

Development of a Suite of Tools for Genome Editing in *Parageobacillus thermoglucosidasius* and Their Use to Identify the Potential of a Native Plasmid in the Generation of Stable Engineered Strains

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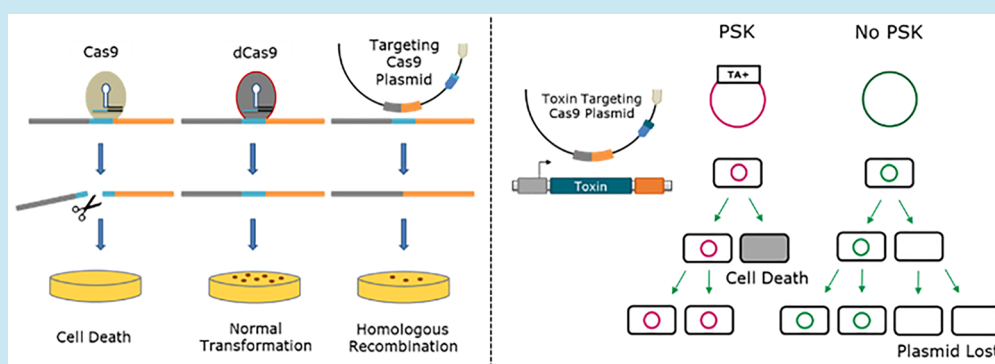
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ABSTRACT: The relentless rise in the levels of atmospheric greenhouse gases caused by the exploitation of fossil fuel necessitates the development of more environmentally friendly routes to the manufacture of chemicals and fuels. The exploitation of a fermentative process that uses a thermophilic chassis represents an attractive option. Its use, however, is hindered by a dearth of genetic tools. Here we expand on those available for the engineering of the industrial chassis *Parageobacillus thermoglucosidasius* through the assembly and testing of a range of promoters, ribosome binding sites, reporter genes, and the implementation of CRISPR/Cas9 genome editing based on two different thermostable Cas9 nucleases. The latter were used to demonstrate that the deletion of the two native plasmids carried by *P. thermoglucosidasius*, pNCI001 and pNCI002, either singly or in combination, had no discernible effects on the overall phenotypic characteristics of the organism. Through the CRISPR/Cas9-mediated insertion of the gene encoding a novel fluorescent reporter, eCGP123, we showed that pNCI001 exhibited a high degree of segregational stability. As the relatively higher copy number of pNCI001 led to higher levels of eCGP123 expression than when the same gene was integrated into the chromosome, we propose that pNCI001 represents the preferred option for the integration of metabolic operons when stable commercial strains are required.

KEYWORDS: CRISPR/Cas9, genetic tools, thermophile, stable plasmid expression, synthetic biology, curing plasmids

The chemicals and fuels on which the world relies are predominately derived from fossil reserves. The greenhouse gas emissions that are generated by exploiting these reserves, however, are the principle cause of climate change. Alternative, more sustainable routes to chemicals and fuels are, therefore, required. Microbial-based fermentations, in which renewable feedstocks derived from plant materials are converted into the desired product, represent one of the most promising solutions. The production of such “green” chemicals via this route remains relatively uncommon as the processes involved are generally uncompetitive compared to their conventional, petroleum-based counterpart. One way to improve the competitiveness is to use a more effective microbial chassis. For this, respective thermophilic chassis

offer a number of attractions over current model organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*.^{1–4}

Thermophilic organisms are often more robust and can withstand fluctuations in temperature, pH, and environmental change, traits that are deemed essential for a commercially viable process. High growth temperatures reduce running costs by limiting contamination by common mesophiles, removing

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the need for antibiotics, eliminating the need for cooling between successive fermentation cycles, and facilitating recovery of volatile chemicals through continuous distillation, thereby avoiding the issue of end-product inhibition.^{3,5–7} A further significant advantage is that the growth temperatures are optimum for enzymatic lignocellulose degradation, and therefore simultaneous hydrolysis, saccharification, and fermentation can be achieved.⁸ As a consequence, these organisms may be exploited as whole cell biocatalysts in a consolidated bioprocess.⁹ However, currently, the widespread use of thermophiles is hindered by the lack of well-developed genome editing tools that are abundant in their mesophilic counterparts.^{10–12}

Parageobacillus thermoglucosidasius (previously called *Geobacillus thermoglucosidasius*)¹³ is a thermophilic, Gram-positive bacterium and is facultatively anaerobic, with a growth range demonstrated to be between 37 and 68 °C.¹⁴ It is one of the few thermophiles that can be successfully genetically manipulated and has demonstrated its capability to produce biofuels from lignocellulose, therefore making *P. thermoglucosidasius* a current target for industry.^{15–18} Like many *Geobacillus* and *Parageobacillus* species, *P. thermoglucosidasius* has both a fast growth rate and can ferment a wide variety of sugars.

In an effort to capitalize on the inherent advantages of thermophiles, Cripps et al. (2009) genetically engineered *P. thermoglucosidasius* to produce ethanol as a primary product during fermentation.¹⁵ The strain created, TM242, produced ethanol at 95% of theoretical yields when grown on cellobiose as the carbon source. More recently, the same strain was shown to produce ethanol at yields equating to 9.9 ± 0.4 g/L (92% yield) in a fermentation that used palm kernel cake (PKC) as the feedstock, a mannan-rich waste product from palm oil processing.¹⁶ As the methods used to create TM242 were relatively laborious, we previously described (2017) a less labor-intensive procedure for the creation of knock-ins (KIs) and knockouts (KOs) that exploit the *pyrE* gene as both a positive and negative selection marker.¹⁹ While an improvement, the method does not significantly reduce the time needed to generate mutant strains or allow for location-independent gene KIs.

