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Article

Antisense-RNA-Mediated Gene Downregulation in *Clostridium pasteurianum*

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Abstract: *Clostridium pasteurianum* is receiving growing attention for its unique metabolic properties, particularly its ability to convert waste glycerol and glycerol-rich byproducts into butanol, a prospective biofuel. Genetic tool development and whole genome sequencing have recently been investigated to advance the genetic tractability of this potential industrial host. Nevertheless, methodologies for tuning gene expression through plasmid-borne expression and chromosomal gene downregulation are still absent. Here we demonstrate plasmid-borne heterologous gene expression and gene knockdown using antisense RNA in *C. pasteurianum*. We first employed a common thermophilic β-galactosidase (*lacZ*) gene reporter system from *Thermoanaerobacterium thermosulfurogenes* to characterize two promoters involved in the central fermentative metabolism of *C. pasteurianum*. Due to a higher level of constitutive *lacZ* expression compared to the ferredoxin gene (*fdx*) promoter, the thiolase (*thl*) promoter was selected to drive expression of asRNA. Expression of a *lacZ* asRNA resulted in 52%–58% downregulation of β-galactosidase activity compared to the control strain throughout the duration of culture growth. Subsequent implementation of our asRNA approach for downregulation of the native hydrogenase I gene (*hydA*) in *C. pasteurianum* resulted in altered end product distribution, characterized by an increase in production of reduced metabolites, particularly butyrate (40% increase) and ethanol (25% increase). Knockdown of *hydA* was also accompanied by increased acetate formation and

lower levels of 1,3-propanediol, signifying a dramatic shift in cellular metabolism in response to inhibition of the hydrogenase enzyme. The methodologies described herein for plasmid-based heterologous gene expression and antisense-RNA-mediated gene knockdown should promote rational metabolic engineering of *C. pasteurianum* for enhanced production of butanol as a prospective biofuel.

Keywords: antisense RNA; biofuels; *Clostridium*; gene reporter; genetic engineering; glycerol; gene knockdown; metabolic engineering

1. Introduction

Clostridium pasteurianum is a strictly anaerobic, Gram-positive, spore-forming bacterium that has received growing biotechnological attention for its capacity to ferment low-value waste feedstocks, particularly crude glycerol, to commodity chemicals and biofuels [1–3]. *C. pasteurianum* is the only known organism capable of converting glycerol to butanol [4], an attractive biofuel that is superior to ethanol [5–7]. Cheap crude glycerol is currently available in abundance as a result of the tremendous growth experienced by global biodiesel and bioethanol industries in recent years [8,9]. Biodiesel production yields 10% (*w*/*w*) crude glycerol, while up to 20 liters of glycerol-rich thin stillage can result from the production of only 1 liter of bioethanol [10]. In 2007, global biodiesel production capacity from a total of 119 countries was estimated at 51 billion liters [11], leading to a plummet in the price of crude glycerol to approximately $0.05/lb [8]. Consequently, *C. pasteurianum* has become an attractive prospective industrial host for the production of butanol from abundant and renewable waste glycerol [3]. Up to now, few genetic tools have been developed for metabolic engineering of this important bacterium. We recently developed a high-efficiency electrotransformation procedure for gene transfer to *C. pasteurianum* [12], in addition to an intron-based method of chromosomal gene disruption [13]. Several genome sequencing projects are underway [14–16], including two completed genome sequences [17,18] and one genomic analysis report [19]. Collectively these developments serve as the initial steps toward extensive metabolic engineering of this unique species [20].

A fundamental facet of metabolic engineering is the ability to decrease the expression of genes involved in competing metabolic pathways through the use of gene knockdown and knockout techniques [21]. Gene knockouts cause complete inhibition of gene expression and, therefore, often lead to lethal phenotypes, particularly for genes involved in central metabolic pathways [22]. For example, disruption of the hydrogenase or thiolase genes [23] in *C. acetobutylicum* is presumed to result in cell death, thus placing significant constraints on rational metabolic engineering strategies employing gene knockout. Antisense-RNA-mediated gene downregulation, on the other hand, is a more refined approach for limiting expression of native genes and metabolic pathways, as production of the target protein is still permitted, though at a substantially decreased level [24,25]. For organisms that carry out highly branched and complex fermentations, such as *C. pasteurianum* [5,26], metabolic engineering is a very useful tool for limiting or entirely abolishing carbon and electron fluxes through competing pathways [20]. Although *C. pasteurianum* naturally produces appreciable quantities of butanol (up to 17 $g \cdot L^{-1}$ [27]) from glycerol, 1,3-propanediol also accumulates in the culture medium, thus limiting flux through the

