum*. Furthermore, high transformation efficiencies ($\sim 1 \times 10^6$ CFU/ μ g DNA) permitted the use of a nonreplicating KO vector (pLOR), avoiding the need for plasmid curing. While we were able to generate four mutant strains using this technique, a counterselection system for the elimination of single-crossover mutants is highly desirable for future gene deletions. It is unknown if our inability to generate a few of the desired KOs was due to allelic exchange inefficiency or the essential nature of the targets. Unfortunately, we were unable to apply a recently published counterselection method based on a *C. perfringens* lactose-inducible promoter (38), as we observed transcription of this promoter with or without lactose addition in *C. saccharoperbutylacetonicum* N1-4 (data not shown). We are currently working to employ a *pyrF*-based selection system (working with the Δ *pyrF* strain developed in this study), as we have since identified additional antibiotic markers effective in *C. saccharoperbutylacetonicum* N1-4. Counterselection is particularly important for DCAE gene deletions in *C. saccharoperbutylacetonicum* N1-4, which seemed to have a relatively low double-crossover rate. Low homologous recombination frequencies could also be addressed by including a resolvase protein (RecU) on the pLOR backbone, as this has proven to be an effective method of improving recombination efficiencies in other *Clostridium* species (50).

To select targets for rational metabolic engineering in *C. saccharoperbutylacetonicum* N1-4, we performed bioinformatic comparisons to published *C. acetobutylicum* and *C. beijerinckii* genomes (Fig. 3B and C). These revealed similarities in fermentative gene clustering (most notably, the *sol* operon), but also a large number of redundant ABE-related genes in the *C. saccharoperbutylacetonicum* N1-4 genome. Curiously, while some redundant genes are highly homologous (e.g., *bcd2* and *bcd4* have 98% identical and 99% similar protein sequences), others showed significant divergence (e.g., *buk1* and *buk2* have only 37% identical and 60% similar protein sequences). The consequences of these gene redundancies in the context of ABE fermentation are currently unclear; further work is required to determine which homologs are actually expressed over the course of a fermentation, evaluate whether any synergy is achieved in expressing genes with redundant function, and assess the impact of deleting “unnecessary” homologs. Toward addressing these questions, we emphasized selecting multiple gene homologs across the ABE metabolic network for our plasmid-based overexpression studies.

The reason for the slightly decreased batch performance of the *thl4* overexpression strain (*C. saccharoperbutylacetonicum* N1-4/pWIS8) is somewhat unclear. The acetate accumulation observed (3.0 g/liter) was unexpected, as thiolase is predicted to direct

carbon flux away from the acetyl-CoA/acetyl-P/acetate cycle (Fig. 3A). One possible explanation is that *thl4* is relatively inefficient compared to the four other predicted copies of the thiolase gene in the N1-4 genome. If valid, *thl4* overexpression may cause nonproductive substrate (acetyl-CoA) titration, placing additional stress on upstream pathways to increase acetyl-CoA pools. The pWIS10 strain (overexpressing *ctfAB2*) demonstrated a similar behavior in batch fermentation (reduced solvent production and increased acid accumulation), although the effect was more pronounced in this strain. A similar substrate titration effect due to a relatively inefficient CtfAB2 (acting on acetoacetyl-CoA) could be a possible explanation for this behavior. Thus, we postulate that CtfAB2 is not the primary CoA transferase in *C. saccharoperbutylacetonicum* N1-4, also supported by the retained ability of the Δ *ctfAB2* strain to reassimilate acetate and butyrate. This example demonstrates the strategy of strain improvement through deletion of potentially inefficient redundant genes, although in this case, *ctfAB2* deletion did not significantly affect the fermentation profile. Deletion of the five other putative CoA transferase genes would serve to explore this strategy further.