One solution both to speed up mutant generation and to make it more efficient would be to implement genome editing based on CRISPR (clustered regularly interspaced short palindromic repeats) technology.²⁰ This prokaryote-adaptive immune system has been shown to have the ability to identify and selectively cut exogenous DNA. This occurs through the identification of a complementary target called a protospacer and the introduction by a CRISPR-associated protein (Cas) of a lethal double-stranded DNA break downstream of this location at a DNA motif called a protospacer-adjacent motif (PAM). In order to manipulate this system to achieve genome editing, a synthetic guide RNA (sgRNA) is designed, which is complementary to the target site, upstream of the specific PAM. This sgRNA can then guide the Cas protein to the specified targeted sequence, which will cut the DNA. The *cas9* from *Streptococcus pyogenes* (spCas9) is the most commonly used Cas9 endonuclease and recognizes a PAM sequence of 5'-NGG-3', making the specific sgRNA sequence 5'-N₂₀-NGG-3'.^{20–24} Genome editing can then be achieved using DNA repair systems, either nonhomologous end joining in eukaryotes or homology-directed repair in prokaryotes.^{25,26}

To date, spCas9 has successfully been exploited for prokaryotic genome editing in several different organisms.^{25,27–30} However, previous studies have demonstrated that spCas9 is inactive at temperatures above 42 °C, making its use in *P. thermoglucosidasius* challenging.³¹ A thermostable Cas9 from *Geobacillus thermodenitrificans* has since been identified.³² Here we decided to exploit well-characterized CRISPR/Cas9 systems from the moderate thermophile *Streptococcus thermophilus*^{33–35} and demonstrate the use of both its Cas9 nucleases, stCas91 and stCas93, to generate gene deletions in *P. thermoglucosidasius*. Exemplification was made possible by the assembly and characterization of a range of regulatory elements to control the expression of the *cas* genes, including a novel reporter gene encoding a thermophilic fluorescent protein, eCGP123. The system developed was used to show that deletion of one or both of the two native plasmids present within *P. thermoglucosidasius*, pNCI001 and pNCI002, had no discernible effects on either the growth of the host or metabolites produced. Through the use of the novel reporter and CRISPR/Cas9-mediated insertion of the encoding gene, plasmid pNCI001 was shown to be remarkably segregationally stable. The production of eCGP123 by cells carrying the modified pNCI001 plasmid was higher than when the same gene was inserted into the chromosome. We propose that when stable inheritance of metabolic operons in commercial strains is required pNCI001 represents a preferred option over the chromosome as its higher relative copy number favors elevated production levels.

RESULTS AND DISCUSSION

Development and Exploitation of a Fluorescence Reporter System for the Characterization of Regulatory Parts. To evaluate the effectiveness of stCas91 and stCas93 in *P. thermoglucosidasius* NCIMB 11955 as a basis for a genome editing system, effective expression of these nucleases, in combination with their corresponding sgRNA, was required. To identify the most effective promoter systems to accomplish this, a novel reporter system was first established in *P. thermoglucosidasius* based on a thermostable fluorescent protein, eCGP123.^{36,37} Purified eCGP123 protein has previously been shown to fluoresce at 80 °C³⁶ under *in vitro* conditions and to function *in vivo* in the thermoacidophilic archaeon *Sulfolobus acidocaldarius*³⁸ but has yet to be evaluated in a bacterium. As superfolder green fluorescent protein (sfGFP) has previously been successfully expressed at high temperature in a number of different thermophilic bacteria,^{39,40} including in *P. thermoglucosidasius*,⁴¹ the performance of eCGP123 was compared to equivalent constructs made with sfGFP. The two reporter genes were placed under the transcriptional control of the previously characterized P_{ldh} promoter and localized to the modular plasmid pMTL61110.¹⁷ *P. thermoglucosidasius* cells carrying either plasmid were shown to fluoresce to a similar level at 60 °C and to maintain their activity up to and including at 75 °C (Figure 1). Compared to sfGFP, eCGP123 showed less variation in fluorescence over the temperature range tested and retained higher activity at temperatures greater than 65 °C (Figure 1).

Having established the suitability of eCGP123 as a reporter system, it was used to compare the effectiveness of a number of different promoters, Shine–Dalgarno (SD), and Rho-independent terminator sequences. Promoters (Table S1) were selected from *P. thermoglucosidasius* genes predicted to be

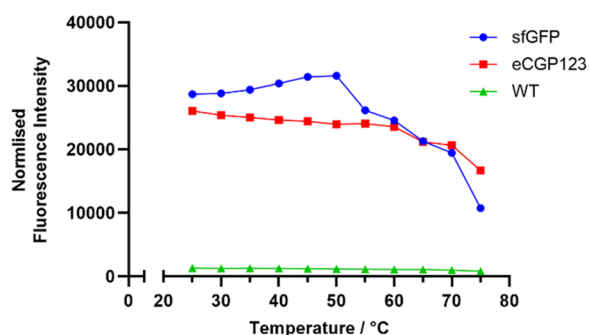


Figure 1. Assay to determine the thermostability of the fluorescent protein markers sfGFP and eCGP123. *P. thermoglucosidarius* was grown in 2SPYNG (50 mL) at 52 °C under aerobic conditions in a baffled conical flask shaking at 250 rpm for 8 h. Protein lysate was then extracted and quantified using a BugBuster 10X protein extraction reagent and Pierce BCA Protein Assay kit, respectively. The protein lysate of each fluorescent reporter was then incubated, and the level of fluorescence intensity per mg/mL of protein was tested at incrementally increasing temperatures.

highly expressed (e.g., from glycolysis or the TCA cycle), whereas the SD (Table S2) and Rho-independent terminator (Table S3) sequences were taken from other organisms, to minimize potential recombination events. Using the fluorescent reporter gene eCGP123, we were able to successfully produce relative strength libraries for promoters, SD sites, and Rho-independent terminators. These libraries have a broad range of expression levels which can be fine-tuned, a necessity for the expression of exogenous genes such as *cas9* (Figure 2).

Construction of CRISPR/Cas9 Genome Editing Vectors for *P. thermoglucosidarius*. To assemble CRISPR/Cas9 genome editing vectors, a similar architecture to that used in clostridia was adopted.³⁰ A crucial difference was that the spCas9 gene was replaced with either the stCas91 or stCas93 genes. Based on the plasmid pMTL61110, these vectors carried the ColEI and pUB110 origins of replication, a thermostable kanamycin resistance gene, the designated *cas* gene, and a locus encoding the specific sgRNA of the gene being targeted. Our experiments had identified that the two strongest promoters tested were that of the *ldh* and *gapDH* genes, P_{ldh} and P_{gapDH} . Accordingly, the stCas genes were placed under the control of P_{ldh} and the sgRNA under the control of P_{gapDH} . To prevent transcriptional readthrough, the terminators T1 and T2 were positional distal to the sgRNA locus. The two terminators are natively found together in the termination region of the *rrnB* gene of *E. coli* and has shown to be an efficient terminator.⁴²