butanol biosynthetic pathway. Gene downregulation is expected to be of great value to metabolic engineering of *C. pasteurianum* [3], as current understanding of glycerol catabolism in *Clostridium* dictates that disruption of the reductive 1,3-propanediol pathway will uncouple biomass formation and glycerol dissimilation [4], leading to inability to metabolize glycerol and subsequent cell death. Therefore, attenuation of competing pathways via antisense-RNA-mediated gene knockdown is a viable option for increasing butanol titer from fermentation of glycerol by *C. pasteurianum*.

Antisense RNAs are designed to possess partial or complete complementarity to a target mRNA, leading to base-pairing and degradation of the resulting RNA duplex prior to mRNA translation [24,28,29]. Alternatively, it has been proposed that mRNA–asRNA complexes sterically block translational machinery, resulting in reduced levels of the target protein [24]. This strategy has been employed in several clostridial species, including *C. acetobutylicum* [24,25,30], *C. cellulolyticum* [31], and *C. saccharoperbutylacetonicum* [32], for downregulation of competing metabolic pathways, including butyrate and acetone formation pathways, to enhance butanol titers, selectivity, and tolerance [24,25,30,31,33,34]. Degree of gene downregulation has been shown to vary, for example between 44% and 92% in one report [25], and is commonly quantified by measuring enzyme activity or production of protein target via Western blot analysis. Downregulation can also be finely tuned by selection of the promoter driving transcription of the asRNA, where stronger promoters presumably generate more asRNA product, leading to a greater degree of downregulation [24]. Promoters utilized in successful asRNA approaches include ones from genes involved in central carbon metabolism, such as phosphotransbutyrylase (*ptb*) and thiolase (*thl*) [35], as well as butanol dehydrogenase (*bdh*) [32].

In this report, we engineered *C. pasteurianum* through manipulation of gene expression. We employed a common clostridial gene reporter system based on the *Thermoanaerobacterium thermosulfurogenes* β-galactosidase gene (*lacZ*) [35] to assess the relative strength of two gene promoters involved in the central fermentative metabolism of *C. pasteurianum*. We then selected the native *C. pasteurianum* thiolase promoter to drive transcription of asRNA targeted to our *lacZ* reporter gene. To assess the efficacy of gene knockdown in *C. pasteurianum*, we targeted the primary hydrogenase gene (*hydA*) for downregulation using our devised asRNA approach. Collectively, we demonstrate that asRNA gene knockdown and plasmid-based heterologous gene expression, along with a complete genome sequence [18,19] and previously developed genetic tools [12,13], should promote the feasibility of metabolic engineering and strain optimization of *C. pasteurianum*.

2. Experimental Section

2.1. Bacterial Strains, Cultivation Conditions, and Electrotransformation

Bacterial strains utilized in this work are listed in Table 1. *C. pasteurianum* ATCC 6013 was cultivated in 2×YTG medium (per liter: 16 g tryptone, 10 g yeast extract, 5 g glucose, 4 g sodium chloride, pH 6.2) and spores were maintained on solidified 2×YTG medium containing 12 $g \cdot L^{-1}$ agar, as described previously [12]. Vegetative cultures were prepared by suspending and heat shocking a sporulated colony at 80 °C for 10 min. Recombinant *C. pasteurianum* strains were selected using 10 $\mu g \cdot mL^{-1}$ thiamphenicol or 6 $\mu g \cdot mL^{-1}$ clarithromycin. Electrotransformation of *C. pasteurianum* was performed according to a previous report [12].

Table 1. Strains, plasmids, and oligonucleotides employed in this study.