The batch fermentation performance of strains *C. saccharoperbutylacetonicum* N1-4/pWIS12 and *C. saccharoperbutylacetonicum* N1-4/pWIS14 (expressing *adh11* and *adh5*, respectively) is consistent with the predicted aldehyde dehydrogenase activity for these proteins, as total alcohol production increased (primarily ethanol) for these strains. *E. coli* heterologous expression and *in vitro* analyses of Adh11 (referred to as "Bld" in these studies) have previously been performed for applications in synthetic 1,3-butanediol (51) and 1,4-butanediol (52) pathways, demonstrating the ability of Adh11 to reduce butyryl-CoA derivatives to their corresponding aldehydes. As evidenced by the increased ethanol production observed for *adh11* and *adh5* overexpression strains, Adh11 and Adh5 appear to also have significant activity toward acetyl-CoA as a substrate. Under this assumption, ethanol (rather than butanol) production may have increased due to increased metabolic flux toward acetaldehyde from acetyl-CoA, outweighing the flux directed from acetyl-CoA toward butyryl-CoA (and eventually butanol). Therefore, protein engineering of Adh11/Adh5 to improve the substrate specificity for butyryl-CoA (and decrease the substrate specificity for acetyl-CoA) could be an effective strategy to improve butanol titers in *C. saccharoperbutylacetonicum* N1-4. From our results alone, we are unable to confirm the bifunctional aldehyde/alcohol dehydrogenase activity predicted for Adh5, as the *C. saccharoperbutylacetonicum* N1-4 genome is predicted to encode several other alcohol dehydrogenases (Fig. 3B). Deletion of *adh5* and *adh11* revealed significant disruptions in solvent production, indicating that multiple dehydrogenases are likely involved in alcohol production in *C. saccharoperbutylacetonicum* N1-4. Efforts to characterize the roles of different aldehyde/alcohol dehydrogenases in *C. acetobutylicum* (most recently described in references 53 and 54) have taken a similar reverse genetics approach and have been successful in identifying butanol-specific dehydrogenases useful for improving butanol selectivities. This approach could easily be extended to *C. saccharoperbutylacetonicum* N1-4 by evaluating mutants/overexpression strains of all 12 predicted *adh* genes (see Table S2 in the supplemental material).

The improved solvent (particularly acetone) production by the pWIS13 strain (overexpressing the hydrogenase gene *hyd1*) was unexpected given that increased hydrogen production has previously been shown to result in decreased butanol production for *C. acetobutylicum* (55). In *C. acetobutylicum*, reduced ferredoxin competitively donates reducing equivalents to either HydA or ferredoxin:NAD(P)H oxidoreductase, leading to increased NAD(P)H production (and thus, butanol production) for strains with reduced hydrogenase activity (56). Quantification of the hydrogen evolution of the *hyd1* overexpression strain versus a control strain could aid in explaining our results, as well as performing gene overexpression/deletion studies for the other three predicted hydrogenase genes in *C. saccharoperbutylacetonicum* N1-4 (Table S2).

In summary, we have demonstrated a methodology for highly efficient transformations, genetic machinery for plasmid-based gene overexpression, targeted gene KOs through allelic exchange using a nonreplicating vector, and successful rational meta-