The PAM sequences of stCas91 and stCas93 have previously been shown to be 5'-NNAGAAW-3' and 5'-NGGNG-3', respectively.^{43–48} In the initial experiments, sgRNAs incorporating these sequences were designed to target the acetate kinase gene, *ack*. When vectors, pCas91_Ack and pCas93_Ack, carrying the sgRNA and their respective stCas genes were used in transformation protocols with competent *P. thermoglucosidarius* cells, no kanamycin-resistant colonies were obtained. This was in contrast to the control plasmid, pCas9, which was lacking in either the sgRNA locus (pMTL575555 and pMTL675555) or both the *cas* gene and the sgRNA (pMTL61110), in which both produced a typical transformation efficiency of around 1×10^4 colonies. These observations are consistent with the fact that the stCas enzymes being produced were being successfully targeted to

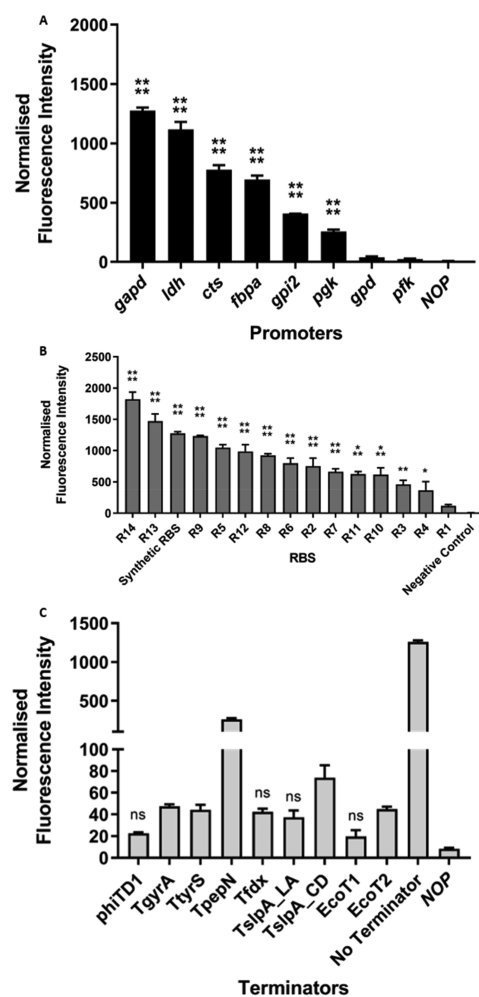


Figure 2. Relative strengths of promoters, ribosomal binding sites (RBSs), and rho-independent terminators tested in *P. thermoglucosidarius* as determined by the relative fluorescence units (RFUs) of eCGP123 expression normalized to protein concentration (mg/mL). *P. thermoglucosidarius* was grown in 2SPYNG (50 mL) at 52 °C under aerobic conditions in a baffled conical flask shaking at 250 rpm for 8 h. (A) Promoters from left to right are as follows: those derived from the genes encoding glyceraldehyde 3-phosphate dehydrogenase (*gapd*), lactate dehydrogenase (*ldh*), citrate synthase (*cts*), fructose-bisphosphate aldolase (*fbpa*), glucose-6-phosphate isomerase 2 (*gpi2*), phosphoglycerate kinase (*pgk*), glycerol-3-phosphate dehydrogenase (*gpd*), 6-phosphofruktokinase (*pfk*), and no promoter (NOP). All promoters originate from *P. thermoglucosidarius* with the exception of *ldh* which originates from *Geobacillus thermodenitrificans*. (B) The RBS library contained Shine–Dalgarno sequences from *Clostridium botulinum* as well as a negative control consisting of no consensus RBS sequence. The promoter, P_{gapd} remained constant throughout each of the RBS constructs. Statistical analysis was carried out using Graphpad Prism 7.03. A one-way analysis of variance (ANOVA) with a Dunnett's method post hoc analysis was used to determine whether the data presented from a sample were statistically different from the control. P values ≤ 0.05 were considered statistically significant and are represented by asterisks. The following asterisk format is used throughout: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. (C) Rho-independent terminators were expressed downstream of the promoter P_{gapd} used to express eCGP123. All the terminators show a statistically significant decrease compared to the sample containing no terminator. The samples showing no statistical difference compared to no promoter (NOP) are denoted by ns. The error bars on the graph denote the standard error of the mean ($n = 3$).

the *ack* gene, and the resultant double-strand DNA breaks generated could not be repaired by *P. thermoglucosidasius*, rendering the transformed cells nonviable. To confirm that the failure to obtain transformants was due to lethal cleavage of the host chromosome, the two *cas9* genes (*stcas91* and *stcas93*) were deliberately inactivated through the introduction of a frame-shift mutation, at their unique *PacI* and *Eco81I* sites, respectively, producing Δ *Cas9* (Materials and Methods). The plasmids carrying these frameshift mutations transformed *P. thermoglucosidasius* at equivalent frequencies to those observed with plasmids not carrying a *cas9* gene, a result also observed by Sapranauskas et al. (2011) in *E. coli*.⁴⁹ Taken together, these data indicated that both *S. thermophilus* enzymes are functional at the elevated temperature used for growth of *P. thermoglucosidasius*, at 52 °C.

Cas9-Induced Knockout of Genes through Homologous Recombination. Having established that both *stCas9* enzymes were active at 52 °C in *P. thermoglucosidasius*, plasmids were assembled equivalent to *pCas91_Ack* and *pCas93_Ack* but which additionally carried an ca. 1000 bp editing template comprising 500 bp homology arms (HAs) that flanked the *ack* gene. Replacement of the WT *ack* gene with this mutant allele by homologous recombination (HR) would result in an in-frame deletion mutant. The two plasmids created, *pCas91_Ack_hr* and *pCas93_Ack_hr*, successfully transformed *P. thermoglucosidasius*. The number of kanamycin-resistant colonies obtained from transformations, however, was significantly lower, <20 colonies, than control plasmids in which the *stCas9* genes were inactivated. PCR screening of colonies using oligonucleotide primers flanking the *ack* gene, following restreak of primary transformants onto selective agar media, demonstrated that a DNA band of a size consistent with deletion of *ack*, 1.2 kb, had occurred in 5 out of 12 colonies for *stCas91* and 10 out of 10 colonies for *stCas93* (Figure 3a). However, a band equivalent to the WT allele, 2.4 kb, was also present. Cells from such colonies were, therefore, subjected to six sequential passages of 12 h at 52 °C in fresh 2SPYNG km media before being screened again, all of which were now composed of pure mutants. Elimination of *ack* was selected due to evidence of successful deletion in many different species, including thermophiles, such as *E. coli*, *C. acetobutylicum*, and *Thermoanaerobacterium saccharolyticum*.^{50–52} Due to the function of *ack* in the mixed acid fermentation pathway, it is often deleted to improve expression of valuable products.