Strains or Plasmids	Relevant Characteristics	Source or Reference
Strains		
Escherichia coli DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG φ80dlacZΔM15* Δ(*lacZYA-argF*)*U169*, *hsdR17*($r_K^- m_K^+$), λ^-	Lab stock
Escherichia coli ER1821	F^- *endA1 glnV44 thi-1 relA1? e14*$^-$(*mcrA*$^-$) *rfbD1? spoT1?* Δ(*mcrC-mrr*)*114::IS10*	Lab stock; New England Biolabs
Clostridium pasteurianum ATCC 6013	Wild-type	American type culture collection
Plasmids		
pFnuDIIMKn	*E. coli* vector expressing the M.FnuDII methyltransferase for plasmid methylation prior to electrotransformation of *C. pasteurianum* (Kn^R; p15A ori)	[12]
pHT3	Promoterless *E. coli-C. pasteurianum* β-galactosidase gene expression reporter vector (Ap^R; ColE1 ori; Em^R; pIM13 ori)	[35]
pMTL85141	*E. coli-C. pasteurianum* shuttle vector (ColE1 ori; Tm^R; pIM13 ori)	[36]
pSY6catP	*E. coli-Clostridium* group II intron expression vector (Ap^R; ColE1 ori; Tm^R; pIM13 ori)	[12]
pHT3catP	Thiamphenicol-resistant derivative of pHT3	This study; [37]
pHT3catP-fdx	Derived by transcriptionally fusing the *C. pasteurianum fdx* promoter with the *lacZ* gene of pHT3catP	This study
pHT3catP-thl	Derived by transcriptionally fusing the *C. pasteurianum thl* promoter with the *lacZ* gene of pHT3catP	This study
pHTaslacZ110	Vector expressing a 110 nt *lacZ* asRNA from the *C. pasteurianum thl* promoter	This study
pHTashydA	Vector expressing a 175 nt *hydA* asRNA from the *C. pasteurianum thl* promoter	This study
Oligonucleotide	**Sequence (5′–3′) ***	
Pfdx.NarI.S	CAGAACGGCGCCGAAGATATAAGAAAAAGACTCCCAAAGG	
Pfdx.XmaI.AS	ATCATACCCGGGCCATAACTTATTGTATCATGTTTTTAAAC	
Pthl.NarI.S	CTGTAGGCGCCGATATAGTCTATAAGCATTTAGATGGAGTTAG	
Pthl.XmaI.AS	AAATACCCGGGTCGATTGTTATTTAATTCACAATTTAATTATAACC	

* Relevant restriction endonuclease recognition sequences are underlined.

2.2. DNA Isolation, Manipulation, and Plasmid Construction

Plasmids and oligonucleotides utilized in this work are shown in Table 1. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA) at the 25 nanomole scale using standard desalting. Restriction endonucleases, Quick Ligation Kit, Standard *Taq* DNA Polymerase, and Phusion DNA Polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Molecular biology kits for purification of plasmid DNA, PCR products, and gel purified DNA and custom gene

synthesis were purchased from Bio Basic Inc. (Markham, ON, USA). Recombinant DNA methods were performed according to standard procedures [38]. Total genomic DNA was isolated from *C. pasteurianum* as previously described [13].

A thiamphenicol derivative of pHT3 [35], named pHT3catP [37], was constructed by subcloning a 1314 bp ClaI + SacI restriction fragment of pSY6catP [12] containing a thiamphenicol-resistance cassette into the corresponding restriction sites of the erythromycin-resistance determinant of pHT3. Transcriptional promoter fusions with *lacZ* in pHT3catP were prepared by amplifying putative *fdx* and *thl* promoter regions from *C. pasteurianum* total genomic DNA using primer pairs Pfdx.NarI.S + Pfdx.XmaI.AS and Pthl.NarI.S + Pthl.XmaI.AS, respectively. The resulting 565 bp (*fdx*) and 511 bp (*thl*) PCR products were digested with NarI + XmaI and ligated into the corresponding sites of pHT3catP to generate pHT3catP-fdx and pHT3catP-thl, respectively. To construct plasmid pHTaslacZ110, a *lacZ* asRNA expression cassette containing the *C. pasteurianum thl* promoter, *lacZ* asRNA, and *C. pasteurianum fdx* transcriptional terminator was obtained as a custom AhdI- and BstAPI-flanked gene synthesis fragment. The resulting 517 bp fragment was digested with AhdI + BstAPI and inserted into the corresponding restriction sites of pHT3catP-thl. Plasmid pHTashydA was constructed by obtaining a 276 bp BamHI- and BstXI-flanked custom gene synthesis product possessing a 175 nt *hydA* asRNA upstream of the *C. pasteurianum fdx* transcriptional terminator. Antisense RNA was designed to target the RBS and 164 nt of the 5' *hydA* coding region. The resulting synthetic product was digested with BamHI + BstXI and ligated into the corresponding sites of pHT3catP-thl for transcriptional fusion of the *hydA* asRNA with the *C. pasteurianum thl* promoter.