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics ^a	Source or reference(s)
Bacterial strains		
<i>E. coli</i> XL1-Blue	Cloning strain	Stratagene/Agilent ^b
<i>C. saccharoperbutylacetonicum</i> strains		
N1-4 (HMT)	Wild-type strain	ATCC 27021
N1-4 Δ pyrF	Δ pyrF::Ery ^r	This study
N1-4 Δ ctfAB2	Δ ctfAB2::Ery ^r	This study
N1-4 Δ adh5	Δ adh5::Ery ^r	This study
N1-4 Δ adh11	Δ adh11::Ery ^r	This study
Plasmids		
pIMP1 (= pWIS_empty)	<i>E. coli</i> - <i>Clostridium</i> shuttle vector; Ery ^r ; <i>repL</i> ; Amp ^r ; <i>colE1</i>	26, 35
pWIS_2 through pWIS_14	pWIS_empty with <i>C. acetobutylicum</i> P _{crit} and T _{hbd} strain N1-4 gene/gene pair to be overexpressed	This study
pKO_mazF	Th ^r Ery ^r ; <i>repL</i> ; <i>colE1</i> ; <i>bgaR</i> and P _{bgaL} upstream of <i>mazF</i>	38
pLOR_empty	P _{crit} upstream of Ery ^r ; Amp ^r ; <i>colE1</i>	This study
pLOR_pyrF	pLOR_empty with Ery ^r flanked by ~2-kb <i>pyrF</i> upstream and downstream homologous regions	This study
pLOR_ctfAB2	pLOR_empty with Ery ^r flanked by ~2-kb <i>ctfA2-ctfB2</i> upstream and downstream homologous regions	This study
pLOR_adh5	pLOR_empty with Ery ^r flanked by ~2-kb <i>adh5</i> upstream and downstream homologous regions	This study
pLOR_adh11	pLOR_empty with Ery ^r flanked by ~2-kb <i>adh11</i> upstream and downstream homologous regions	This study

^aEry^r, erythromycin resistance; *repL*, pM13 Gram-positive origin of replication; Amp^r, ampicillin resistance; *colE1*, *E. coli* replication origin; P_{crit}, crotonase promoter; T_{hbd}, 3-hydroxybutyryl-CoA dehydrogenase terminator; Th^r, thiamphenicol resistance; P_{bgaL}, *C. perfringens* *bgaL* promoter; *bgaR*, *C. perfringens* divergent regulator of P_{bgaL}; *mazF*, toxin gene.

^bAgilent Technologies, Santa Clara, CA.

bolic engineering approaches for use in the industrial ABE producer *Clostridium saccharoperbutylacetonicum* strain N1-4 (HMT) ATCC 27021. This study serves as a foundation for future metabolic engineering efforts of this prominent solvent producer to not only improve fermentation performance metrics (e.g., solvent titers, productivities, and yields, oxygen tolerance, substrate specificity, cell viability), but also to serve as a platform for the production of other high-value products (e.g., higher alcohols and petrochemical precursors). Additionally, this study opens the door for advanced genetic manipulations of this organism using recent achievements in adapting CRISPR/Cas9 systems for use in clostridia (40, 41). Before this is possible, future studies are required to identify additional promoters, antibiotic markers, and counterselection methods able to function in *C. saccharoperbutylacetonicum* N1-4. With these tools and techniques in hand, we believe that future engineered strains of *C. saccharoperbutylacetonicum* N1-4 will be better able to meet the stringent demands required for an economically viable biofuel production process.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2.

Culture media and growth conditions. *Clostridium saccharoperbutylacetonicum* strain N1-4 (HMT) ATCC 27021 was cultured at 34°C in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) containing an atmosphere of 97% nitrogen and 3% hydrogen. For transformation procedures, strains were grown in 2× YTG medium (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, 10 g/liter glucose with an additional 15 g/liter agar for solid media) with the pH adjusted to 6.5. For comparing colony morphologies, solid TYA medium was used (6 g/liter tryptone, 2 g/liter yeast extract, 3 g/liter ammonium acetate, 0.2 g/liter MgSO₄, 0.01 g/liter FeSO₄, 40 g/liter glucose, 0.5 g KH₂PO₄, and 15 g/liter agar) with the pH adjusted to 6.5. For growing overnight cultures and preparing glycerol stocks, PL7G media, a derivative of clostridial growth medium (CGM) (57) was used (30 g/liter glucose, 5 g/liter yeast extract, 2.67 g/liter ammonium sulfate, 1 g/liter NaCl, 0.75 g/liter monobasic sodium phosphate, 0.75 g/liter dibasic sodium phosphate, 0.5 g/liter cysteine-HCl, 0.7 g/liter magnesium sulfate heptahydrate, 20 mg/liter manganese sulfate heptahydrate, and 20 mg/liter iron sulfate heptahydrate, with the initial pH set to 6.3).