Having demonstrated that both *stCas9* enzymes could be used for genome editing, two further genes were targeted, lactate dehydrogenase (*Ldh*) and alcohol dehydrogenase (*AdhE*). In this case, only plasmids based on *stCas93* were constructed, as its PAM is more ubiquitous than that of *stCas91* and, therefore, provides greater scope for mutant generation. In the case of *ack*, for instance, 35 targets were present for *stCas93* compared to 4 for *stCas91*. Furthermore, *stCas93* demonstrated an increased efficiency to produce the *ack* mutant than *stCas91*, a result that has also previously been observed by Muller et al. (2016) in human cells.⁴⁶ Accordingly, vectors equivalent to those used to knockout *ack* were constructed for both *ldh* and *adhE*, and a similar protocol was used to generate in-frame deletion mutants (Figure 3b), as evidenced by the presence of a foreshortened DNA fragment. To rule out the possibility of a translocation event, a PCR containing internal primers was performed to ensure deletion of the gene in question (Figure 3c). Like *ack*, *ldh* and *adhE* are often key targets in the production of industrially significant

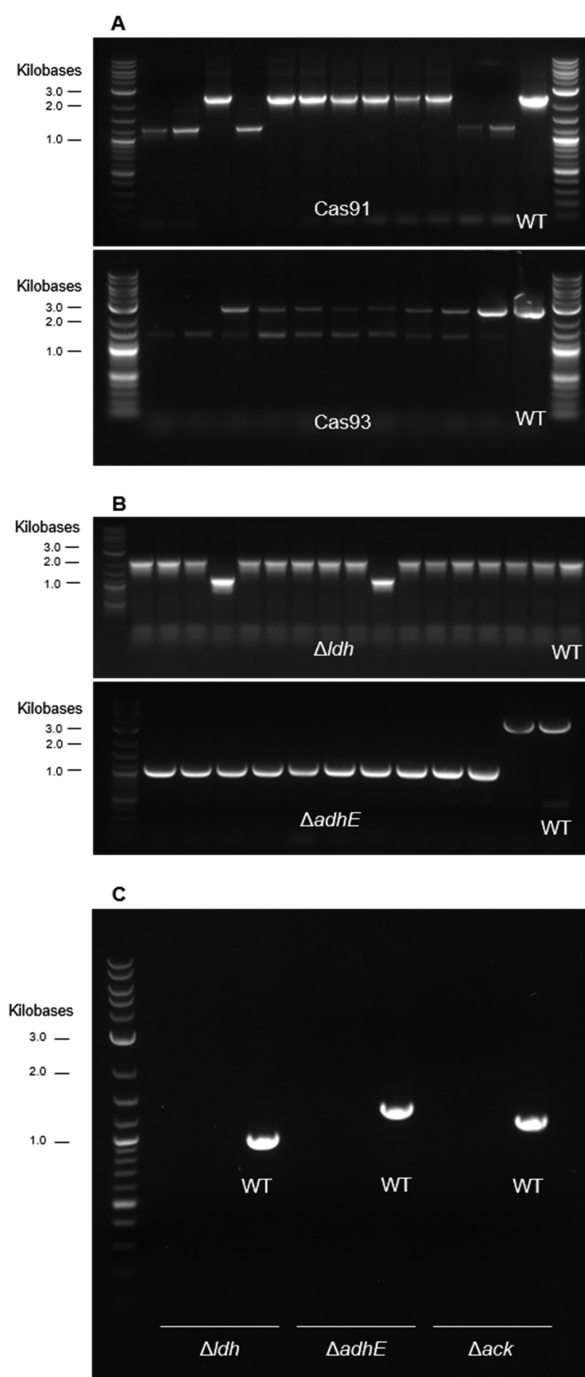


Figure 3. (A) Gel electrophoresis image of a colony PCR, utilizing *Ack_F* and *Ack_R* primers, to assess whether the acetate kinase (*ack*) gene had been successfully knocked out of the *P. thermoglucosidasius* genome using *stCas9-1* and *stCas9-3*. A PCR of WT *ack* was performed as a control (~2.4 kb). Colonies that have the Δ *ack* would yield a band at ~1.2 kb. A 2-log DNA ladder was used to determine the size of each band. (B) A gel electrophoresis image of a colony PCR performed to assess whether the genes encoding lactate dehydrogenase (*ldh*) and aldehyde-alcohol dehydrogenase (*adhE*) had been knocked out of the *P. thermoglucosidasius* genome. The primers used were (*ldh*) *CP_LDH_F* and *CP_LDH_R* and (*adhE*) *CP_ADH_F* and *CP_ADH_R*. A PCR of each WT gene was performed as a control (~2.1 kb and ~3.7 kb, respectively). Colonies that had the successful gene deletion would yield a band at ~1.1 kb. A 2-log DNA ladder was used to determine the size of each band. (C) A gel electrophoresis image of a colony PCR was performed to confirm loss of the genes, lactate dehydrogenase (*ldh*), aldehyde-alcohol

Figure 3. continued

dehydrogenase (*adhE*), and acetate dehydrogenase (*ack*), from the *P. thermoglucosidasius* genome. The internal gene primers were (*ldh*) LDH_Inter_F LDH_Inter_R, (*adhE*) ADH_Inter_F ADH_Inter_R, and (*ack*) ACK_Inter_F ACK_Inter_R. Amplification of the internal primers yields bands at ~0.9 kb, ~1.3 kb, and ~1.2 kb, respectively. A 2-log DNA ladder was used to determine the size of each band.

strains, to manipulate the metabolism of an organism, due to their role in mixed-acid fermentation, hence validating their inclusion into the study.

Curing of *P. thermoglucosidasius* Native Plasmids Using CRISPR/Cas9. Like many species of *Geobacillus* and *Parageobacillus*, *P. thermoglucosidasius* NCIMB 11955 contains two large plasmids, pNCI001 (~85 kb) and pNCI002 (~49 kb). As the maintenance of plasmid DNA by a cell has a metabolic cost,^{53,54} it would be expected that the burden imposed is counteracted by a plasmid-endowed attribute of benefit to the cell. The role of pNCI001 and pNCI002, however, remains cryptic as neither plasmid sequence has provided clues as to their function.⁵⁵ One way to ascribe function would be to isolate cured derivatives of *P. thermoglucosidasius* that no longer carry the plasmids and compare their phenotypes to the WT.^{56–58} Normally achieved using negatively selectable markers,^{56,57,59} this could now be achieved by using the developed CRISPR system to target the genes encoding the replication proteins of pNCI001 and pNCI002, RepB (BCV53_19210, BCV53_19380), and RepA (BCV53_19650), respectively.⁵⁵ Accordingly, stCas93-based KO plasmids were designed, carrying appropriate sgRNAs and editing templates comprised of a mutant *rep* gene allele. Following the transformation of *P. thermoglucosidasius* with the CRISPR plasmid targeting *repA*, 16 kanamycin-resistant colonies were screened, and all 16 were shown to be lacking plasmid pNCI002, as evidenced from the absence of an appropriately sized amplified DNA fragment in a colony PCR using oligonucleotide primers specific to the plasmid (Figure 4a). In contrast, no transformants were obtained using the CRISPR plasmid that targeted the *repB* gene of plasmid pNCI001.