2.3. Strain Cultivation

β-Galactosidase expression and enzymatic assay were performed as previously described [35,39], with slight modifications. For expression of recombinant *C. pasteurianum* ATCC 6013 harboring pHT3catP, pHT3catP-fdx, pHT3catP-thl, or pHTaslacZ110, 45 mL samples were removed from each flask in duplicate every 3–5 h starting at an A_{600} of ~0.5. Cells were harvested from cultures by centrifugation at 10,000× *g* for 10 min at 4 °C, and the resulting cell pellets were stored at −80 °C. Cell pellets were thawed on ice, resuspended in Z buffer to an OD_{600} of 20 in a minimum volume of 1 mL, and lysed by incubation in the presence of 1 mg/mL lysozyme for 1 h at 37 °C, unless specified otherwise. One mL of the resulting lysates was cleared of cell debris by centrifugation at 16,000× *g* for 15 min at 4 °C and the supernatant was heated in a 60 °C water bath for 30 min. Denatured heat-labile proteins were cleared by centrifugation at 16,000× *g* for 30 min at 4 °C. Supernatants were stored at 4 °C prior to enzymatic assays.

Shake flask cultures expressing *hydA* asRNA (pHTashydA) or the control plasmid (pMTL85141) were cultivated in 50 mL of semi-defined medium [4,40] containing per liter: 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 7.35 g $(NH_4)_2SO_4$, 3.0 g $CaCO_3$, 5.08 g Bacto yeast extract, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.06 g $FeSO_4 \cdot 7H_2O$, 1 mg resazurin, and 2 mL trace element solution SL 7. The initial pH of the medium was adjusted to 6.0 prior to sterilization. Glycerol was sterilized separately as a stock solution and added to culture flasks to attain a final concentration of 50 g L^{-1}. Cysteine–HCl (0.5 g L^{-1}) was used to reduce growth medium. Cultures were incubated anaerobically with slight agitation (150 rpm) for 40–50 h. Seed cultures were prepared in 2×YTG medium from sporulated heat-shocked colonies.

2.4. Analytical Methods

Cell growth was quantified spectrophotometrically by measuring culture optical density at 600 nm (OD_{600}). β-Galactosidase activity was assayed as described [35] at 60 °C by measuring optical density at 420 nm (OD_{420}) following development of yellow color resulting from cleavage of ortho-nitrophenyl-β-galactoside (ONPG) to orthonitrophenol. Specific β-galactosidase activity is expressed in Miller units [39].

Shake flask culture supernatants were analyzed for metabolite production by LC-10AT HPLC analysis (Shimadzu, Kyoto, Japan) containing a RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA). The mobile phase consisted of 0.005 M H_2SO_4 (pH 2.0) at a flow rate of 0.6 $mL \cdot min^{-1}$. Data processing was performed using Clarity Lite (DataApex, Prague, Czech Republic). Metabolite titers reported represent the average of two biological replicates.