Escherichia coli XL1-Blue (Agilent Technologies, Santa Clara, CA) was grown aerobically in Luria-Bertani (LB) medium at 37°C. *Clostridium* and *E. coli* strains were maintained as 20% (vol/vol) glycerol stocks stored at -80°C.

TABLE 2 List of native *C. saccharoperbutylacetonicum* N1-4 genes selected for gene overexpression studies^a

Vector	Locus tag	Gene locus	Gene annotation
pWIS2	<i>ptb2</i>	CSPA_RS01245	Phosphate butyryltransferase
	<i>buk1</i>	CSPA_RS01250	Butyrate kinase
pWIS3	<i>crt1</i>	CSPA_RS02130	3-Hydroxybutyryl-CoA dehydratase
	<i>bcd2</i>	CSPA_RS02135	Acyl-CoA dehydrogenase
pWIS4	<i>hbd1</i>	CSPA_RS02150	3-Hydroxybutyryl-CoA dehydrogenase
	<i>adh1</i>	CSPA_RS02155	NADPH-dependent butanol dehydrogenase
pWIS6	<i>ldh1</i>	CSPA_RS05375	L-Lactate dehydrogenase
pWIS7	<i>hbd2</i>	CSPA_RS10210	3-Hydroxybutyryl-CoA dehydrogenase
pWIS8	<i>thl4</i>	CSPA_RS10215	Acetyl-CoA acetyltransferase
pWIS9	<i>bcd4</i>	CSPA_RS10220	Acyl-CoA dehydrogenase
pWIS10	<i>ctfA2</i>	CSPA_RS10270	CoA transferase subunit alpha
pWIS10	<i>ctfB2</i>	CSPA_RS10275	CoA transferase subunit beta
pWIS11	<i>adh2</i>	CSPA_RS10510	Aldehyde-alcohol dehydrogenase
pWIS12	<i>adh11</i>	CSPA_RS27680	Aldehyde dehydrogenase
pWIS13	<i>hyd1</i>	CSPA_RS11980	[FeFe] hydrogenase group A
pWIS14	<i>adh5</i>	CSPA_RS18045	Aldehyde-alcohol dehydrogenase

^aGene annotations are based on NCBI designations. Genes/vectors used in detailed batch fermentations are highlighted in boldface.

For appropriate *Clostridium* strains, culture medium was supplemented with erythromycin (40 µg/ml). Antibiotic was omitted in flask fermentation cultures to avoid negative effects on the fermentation cycle. Carbenicillin (100 µg/ml) was added to *E. coli* culture medium as needed. 5-FOA was purchased as a 100-mg/ml solution (dimethyl sulfoxide [DMSO]) from Zymo Research (Irvine, CA); 500 mg/liter 5-FOA was added to growth media as indicated.

Flask fermentations. For performing flask fermentations, PL7S medium was used, which is identical to PL7G medium with the following exceptions: 70 g/liter sucrose was used in place of glucose, and 300 mg/liter adenine was added. Additionally, Antifoam 204 (10 µl antifoam/70 ml culture; Sigma, St. Louis, MO) and solid CaCO₃ (6 g/liter, for pH control) were added to flask fermentation cultures just prior to inoculation. All overnight cultures, subcultures, and fermentation cultures of *Clostridium* were cultured anaerobically at 34°C without agitation. Initial overnight cultures were started from freshly streaked (<5 days old) individual colonies on 2× YTG plates. To perform flask fermentations, a mid-/late-exponential-phase overnight culture (OD₆₀₀ 0.6 to 1.0; PL7G medium) was used to inoculate a subculture in PL7S medium (pH 6.3) with a 10% inoculum. At mid/late exponential phase (OD₆₀₀ 0.6 to 1.0), 7 ml of subculture was used to inoculate 63 ml of fresh PL7S medium (supplemented with antifoam and CaCO₃) in a 125-ml glass shake flask with foil caps. All flask fermentations were performed as biological triplicates. Endpoint samples for high-performance liquid chromatography (HPLC) analysis (1 ml) were taken 66 h postinoculation.

