

# 1 **Multiplex genome editing for synthetic biology in *Vibrio*** 2 ***natrie*gens**

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12 *Vibrio natrie*gens has recently emerged as an alternative to *Escherichia coli* for molecular  
13 biology and biotechnology, but low-efficiency genetic tools hamper its development. Here,  
14 we uncover how to induce natural competence in *V. natrie*gens and describe methods for  
15 multiplex genome editing by natural transformation (MuGENT). MuGENT promotes  
16 integration of large genome edits at high-efficiency on unprecedented timescales, which  
17 will extend the utility of this species for diverse applications.

18

19 *V. natrie*gens is the fastest growing organism known, with a doubling time of <10 min<sup>1,2</sup>.  
20 With broad metabolic capabilities, lack of pathogenicity, and its rapid growth rate, it is an  
21 attractive alternative to *E. coli* for diverse molecular biology and biotechnology  
22 applications<sup>3</sup>. Methods for classical genetic techniques have been developed for *V.*  
23 *natrie*gens, but these are relatively laborious, require multiple steps, and must be used  
24 sequentially to generate multiple genome edits<sup>3</sup>. The challenges of these techniques  
25 contrast with the ease of genetics in *Vibrio* species that are naturally transformable.  
26 Competent *Vibrios* can take up DNA from the environment and integrate it into their  
27 genome by homologous recombination; processes known as natural competence and  
28 natural transformation, respectively<sup>4-7</sup>. The inducing cue for natural transformation in  
29 competent *Vibrios* is growth on the chitinous shells of crustacean zooplankton, which are  
30 commonly found in the aquatic environment where these microbes reside<sup>4</sup>. Chitin induces

1 the expression of the competence regulator TfoX<sup>8,9</sup>. In fact, overexpression of TfoX obviates  
2 the need for chitin induction, allowing competent *Vibrios* to be naturally transformed in  
3 rich media<sup>4,8</sup>.

4  
5 As no reports of natural transformation existed for *V. natriegens*, we first sought to  
6 establish whether this was possible. Unlike naturally competent *V. cholerae*, incubation on  
7 chitin did not lead to detectable transformation in *V. natriegens* (data not shown). However,  
8 ectopic expression of TfoX (either the endogenous *tfoX* gene or one from *Vibrio cholerae*)  
9 on an IPTG-inducible plasmid (pMMB) supported high rates of natural transformation (**Fig.**  
10 **1a**). This was tested using a linear PCR product that replaces the gene encoding the DNA  
11 endonuclease Dns with an antibiotic resistance (Ab<sup>R</sup>) marker. The *dns* locus was used as a  
12 target for transformation assays throughout this manuscript because loss of this gene does  
13 not impact growth or viability in rich medium. Under optimal conditions ~1-10% of the  
14 population had integrated the transforming DNA (tDNA), which matches the highest rates  
15 of transformation observed among competent species<sup>10</sup> (**Fig. 1a-c**). Natural transformation  
16 of *V. natriegens* required very little transforming DNA (tDNA) (highly efficient with even 1  
17 ng / 10<sup>8</sup> CFU) and was dependent on the length of homologous sequence surrounding the  
18 mutation (**Fig. 1b and c**). This method could also be used to introduce point mutations into  
19 *V. natriegens* (tested with tDNA containing an *rpsL* K43R Sm<sup>R</sup> allele); however, this activity  
20 was partially suppressed by the mismatch repair system (**Fig. 1d**).

21  
22 Having demonstrated *V. natriegens* is naturally competent, we sought to determine if we  
23 could use natural transformation to perform scarless multiplex genome editing by natural  
24 transformation (MuGENT)<sup>11</sup>. MuGENT operates under the premise that under competence  
25 inducing conditions, only a subpopulation of cells is transformable. Those cells that can be  
26 transformed, however, have the capacity to take up and integrate multiple tDNAs<sup>11,12</sup>. Thus,  
27 during MuGENT, cells are incubated with two types of linear tDNA; (1) a selected product  
28 that introduces an antibiotic resistance marker into the genome and (2) unselected  
29 products that introduce scarless genome edits of interest at one or more loci.

30

1 We first tested the ability of MuGENT to introduce a single unmarked genome edit (also  
2 known as cotransformation). To facilitate measurement of cotransformation, we noted this  
3 species forms opaque colonies on agar plates (**Fig. S1**), which could be due to the  
4 production of a capsular polysaccharide. Consistent with this, inactivating a homolog of the  
5 essential capsule biosynthesis gene *wbfr*<sup>13</sup> resulted in the formation of transparent  
6 colonies on agar plates and loss of expression of a high molecular weight polysaccharide  
7 (**Fig. S1**). Thus, to test cotransformation we used an unselected product to replace ~500 bp  
8 of the 5' end of the *wbfr* gene with a premature stop codon and scored cotransformation  
9 via colony morphology (opaque vs. transparent) on agar plates. We found that  
10 cotransformation was remarkably efficient in *V. natriegens* (up to ~80%), even with low  
11 amounts (~25-50 ng / 10<sup>8</sup> CFU) of the unselected product (**Fig. S2a**). Also,  
12 cotransformation with 1 kb flanks on the unselected product was possible, but at ~6-fold  
13 lower frequencies than with 3 kb flanks (**Fig. S2a**).