3. Results

A modified thiamphenicol-selectable reporter system, pHT3catP, containing a thermophilic β-galactosidase gene, *lacZ* [35], was utilized to assess the function of two promoters involved in the central metabolism of *C. pasteurianum*. Putative promoter regions from the native ferredoxin (*fdx*) [41] and thiolase (*thl*) [42] genes of *C. pasteurianum* were cloned upstream of *lacZ* in pHT3catP to generate transcriptional fusions (P_{fdx}-*lacZ* and P_{thl}-*lacZ*, respectively). The respective plasmids, pHT3catP-fdx and pHT3catP-thl, in addition to the promoterless pHT3catP control vector, were electrotransformed to *C. pasteurianum* ATCC 6013 and the resulting recombinant strains were used to assess expression of *lacZ* (Figure 1). The control plasmid pHT3catP failed to generate β-galactosidase activity (<8 MU) throughout the duration of cell growth. Conversely, both pHT3catP-fdx and pHT3catP-thl yielded significant β-galactosidase activity. Specifically, the P_{fdx}-*lacZ* construct generated a lower maximal β-galactosidase activity (144 MU; Figure 1A), compared to the P_{thl}-*lacZ* strain (439 MU; Figure 1B). Although the *fdx* promoter has been widely used for gene expression in a range of clostridia [43,44], we opted for the stronger *thl* promoter for subsequent expression of asRNA in *C. pasteurianum*.

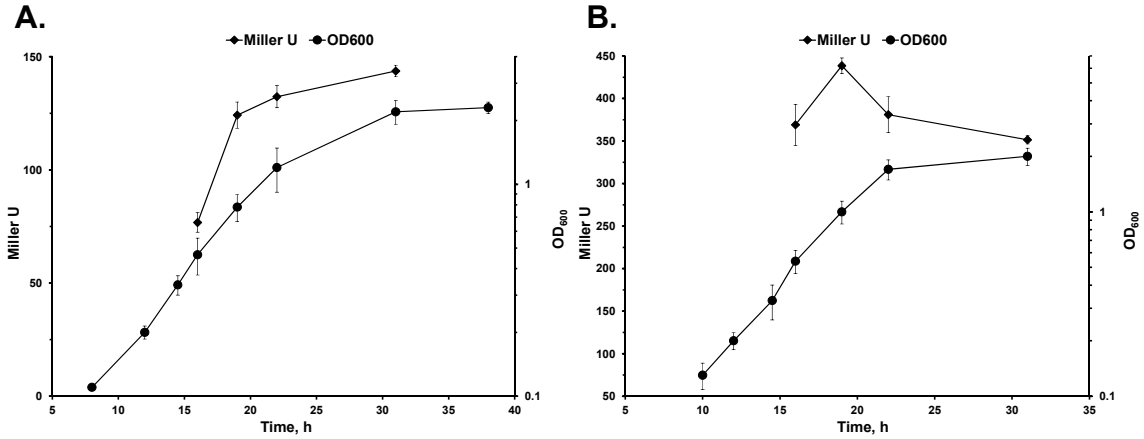

Figure 1. Time course β-galactosidase activities of static flask cultures harboring *lacZ* transcriptional fusion constructs. (**A**) P_{fdx}-*lacZ* cassette (pHT3catP-fdx); (**B**) P_{thl}-*lacZ* cassette (pHT3catP-thl).

To assess the efficacy of asRNA-mediated gene downregulation in *C. pasteurianum*, we again utilized thermophilic *lacZ* as a reporter gene. An asRNA cassette was designed by placing a 110 nt *lacZ* asRNA under transcriptional control of the *thl* promoter (P_{thl}-as*lacZ*110) and a transcriptional terminator derived from the *C. pasteurianum fdx* gene immediately downstream of the asRNA sequence. The 110 nt *lacZ* asRNA was designed to target 66 bp upstream of the initiation codon, including the ribosome binding site, and the first 44 bp of the *lacZ* coding sequence. The resulting P_{thl}-as*lacZ*110 cassette was inserted into plasmid pHT3catP-thl, generating pHTaslacZ110. In this configuration, the *lacZ* asRNA cassette was expressed from a plasmid backbone also containing the full-length P_{thl}-*lacZ* gene, resulting in a plasmid harboring two copies of the same *thl* promoter (Figure 2A). Following electrotransformation, recombinant *C. pasteurianum* strains respectively harboring pHT3catP, pHT3catP-thl, or pHTaslacZ110 were cultivated in parallel and cells were harvested and assayed for β-galactosidase activity at various culture time points (Figure 2B). The P_{thl}-as*lacZ*110 asRNA strain produced only 52%–58% of the β-galactosidase activity generated by the P_{thl}-*lacZ* control strain, thus demonstrating appreciable downregulation of plasmid-encoded *lacZ*.