Analytical procedures. Cell concentrations were monitored via absorbance using a spectrophotometer to determine the OD₆₀₀. Sucrose, acetone, butanol, ethanol, acetate, butyrate, and lactate concentrations were measured using a Shimadzu Prominence UFLC system with refractive index and diode array detection (Shimadzu America, Inc., Columbia, MD). Prior to analysis, samples of culture supernatant were filtered with 0.22 µm polyvinylidene difluoride (PVDF) syringe filters. Compounds were separated with a Bio-Rad Aminex HPX-87H column (300 mm by 7.8 mm) and detected by measurement of refractive index (sucrose, butanol, ethanol, acetate, lactate) or UV absorbance (acetone, 265 nm; butyrate, 208 nm). The following operating conditions were used: mobile phase, 0.01 N sulfuric acid; flow rate, 0.7 ml/min; run time, 35 min; column temperature, 35°C.

Bright-field microscopy. Bright-field microscopy of the three subtypes of *C. saccharoperbutylacetonicum* N1-4 was performed with a Leica DM5000 B fluorescence microscope (Wetzlar, Germany) fitted with a Leica DFC490 camera. Samples of liquid culture (OD₆₀₀ ~1.0) started from colonies of the three subtypes were diluted 100-fold in 0.85% NaCl. Samples were observed using a 40× objective lens.

Motility assays. Glycerol stocks of subtypes R, I, and S were inoculated into liquid medium (2× YTG). After overnight incubation (OD₆₀₀ ~0.6), the cultures were plated on solid TYA and incubated anaerobically for 48 h. Single colonies of each subtype (confirmed visually) were picked with pipette tips and stabbed into the center of fresh soft agar (5 g/liter agar instead of the normal 15 g/liter) TYA plates. Plates were incubated anaerobically for 48 h at 34°C before imaging.

Plasmid construction. Oligonucleotides were synthesized by IDT (Coralville, IA). Table S1 in the supplemental material lists all relevant oligonucleotide sequences used. Vector pWIS_empty was identical to the received pIMP_empty (25, 35). All derivatives of pWIS_empty included the *C. acetobutylicum* ATCC 824 crotonase (*crt*) promoter with a synthetic ribosome binding site (RBS), followed by the coding region for the selected gene(s) to be overexpressed, and finally, the *C. acetobutylicum* ATCC 824 hydroxybutyryl-CoA dehydrogenase (encoded by *hbd*) rho-independent terminator. This expression cassette was inserted downstream of the Gram-positive replicon RepL-coding sequence on the pWIS_empty backbone using Gibson assembly (58). All native *C. saccharoperbutylacetonicum* N1-4 genes used for gene overexpression studies were PCR amplified from purified *C. saccharoperbutylacetonicum* N1-4 genomic DNA. DNA primers used to amplify these genes contained 27-bp overhangs to permit

Gibson assembly with the *crt* promoter region (forward primer) and *hbd* terminator region (reverse primer). The pWIS vector backbone was amplified as two regions (pWIS_bk A, 3.0 kb, primers pWIS_bk_F and pWIS_bk_R2; pWIS_bk B, 2.0 kb, primers pWIS_bk_F2 and pWIS_bk_R), which overlap at a 24-bp sequence. Gibson assembly for pWIS series vectors included pWIS_bk A, pWIS_bk B, and the amplified native N1-4 gene containing 27-bp 5' and 3' overhangs (Table S1). After transformation into *E. coli* XL1-Blue, putative transformants were cultured and underwent plasmid Miniprep purification (Qiagen North America, Germantown, MD). Purified plasmid DNA was screened by restriction digest testing, followed by Sanger sequencing.