On the face of it, these experiments demonstrate that pNCI002 is not essential and that pNCI001 may play an essential role under the conditions tested. A closer inspection of the pNCI001 sequence, however, indicated the presence of a putative toxin/antitoxin system. These typically ensure plasmid survival through post segregationally killing (PSK) as a consequence of the significant differences in the half-lives of its two components.⁶⁰ In essence, the lethal effects of the toxin are nullified by the antidotal antitoxin. However, if the cell simultaneously loses the ability to produce both components as a consequence of losing the plasmid/genes, then the significantly reduced half-life of the antitoxin compared to that of the toxin means that the cell is rapidly killed.^{60,61} Hence, no transformants were obtained when *P. thermoglucosidasius* was transformed with a CRISPR plasmid that prevented the replication of plasmid pNCI001 and by inference replenishment of cellular levels of the antitoxin. Plasmid maintenance in this way is a commonly reported phenomenon to maintain DNA, be it through maintaining plasmids or the stabilization of specific areas of chromosomal DNA.^{60–65}

If the toxin/antitoxin system prevents the loss of pNCI001, then the prior inactivation of the toxin gene, while maintaining

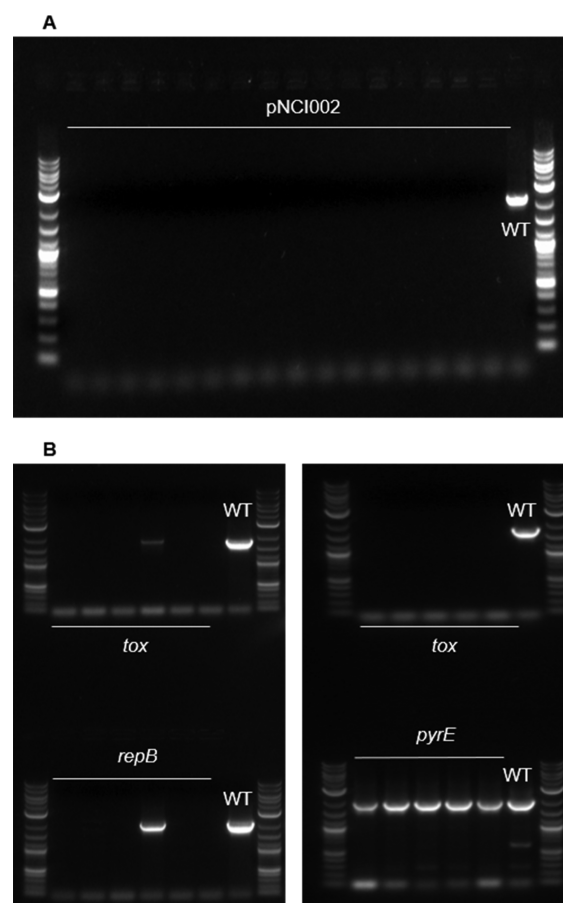


Figure 4. Gel electrophoresis image of colony PCR to assess the presence of the plasmids in *P. thermoglucosidasius*. (A) SMPR_CP_F and SMPR_CP_R primers were used to assess whether the pNCI002 replication gene (*repA*) had been knocked out. A PCR of the WT *repA* was performed as a control (~2.2 kb). Colonies containing $\Delta repA$ would yield a band at ~1.2 kb. However, no PCR product was observed when run on a 1% agarose gel, indicating the complete loss of the small plasmid (pNCI002). (B) A colony PCR to show the loss of pNCI001 from *P. thermoglucosidasius*. Two separate PCR reactions were carried out on the same colony. PCR of the *tox* gene utilized the primers Tox_CP_F and Tox_CP_R, and the *repB* replication gene used the primers BMPR_CP_F and BMPR_CP_R. A PCR of the WT *tox* gene and the *repB* was performed as a control (~1.8 kb and ~2.3 kb, respectively). Colonies containing Δtox would yield a band at ~1.3 kb. However, no PCR product was observed when run on a 1% agarose gel, indicating the complete loss of the pNCI001. The unsuccessful PCR of the *repB* further confirmed this finding. A colony PCR of the $\Delta repB$ mutants was performed to confirm the strain as *P. thermoglucosidasius*. PCR of *pyrE* was performed, utilizing the primers PyrE_F and PyrE_R, alongside a PCR of *tox*, as performed previously. PCR of WT *pyrE* was implemented as a control (~1.7 kb). A 2-log DNA ladder was used to determine the size of each band.

a functional antitoxin gene, would allow the plasmid to be subsequently cured. Accordingly, a CRISPR KO plasmid was designed that would introduce the desired mutation into the toxin gene. It was anticipated that the mutant plasmid generated could be subsequently cured from the cell by targeting *repB* with the previously constructed CRISPR KO vector. Surprisingly, when CRISPR/Cas vectors that targeted the toxin gene were introduced into *P. thermoglucosidasius*, the plasmid pNCI001 was lost immediately. Post-transformation, 5 out of 6 colonies tested showed clean mutants, without any

further action to target *repB*, or any additional passages, being necessary (Figure 4b). Hence, it would appear that pNCI001 is inherently unstable and that its maintenance is entirely dependent on the toxin/antitoxin system.⁶⁶ It is also apparent that the plasmid is not essential. Having cured *P. thermoglucosidasius* of pNCI001, the derivative strain was transformed with the CRISPR plasmid targeting the *repA* gene of pNCI002 to generate a derivative plasmid-free strain lacking both large plasmids.

Phenotypic characterization of *P. thermoglucosidasius* strains lacking one or other of the plasmids, or both, failed to find any differences to the WT. Thus, there were no discernible differences in the growth rates of the four strains (Figure 5).