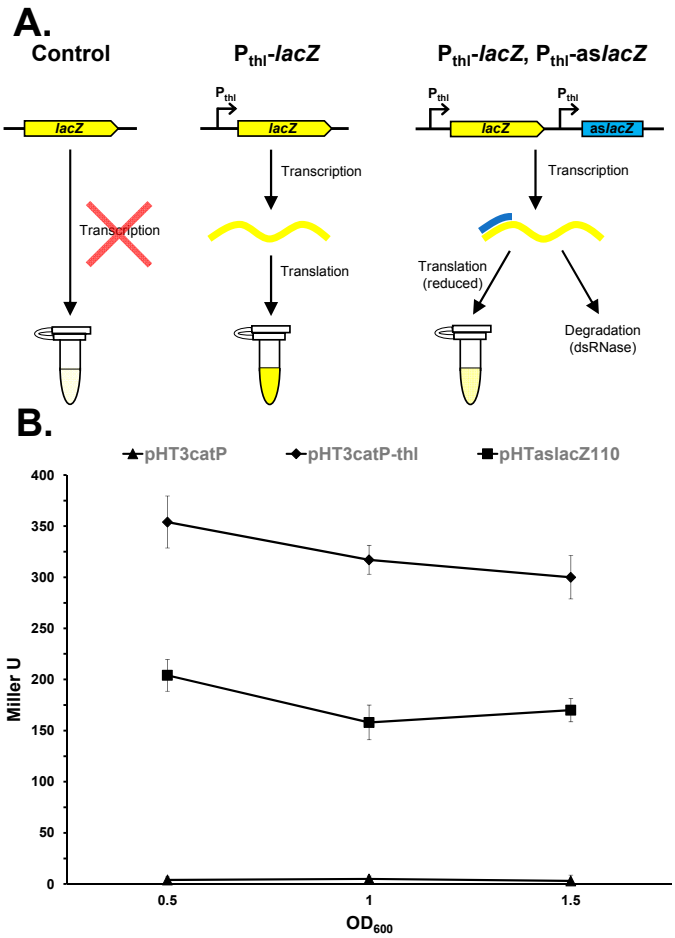

Figure 2. Antisense-RNA-mediated gene downregulation in *C. pasteurianum*. (**A**) Antisense RNA strategy and proposed mechanism of gene downregulation based on β-galactosidase enzymatic assay. dsRNase refers to putative host RNase(s) recognizing double-stranded RNA molecules; (**B**) β-Galactosidase activities of static flask cultures harboring promoterless *lacZ* plasmid pHT3catP, P_{thl}-*lacZ* plasmid pHT3catP-thl, and P_{thl}-as*lacZ*110 antisense RNA plasmid pHTaslacZ110.

Based on successful downregulation of heterologous *lacZ* using asRNA, we aimed to extend our approach to a native *C. pasteurianum* gene involved in the organism's central fermentative metabolism. Accordingly, we designed a 175 nt asRNA cassette targeted to the *C. pasteurianum* hydrogenase I gene (*hydA*). The resulting plasmid (pHTashydA175), in addition to the control plasmid lacking asRNA (pMTL85141), were transferred to *C. pasteurianum* and shake flask cultivations were performed using a semi-defined growth medium containing 50 g L^{-1} glycerol. Compared to the control strain, the *hydA* asRNA strain produced markedly less gas, as evidenced from a reduced amount of bubbles and foam at the surface of shake flasks. This outcome is consistent with decreased hydrogen gas evolution resulting from hydrogenase inhibition. Cultures harboring pHTashydA175 also reached a higher final cell density and exhibited a shorter cultivation time (Table 2). End product analysis of cultures harboring pMTL85141 control plasmid and pHTashydA asRNA plasmid revealed dramatic differences in product distribution. The *hydA* asRNA strain produced enhanced levels of acetate (137% increase), butyrate (40% increase), ethanol (25% increase) and, to a lesser extent, butanol (12% increase) (Table 2). 1,3-Propanediol was the only metabolite that was produced at a lower level (43% decrease) following expression of asRNA relative to the control strain. Overall, *hydA* asRNA expression increased total acid production by 108%, while total alcohol formation was largely unaffected.

Table 2. End product analysis of shake flask cultures harboring pMTL85141 and pHTashydA.