The pLOR vector series for performing targeted gene deletions in *C. saccharoperbutylacetonicum* N1-4 was derived from elements of pIMP1 and pKO_mazF. A 677-bp region containing the *E. coli* origin of replication ColE1 was amplified from pIMP1 using primers pLOR_P1_F & pLOR_P1_R. A 1,233-bp region containing the ampicillin resistance gene (*Amp^r*) was amplified from pIMP1 using primers pLOR_P2_F and pLOR_P2_Ro. An 858-bp region containing the erythromycin resistance (*Ery^r*) gene driven by the *C. acetobutylicum* thiolase (*thl*) promoter was amplified from pIMP1 using primers pLOR_P3_Fo and pLOR_P3_R. A 44-bp region containing a spacer sequence present in pKO_mazF was included upstream of *P_{thl}-Ery^r* by addition of an overlapping sequence to the 5' end of pLOR_P3_F. A 287-bp region containing a terminator sequence was amplified from pKO_mazF to follow the *P_{thl}-Ery^r* cassette using primers pLOR_P4_Fo and pLOR_P4_R. These four PCR products underwent Gibson assembly along with PCR-amplified upstream homologous region (UHR) and downstream homologous region (DHR) (both approximately 2.0 kb) designed to target the *pyrF* gene (using primers UHR_pyrF_F/UHR_pyrF_R, and DHR_pyrF_F/DHR_pyrF_R to amplify the *pyrF* UHR and DHR, respectively); 24-bp overhang sequences were included on primers of neighboring PCR products to permit assembly. Following transformation, plasmid purification, confirmation by test digestion, and sequencing, pLOR_pyrF served as the template for all other pLOR assemblies. For assembly of other pLOR constructs, appropriate UHR and DHR PCR products were assembled with the ColE1/AmpR region from pLOR_pyrF (with primers pLOR_P2_F and pLOR_P1_R), and the *P_{thl}-Ery^r* cassette (with primers pLOR_P3_Fs and pLOR_P4_R) (to be flanked by the UHR and DHR regions); 24-bp overhang sequences were included on all primers for the UHR and DHR PCRs to permit annealing to the pLOR vector backbone elements. As above, all vectors were confirmed by test digestion and sequencing.

Plasmid and genomic DNA isolation in *C. saccharoperbutylacetonicum* N1-4. For plasmid DNA isolation in *C. saccharoperbutylacetonicum* N1-4, 5 ml of stationary-phase liquid cultures ($OD_{600} > 2.0$) underwent plasmid Miniprep purification according to the manufacturer's instructions (Qiagen North America, Germantown, MD). Genomic DNA isolation of *C. saccharoperbutylacetonicum* N1-4 was performed using a modified alkaline lysis method. Stationary-phase culture (10 ml) was prepared as for plasmid purification and centrifuged (room temperature, $3,500 \times g$, 15 min). The pellet was resuspended in 5 ml SET buffer (75 mM NaCl, 25 mM EDTA pH 8.0, 20 mM Tris-HCl [pH 7.5]), and lysozyme was added to a final concentration of 2 mg/ml. The mixture was incubated for 60 min at 37°C with gentle mixing every 15 min, and lysis was induced by addition of 660 μ l of 1 M NaOH solution containing 10% (wt/vol) SDS. The resulting solution was mixed by inversion, proteinase K was added to a final concentration of 0.5 mg/ml, and the mixture was incubated for 1 h at 55°C. Phenol-chloroform (equal volume, 1:1; room temperature) was added, and the solution was mixed by inversion for 5 min. After centrifugation (room temperature, $3,500 \times g$, 10 min), the aqueous phase was removed by pipetting, and 3 M sodium acetate (10% by volume in the resulting mixture) was added. After mixing by inversion, 2 volumes of ethanol were added to precipitate genomic DNA, and the DNA was harvested with a glass hook and washed with 70% ethanol. After 15 min of drying, the precipitated DNA was resuspended in low Tris-EDTA (TE) buffer (10 mM Tris, 0.1 mM EDTA [pH 8.0]) and incubated at 50°C until dissolved. This procedure resulted in 1 ml of approximately 1 μ g/ μ l genomic DNA.