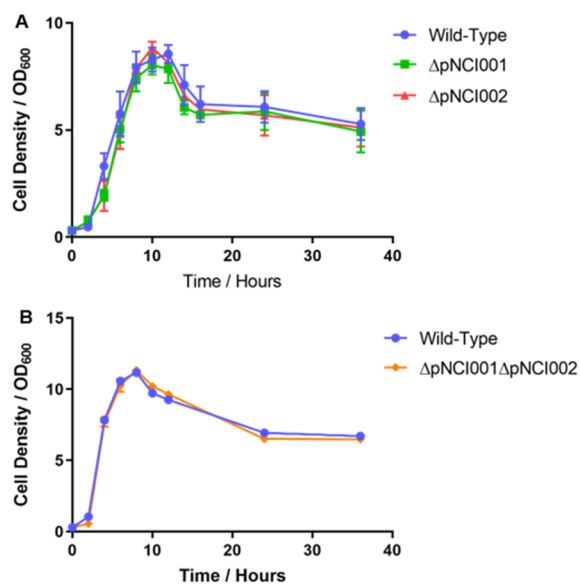


Figure 5. Growth curves of the plasmid-deficient strains of *P. thermoglucosidasius* in a defined medium, monitoring the effect on growth over a 36 h time period resulting from the (A) single and (B) double plasmid loss, compared to wild-type. The error bars on the graph denote the standard error of the mean ($n = 3$). Growth of *P. thermoglucosidasius* was performed under aerobic conditions at 60 °C in a baffled conical flask in modified ASYE (50 mL) and supplemented with 1% glycerol.

Accordingly, neither plasmid, alone nor in combination, conferred either a growth advantage or a metabolic burden on the cell. Furthermore, high-performance liquid chromatography-ultraviolet (HPLC-UV) analysis of the culture supernatant indicated no apparent change in metabolite production or in the extent of glycerol utilization between the mutants and the WT (Figure S1).

Exploiting pNCI001 as a Stable Locus for Heterologous Genes. Indirect evidence suggests that the two large plasmids exhibit a high degree of segregational stability in *P. thermoglucosidasius* NCIMB 11955. Thus, derivatives of NCIMB 11955 that have lost either plasmid have not been reported, despite the fact that they apparently confer no advantage on the host. Such a degree of segregational stability is not a feature of the small autonomous plasmids routinely used to clone the heterologous genes into NCIMB 11955, where plasmid retention is reliant on the supplementation of media with selective antibiotics, principally kanamycin. The segregational stability of introduced genes without the need to supplement media is a fundamental requirement of any

industrial process. Traditionally, such stability is achieved through the introduction of genes into the chromosome, where gene loss is unlikely to occur but productivity is significantly reduced compared to the use of small cloning vectors due to the decreased gene dosage. In this respect, plasmids pNCI001 and pNCI002 may represent superior alternatives to the chromosome as the coverage data obtained previously⁵⁵ suggest their copy number is higher than that of the chromosome.⁶⁷

To test our hypothesis, we designed a series of stCas9-based CRISPR vectors, carrying: (i) a sgRNA under the transcriptional control of the P_{gapdh} promoter targeting the region downstream of *pyrE* or pseudogenes within plasmids pNCI001 and pNCI002, BCV53_19490, and BCV53_19815, respectively; (ii) the eCGP123 gene under the control of the P_{ldh} promoter and the synthetic RBS sequence; and (iii) the requisite HAs flanking the eCGP123 to support its insertion into the targeted locus (see Materials and Methods for details). Following transformation of the various vectors into *P. thermoglucosidasius* as previously described, kanamycin-resistant cells were passaged twice to produce the desired mutants and then screened. The editing plasmid was then cured, and the successful KI was detected by PCR and also identified by detecting the fluorescence produced by the reporter protein. The generation of mutant cell lines in which the eCGP123 was inserted into the chromosome downstream of *pyrE* was readily achieved (Figure 6a and b). Similarly, it proved possible to readily obtain derivatives of pNCI001 carrying the eCGP123 gene inserted within the pseudo gene BCV53_19490. However, despite numerous attempts, it did not prove possible to insert the gene encoding eCGP123 into the pseudogene BCV53_19815 of plasmid pNCI002. This unsuccessful insertion into pNCI002 validates the already indicated nonessential nature of pNCI002. It is proposed that, instead of undergoing HR to replace the protospacer targeted by the Cas9, pNCI002 is instead cured by the double-stranded break caused by the Cas9. This signifies that there is no selection pressure conserving pNCI002. Unlike pNCI001, pNCI002 contains no observed toxin–antitoxin module to ensure the survival of the plasmid.

Having generated strains in which the eCGP123 gene had been localized to either the chromosome, plasmid pNCI001, and through the previous standard cloning procedures, to the multicopy cloning vector pMTL61110, it was possible to evaluate the relative effects of gene dosage on heterologous gene expression. The levels of fluorescence of the three strains are illustrated in Figure 7a. As predicted, the highest levels of fluorescence were seen in cells where the eCGP123 was localized to the multicopy pMTL61110, and the lowest levels were seen when the gene had been inserted into the chromosome. While cells in which the eCGP123 gene had been inserted into pNCI001 exhibited much lower fluorescence than those cells carrying pMTL61110::eCGP123, they exhibited significantly higher levels of expression than when the gene was in the chromosome (Figure 7a). Furthermore, the suggestion that pNCI001 is stability maintained was confirmed through screening of 14 colonies which were confirmed to maintain the plasmid pNCI001 and the inserted eCGP123 gene (Figure 7b).

■ MATERIALS AND METHODS

Biological Material and Growth Conditions. The bacterial strains used in this study are listed in Table S4.