Strain	Culture time (h)	OD_{600}	Substrate and product titers (g L^{-1})							
			Glycerol	Acetate	Butyrate	Ethanol	Butanol	1,3-PDO	Total acids	Total alcohols
WT (pMTL85141)	52.5	7.6 ± 0.4	11.4 ± 0.06	0.38 ± 0.0	0.15 ± 0.02	1.6 ± 0.04	8.9 ± 0.3	3.3 ± 0.3	0.53 ± 0.02	13.8 ± 0.05
WT (pHTashydA)	43.0	10.3 ± 0.07	12.3 ± 0.1	0.90 ± 0.4	0.21 ± 0.0	2.0 ± 0.1	10.0 ± 0.0	2.3 ± 0.03	1.1 ± 0.4	14.4 ± 0.07

4. Discussion

Fundamental genetic techniques to modulate host gene expression through either gene upregulation (*i.e.*, overexpression) or downregulation (*i.e.*, knockdown) have found widespread use within the clostridia for various strain construction applications [21]. Since a method of gene transfer has only recently been reported for *C. pasteurianum* [12], techniques to manipulate gene expression are lacking for this species. Here we demonstrate the utility of both plasmid-based heterologous gene expression and asRNA-mediated gene knockdown in *C. pasteurianum*.

By generating transcriptional fusions, gene reporter systems facilitate characterization of a range of host promoters, resulting in a diverse promoter library, ideally exhibiting a broad spectrum of expression strengths. Most frequently, strong constitutive promoters are desired to maximize expression of a target gene or metabolic pathway throughout the duration of culture growth [20]. To compare promoters and provide a quantitative means of assessing relative promoter strength in *C. pasteurianum*, we selected a thermophilic β-galactosidase gene reporter system [35], since it has found widespread use in the clostridia [20]. We selected promoters from the *C. pasteurianum thl* and *fdx* genes, since the *C. acetobutylicum thl* gene promoter leads to high level constitutive gene expression [35] and the ferredoxin protein accounts for up to 2% of total soluble protein in growing cultures of *C. pasteurianum* [45].

We showed that the *C. pasteurianum thl* promoter generated roughly three-fold higher maximal β-galactosidase activity compared to the native *fdx* promoter (Figure 1). A similar result was obtained using *C. acetobutylicum*, as the native *thl* promoter out-performed promoters from the *C. acetobutylicum* phosphotransbutyrylase (*ptb*) and acetoacetate decarboxylase (*adc*) genes [35]. Thiolase, which catalyzes the transition from C_2 to C_4 intermediates in clostridial metabolism [5,26], would be expected to be expressed at a high level in both of these organisms, since the preferred products of glucose fermentation are butyrate (C_4) in the case of *C. pasteurianum* [46] and butyrate and butanol (C_4) in the case of *C. acetobutylicum* [5]. Although the *thl* promoter proved superior in *C. pasteurianum*, the *C. pasteurianum fdx* promoter still produced appreciable β-galactosidase activity owing to ferredoxin's central role in clostridial fermentative metabolism. Further, the *C. pasteurianum fdx* promoter has been shown to function in *C. acetobutylicum* and *C. sporogenes* [44], in addition to *C. cellulolyticum* [43,47] and *E. coli* [41].

In addition to promoter characterization, expression of *lacZ* in this study exemplifies the first demonstration of quantifiable heterologous gene expression in *C. pasteurianum*. Accordingly, the *thl* and *fdx* promoters, along with the associated pHT3 vector backbone, which is based on pIMP1 [48] and contains the commonly employed pIM13 Gram-positive replication module, together constitute the fundamental genetic components necessary for expressing heterologous genes and pathways in *C. pasteurianum*. In this context, the *thl* promoter is favored for applications demanding high-level gene expression, while the *fdx* promoter could be better suited in instances where maximal levels of gene expression are not paramount or desirable. Further, although not investigated in this report, the vector elements required for heterologous gene expression can also be utilized to enhance expression of native *C. pasteurianum* chromosomal genes through plasmid-based gene overexpression. Both heterologous and homologous gene expression strategies are vital to producing superior host strains through metabolic engineering and strain optimization [20].