Transformation procedure for *C. saccharoperbutylacetonicum* N1-4. To prepare competent cell stocks of *C. saccharoperbutylacetonicum* N1-4, anaerobic overnight cultures (10 ml PL7G, 34°C) were started from 20% (vol/vol) glycerol stocks stored at -80°C. After reaching an OD_{600} of ~0.6, overnight cultures were subcultured in 60 ml liquid 2 \times YTG (10% inoculum) and incubated for 3 to 5 h until reaching an OD_{600} of ~0.6. The subcultures were then centrifuged (room temperature, $3,500 \times g$, 15 min), the supernatant was removed, and the pellet was resuspended in 10 ml room temperature electroporation buffer (EPB) (270 mM sucrose, 5 mM NaH₂PO₄ [pH 7.4]). Resuspended cells were centrifuged again (room temperature, $3,500 \times g$, 10 min), and the pellets were resuspended in 3 ml room temperature EPB, yielding the concentrated (20 \times) competent cells. The competent cell stock was aliquoted into prechilled 4-mm electroporation cuvettes (500- μ l aliquots), 2 μ g plasmid DNA was added to each cuvette, and the mixtures were incubated in ice for 30 min. Exponential decay electroporations were performed using a Bio-Rad Gene Pulser Xcell (Hercules, CA) with parameters as follows: voltage, 2.0 kV; resistance, 200 Ω ; capacitance, 25 μ F. Following electroporation (yielding time constants of ~4.0 ms), cells were immediately resuspended in 10 ml liquid 2 \times YTG and were allowed to recover at 34°C for 4 h. Recovery cultures were then centrifuged (room temperature, $3,500 \times g$, 15 min), the supernatant was removed, and pellets were resuspended in 500 μ l fresh 2 \times YTG. The mixture (100 μ l) was spread on solid 2 \times YTG + Ery40 plates and incubated at 34°C for 3 to 4 days. Potential transformant colonies were grown in overnight liquid cultures (2 \times YTG + Ery40) and replated on solid 2 \times YTG + Ery40 before undergoing verification.

Targeted gene KO procedure and verification. The same electroporation method was used to introduce KO vectors (pLOR series) into *C. saccharoperbutylacetonicum* N1-4, except that 5 μ g of plasmid DNA was used. Initial transformant colonies (4–6) were grown in overnight liquid cultures (2 \times YTG + Ery40) and replated on solid 2 \times YTG + Ery40. Twelve colonies (2 or 3 per restreaked transformant) were screened via colony PCR using three primer sets (in three separate reactions) (Fig. 2). Two sets were

designed to amplify regions spanning from inside the erythromycin resistance gene to outside the regions of homology. Both bands should be present for double-crossover KO strains. The third primer set was designed to amplify a region from inside the wild-type copy of the target gene to outside the regions of homology. This third set was used to rule out contamination of the wild type, as this band should be present only for wild-type strains. After colony PCR screening of the 12 colonies along with one wild-type control (three reactions each), approximately 1 or 2 transformant colonies showed the desired band patterns and were considered valid double-crossover mutants. Sanger sequencing of the colony PCR products associated with the KO strains was also performed to confirm the desired deletion event. If no colonies showed the desired band pattern, colonies that displayed PCR products for all three primer sets (indicating a mixed genotype) were twice restreaked on solid 2× YTG + Ery40. Twelve of the resultant colonies were again screened with the same colony PCR primer sets, and approximately 2 or 3 of these colonies were validated as successful KO strains.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02942-16>.

TEXT S1, PDF file, 1.8 MB.

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