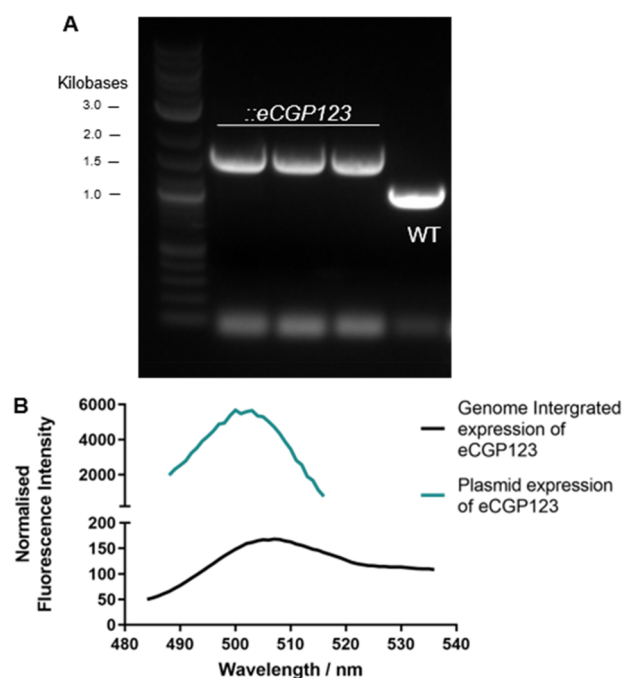


Figure 6. (A) Gel electrophoresis image of a colony PCR, utilizing CP_Genome_eC_F and CP_Genome_eC_R primers, to assess whether the eCGP123 reporter gene had been knocked-in to the genome of *P. thermoglucosidasius*, downstream of *pyrE*. A PCR of WT *P. thermoglucosidasius* was performed as a control (~1.1 kb). Colonies that have the::eCGP123 would yield a band at ~1.6 kb. A 2-log DNA ladder was used to determine the size of each band. (B) A spectral scan (484–536 nm) of the protein lysate extracted from the strain::eCGP123, demonstrating an increase in normalized relative fluorescent units (RFUs), indicating the presence of the eCGP123 gene. The normalized fluorescence intensity of plasmid-based expression of eCGP123 is also displayed to demonstrate the difference in expression level.

E. coli Top10 strain (Invitrogen) was used as a cloning host and grown in LB media (5 mL) or on LB agar plates at 37 °C and supplemented with kanamycin (50 μg/mL). LB broth contains tryptone (10 g), yeast extract (5 g), and NaCl (5 g) per liter of deionized water. The pH of the media was adjusted to 7.5 using HCl or NaOH prior to autoclaving. LB agar was prepared by the addition of No. 1 bacteriological agar (10 g) per liter of deionized water before being autoclaved.

For general growth, *P. thermoglucosidasius* NCIMB 11955 (TMO Renewables) was grown in 2SPYNG (5 mL) at 52 °C shaking (250 rpm) O/N or on TSA agar plates at 52 °C, O/N. Where appropriate, kanamycin (12.5 μg/mL) was used for selection of plasmids. For development of tools, *P. thermoglucosidasius* was grown in 2SPYNG (50 mL) at 52 °C in a baffled conical flask for 8 h. For the generation of mutants using CRISPR/Cas9 technology, *P. thermoglucosidasius* was grown in 2SPYNG (10 mL) at 52 °C in a falcon tube for 12 h before being passaged into fresh media, until a clean mutant was produced. Plasmid loss was carried out using 2SPYNG (10 mL) without antibiotic selection at 60 °C. 2SPYNG contained soy peptone (16 g), yeast extract (10 g), and NaCl (5 g) per liter of deionized water. The pH was adjusted to 7.0 using 5 M KOH prior to autoclaving. TSA agar was prepared using TSA (40 g) (Sigma-Aldrich) per liter of deionized water and autoclaved.

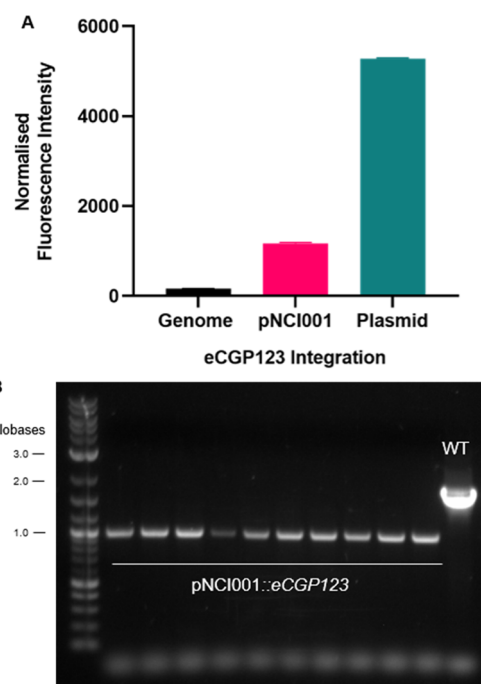


Figure 7. (A) Graph demonstrating the normalized fluorescence intensity of eCGP123 which was expressed on the genome, the plasmid pNCI001, and the plasmid pMTL6110. (B) A gel electrophoresis image of a colony PCR, utilizing CP_BMP_eC_F (outside of HC) and CP_BMP_eC_R (inside of HC) primers, to assess whether the eCGP123 reporter gene had been knocked-in to the pseudo gene, BCV53_19490, in the plasmid pNCI001 of *P. thermoglucosidasius* (~1 kb). A PCR of WT *P. thermoglucosidasius* was performed as a control reaction using the primers CP_BMP_eC_F and CP_BMP_eC_R2 (~1.6 kb). A 2-log DNA ladder was used to determine the size of each band.

Growth of bacterial cultures in liquid medium was monitored by measuring optical density at 600 nm (OD_{600}) using a Jenway 6300 (Cole-Parmer). Samples measured were diluted to 1×10^{-1} .

For transformation of *P. thermoglucosidasius*, 2SPY was used. This was produced in the same way as 2SPYNG but included glycerol (10 g). The pH was adjusted to 7.0 using 5 M KOH prior to autoclaving.

For characterization of the plasmid-deficient strains, *P. thermoglucosidasius* was grown in modified ASYE (50 mL) at 60 °C in a baffled conical flask. Modified ASYE was prepared according to Lin et al. (2014),⁸ consisted of 0.5% yeast extract, 2 mM MgSO₄ (0.24 g), thiamine (0.01 g), citric acid (0.384 g), FeSO₄·7H₂O (27.8 mg), NiCl₃·6H₂O (4.6 mg), 0.2 M HEPES buffer (47.6 g), biotin (3.05 mg), 1X dilution Trace Metal Mix A5 (Sigma), and 1X M9Media (Sigma), per liter of deionized water, and was supplemented with 1% glycerol.

For curing of editing plasmids, the growth conditions of *P. thermoglucosidasius* NCIMB 11955 were changed. The temperature was raised from 52 °C to 60 °C, and the selection pressure of kanamycin was removed. The cultures were grown in 2SPYNG (10 mL) at 60 °C in a falcon tube for 12 h before being passaged into fresh media six successive times. The cultures were then serially diluted to 1×10^{-4} and plated onto TSA plates and grown O/N to produce single colonies. These single colonies were then screened to identify the plasmid-deficient strain by replica plating onto TSA plates with and without kanamycin.

Reagents. All PCR reactions were performed using Phusion 2X Master Mix (New England Biolabs) or DreamTaq Green PCR Master Mix (Thermo Fisher Scientific). T4 ligase (Promega) was used for DNA ligation reactions. Restriction enzymes were purchased from Thermo Fisher Scientific. A Q5 Site-Directed Mutagenesis Kit (New England Biolabs) was used for site-directed mutagenesis.