Based on time course studies of β-galactosidase activity (Figure 1), we selected the *thl* promoter to drive transcription of asRNA in *C. pasteurianum* (Figure 2A). Using this approach, we achieved greater than 50% knockdown of *lacZ* at multiple time points throughout culture growth (Figure 2B), demonstrating that asRNA gene downregulation represents a viable option for controlling gene expression in *C. pasteurianum*. Since asRNA size and secondary structure are important design considerations [25] that were not investigated in this study, we advise the use of transcriptional terminators to avoid generation of superfluous non-homologous sequence at the 3′ end of asRNAs that may interfere with hybridization to target mRNAs. We also recommend testing multiple asRNAs in parallel for each knockdown target, as predicting asRNA effectiveness could prove challenging without assaying multiple designs.

The efficacy of our asRNA gene knockdown methodology was verified by targeting the primary hydrogenase gene (*hydA*) in *C. pasteurianum*. We observed a dramatic shift in end product distribution characterized by increased production of acids (acetate and butyrate) and ethanol (Table 2). Based on current models of clostridial metabolism, inhibition of the hydrogenase enzyme is expected to result in a buildup of reduced ferredoxin, which is subsequently utilized to drive reduction of NAD^+ to NADH by the ferredoxin:NAD^+ oxidoreductase enzyme. The NADH formed in this reaction is then employed for generation of reduced end products, such as ethanol and butanol. This shift in carbon and electron flow has been widely documented using carbon monoxide gassing in *C. acetobutylicum* [49] and

C. pasteurianum [27], which also results in inhibition of the hydrogenase system. While we observed higher levels of butyrate and ethanol, both NADH-consuming pathways, the butanol pathway was only slightly stimulated, suggesting that excess NADH formed from the ferredoxin:NAD^+ oxidoreductase enzyme was not utilized for production of butanol. Strikingly, acetate formation, which is not a redox pathway, was dramatically increased upon expression of *hydA* asRNA, exemplifying an unexpected shift in acid production. In total, combined acid production (acetate and butyrate) more than double in the *hydA* asRNA strain, suggesting that hydrogenase knockdown affects cellular energetics, as acetate and butyrate pathways result in ATP formation via substrate level phosphorylation. Indeed, the *hydA* knockdown strain exhibited a shorter cultivation time and increased biomass production (Table 2), which are consistent with a higher ATP yield. Finally, expression of *hydA* asRNA also exhibited an unexpected decline in 1,3-propanediol production, which ostensibly contrasts the presumed flow of electrons outlined above. Overall, however, we have observed substantial variability in 1,3-propanediol titer across fermentations conducted under seemingly identical conditions (data not shown), as 1,3-propanediol appears to be the most variable metabolite produced by *C. pasteurianum*. A similar lack of pathway control has been noted by others [3,4]. Therefore, the decreased production of 1,3-propanediol observed in this study may not represent a direct consequence of hydrogenase downregulation. Collectively, the findings unveiled in this study through downregulation of *hydA* underpin the complex and highly branched fermentative metabolism exhibited by *C. pasteurianum* and provide a groundwork for rational metabolic engineering of this important industrial bacterium.

5. Conclusions

In this work, we show that plasmid-based heterologous gene overexpression and asRNA-mediated gene downregulation are effective tools for altering gene expression in *C. pasteurianum*. The constitutive *fdx* and *thl* transcriptional promoters characterized in this study should find use in future *C. pasteurianum* gene expression applications, including plasmid-based expression of non-native pathways and cellular activities, as well as overexpression of key *C. pasteurianum* genes. We also show that central fermentative metabolism in *C. pasteurianum* can be dramatically altered through expression of asRNA targeted to the primary hydrogenase mRNA. Our devised asRNA methodology should expand the available set of genetic tools towards metabolic engineering of *C. pasteurianum,* through systematic knockdown of undesirable and unproductive chromosomal genes and metabolic pathways.

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Author Contributions

Michael E. Pyne helped conceived of the study, participated in its design and coordination, carried out the experiments, and drafted the manuscript. Murray Moo-Young participated in the study design and coordination. Duane A. Chung and C. Perry Chou helped conceive of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

Duane A. Chung is a founder and employee of Algaeneers Inc., at which Michael E. Pyne has also been employed. Algaeneers Inc. has a financial interest in production of biofuels using microorganisms.

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