Plasmid Design and Construction. Oligonucleotide primers were synthesized by Sigma-Aldrich and are listed in Table S5. Plasmids were constructed by restriction enzyme-based cloning procedures.⁶⁸ Constructs were verified by DNA sequencing (Source Bioscience UK Limited). All the plasmids used in this study are listed in Table S6 and may be sourced from www.plasmidvectors.com. The sequences of plasmids pMTL575555 and pMTL675555 are available from GenBank, Accession Numbers MZ182078 and MZ182077, respectively. The plasmid pMTL61110¹⁷ was used as the base chassis for all of the plasmids constructed in this study. To test the different Shine–Dalgarno sequences, site-directed mutagenesis was used to alter the sequences accordingly. To test the different terminators, a forward and reverse oligonucleotide sequence corresponding to each terminator was annealed to produce double-stranded construct. This construct was then cloned into the modular vector using standard restriction based cloning procedures. Homology cassettes used in this study were produced using splicing by overlap extension polymerase chain reaction (SOE-PCR).

Oligonucleotide Design, Analysis, and Synthesis. Oligonucleotides for synthetic guide RNA (sgRNA), for polymerase chain reaction (PCR), and for sequencing of DNA sequences were designed manually. Secondary structure of oligonucleotides was analyzed for the production of primers and for calculating the free energy of Rho-independent terminators. DNA sequences were analyzed using the OligoAnalyzer tool (<https://www.idtdna.com/calc/analyzer>) of Integrated DNA Technologies. Oligonucleotide synthesis was performed by Sigma-Aldrich. Spacer acquisition for sgRNA was completed using Benchling CRISPR Guide Design software (www.benchling.com).

Transformation of Plasmid DNA. All *E. coli* strains were transformed using the heat-shock method. Plasmid DNA (1–2 μL) (100–500 $\text{ng}/\mu\text{L}$) was added to chemical competent *E. coli* Top10 cells (60 μL). This mixture was left on ice for 30–45 min, then transferred to 42 °C for 30 s, and then returned back to ice for 3–5 min. LB media (900 μL) was then added to the cells and left to recover for 1.5 h, incubated at 37 °C, and shaken (200 rpm). After recovery, the cells (200 μL) were plated onto LB agar plates containing the appropriate antibiotic selection.

Transformation of *P. thermoglucosidasius* was conducted following the procedure described by Cripps et al. (2009).¹⁵ Plasmid DNA (5 μL) (100–500 $\text{ng}/\mu\text{L}$) was added to electrocompetent *P. thermoglucosidasius* NCIMB 11955 cells (60 μL) in a precooled 1 mm gap electroporation cuvette. Electroporation was carried out using a GenePulser (BioRad), set at 600 Ω , 2.5 kV, and 10 μFD . Immediately following the pulse, prewarmed 2SPY (1 mL) was added to the cells and left to recover for 2.5–4 h, incubated at 52 °C shaking (250 rpm). After recovery, the cells were concentrated by centrifugation (5000g, 5 min) and resuspended in 2SPY media (200 μL) before being plated onto prewarmed TSA agar plates containing kanamycin (12.5 $\mu\text{g}/\text{mL}$).

Production of p Δ Cas9. For the vector p Δ Cas9, we produced a frame shifted variant of each *cas9*. This was achieved through the use of the restriction endonuclease enzymes *PacI* and *Eco81I*, respectively, to cut the genes. The genes were then subjected to T4 DNA polymerase, which has the ability to form blunt ends by the removal of 3' overhangs or by filling-in 5' overhangs. These blunt ends were then ligated together to produce the complete but inactivated *cas9* gene.

Analytical Methods. The eCGP123 assay used to determine the relative levels of gene expression was carried out as follows. The constructed gene expression vectors were transformed into *P. thermoglucosidasius* competent cells. After recovery, the transformed cells were plated onto TSA plates containing kanamycin (12.5 $\mu\text{g}/\text{mL}$) and grown O/N. A single colony was picked and restreaked onto TSA plates containing kanamycin. A single colony was picked and cultured in a baffled conical flask for 8 h at 250 rpm, in 2SPYNG media containing kanamycin (12.5 $\mu\text{g}/\text{mL}$), before extracting the protein lysate. The level of eCGP123 was measured using a microplate reader (CLARIOstar) using the excitation and emission values determined by Don Paul et al. (2011).³⁷ The relative fluorescence intensities (RFUs) were then normalized using the protein concentration of each sample. Protein concentration was calculated using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein extraction was carried out using BugBuster 10X protein extraction reagent (Merck). The protein extraction buffer (1X) consisted of BugBuster 10X (2 mL), sodium phosphate buffer 50 mM (18 mL), EDTA-free protease inhibitor tablet (Roche), and lysozyme (20 mg/mL).

Metabolites produced by *P. thermoglucosidasius* were analyzed using high-performance liquid chromatography (HPLC-UV), performed using the Dionex UltiMate 3000 System. 5 mM H_2SO_4 was used as the mobile phase. The sample supernatant was mixed, in a 1:1 ratio, with diluent, which consisted of the mobile phase mixed with 50 mM valeric acid. Samples were filtered to remove particulate matter through 0.2 μm syringe filters directly into HPLC vials containing 300 μL inserts with split caps. The samples were run on the Bio-Rad Aminex HPX-87H 300 mm \times 7.8 mm \times 9 μm column, at a flow rate of 0.5 mL/min, at 35 °C for 55 min.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssynbio.1c00138>.

Promoter sequences (Table S1); Shine–Dalgarno sequences (Table S2); terminator sequences (Table S3); strains used in this study (Table S4); primers used in this study (Table S5); plasmids used in this study (Table S6); and metabolite and glycerol utilization profile of the plasmid-deficient strains (Figure S1) (PDF)

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Author Contributions

N.P.M. conceived the study; M.S.H.L., Y.Z., L.S., and N.P.M. designed the experiments; M.S.H.L. performed the experiments; and M.S.H.L. and N.P.M. wrote the manuscript. All authors approved the final version.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

KIs, knock-ins; KOs, knockouts; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated protein; PAM, protospacer-adjacent motif; sgRNA, synthetic guide RNA; spCas9, *Streptococcus pyogenes cas9*; stCas9, *Streptococcus thermophilus cas9*; sfGFP, superfolder green fluorescent protein; SD, Shine–Dalgarno; HA, homology arms; HR, homologous recombination; HPLC–UV, high-performance liquid chromatography–ultraviolet; SOE-PCR, splicing by overlap extension polymerase chain reaction

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