14  
15 We next tested the full multiplex genome editing capacity of MuGENT to simultaneously  
16 cotransform multiple scarless genome edits into the genome in a single step<sup>11,14</sup>. Since  
17 there is no selection for integration of the unselected genome edits *in cis* during MuGENT,  
18 output populations are highly heterogeneous and individual mutants contain any number  
19 and combination of the multiplexed genome edits. Also, this process can be carried out in  
20 multiple iterative cycles to further increase the complexity of genome edits in the  
21 population (**Fig. 2a**)<sup>11</sup>.

22  
23 As an initial test of multiplex genome editing, we targeted 5 genes whose mutagenesis was  
24 considered unlikely to affect viability or growth in LB. These targets included four  
25 carbohydrate transporters (specific for mannitol, fructose, sucrose, and trehalose – all of  
26 which are absent in LB) and the *dns* gene. All genes were targeted for inactivation by  
27 replacing ~500 bp of the 5' end of each gene with a premature stop codon. Integration of  
28 genome edits was determined by multiplex allele-specific colony PCR (MASC-PCR)<sup>15</sup> (**Fig.**  
29 **S2b**). Following one cycle of MuGENT, we found that ~70% of the population contained at  
30 least 1 genome edit, with ~25% of the population containing 3-4 genome edits (**Fig. S2c**). A  
31 quadruple mutant from this experiment was isolated and whole genome sequencing did

1 not reveal any off target mutations in this strain. Thus, MuGENT rapidly generated *V.*  
2 *natriegens* strains with multiple large (0.5 kb) scarless genome edits at high-efficiency  
3 without off-target effects, and can be used to make highly complex mutant populations.  
4  
5 As a second demonstration of multiplex genome editing, we demonstrated its utility in  
6 metabolic engineering by attempting to rapidly enhance production of a value-added  
7 chemical in *V. natriegens*. This species naturally accumulates low levels of the bioplastic  
8 precursor poly- $\beta$ -hydroxybutyrate (PHB) as a storage polymer<sup>16</sup>. PHB is derived from the  
9 condensation and subsequent NADPH-dependent reduction of acetyl-CoA precursors<sup>17</sup>.  
10 Thus, for our targets, we tuned the expression (swap  $P_{native}$  for IPTG-inducible  $P_{tac}$ ) or  
11 inactivated genes that we hypothesized would affect NADPH and/or acetyl-CoA availability.  
12 The targets for promoter swaps were the PHB synthesis operon (*phaBAC*), NAD kinase  
13 (*nadK*), and two transhydrogenases (*pntAB* and *udhA*), while targets for inactivation were  
14 phosphoglucose isomerase (*pgi*), citrate synthase (*gltA*), phosphotransacetylase (*pta*),  
15 isocitrate lyase (*aceA*), and lactate dehydrogenase (*ldhA*) (**Fig. 2b**). Thus, there were 512  
16 possible combinations for these 9 genome edits. We performed multiple cycles of MuGENT  
17 to introduce these genome edits into a competent population of *V. natriegens*. At each cycle,  
18 the selected product was designed to swap the Ab<sup>R</sup> marker at the *dns* locus to maintain  
19 coselection at each step. Following four cycles of MuGENT, which took just 5 days to  
20 perform, ~50% of the population had 3 or more genome edits and ~10% contained 5+  
21 genome edits (**Fig. 2c**). To select mutants with increased PHB production, we then plated  
22 this output population onto media containing Nile red, which stains PHB granules<sup>18</sup>. Nile  
23 red fluorescence on these plates was highly heterogeneous, suggesting that some  
24 genotypes produced more PHB than the parent isolate (**Fig. 2d**). A number of highly  
25 fluorescent colonies were picked and the genotypes determined by MASC-PCR. Also, PHB in  
26 these select strains was directly measured by HPLC. Cumulatively, these analyses rapidly  
27 revealed genotypes that produced ~100-fold more PHB than the parent and ~3.3-fold  
28 more than a strain with just the  $P_{tac}$ -*phaBAC* mutation (**Fig. 2e**).  
29  
30 While many methods for multiplex genome editing in bacterial systems have been  
31 described<sup>19</sup>, many of these are limited to small changes such as SNPs. MuGENT, on the

1 other hand, can efficiently swap, insert, or remove whole promoters or coding sequences as  
2 demonstrated above. Furthermore, one of the major limitations to other multiplex genome  
3 editing methods is that mutagenesis must be performed in strains lacking DNA repair  
4 pathways to allow for high-efficiency integration of genome edits, which results in a large  
5 number of off-target mutations<sup>15,19</sup>. MuGENT in *V. natriegens* is performed in DNA repair  
6 sufficient backgrounds, thus, little to no off target mutations are introduced during the  
7 procedure as indicated above. Also, unlike other multiplex editing approaches, MuGENT  
8 requires no specialized equipment and, thus, has the potential to make multiplex genome  
9 editing commonplace.

10

11 In conclusion, this study demonstrates that MuGENT is a rapid, efficient, and simple tool for  
12 engineering the *V. natriegens* genome. This microbe is already being developed as an  
13 alternative to *E. coli*, and we believe that the ease and speed of MuGENT will extend the use  
14 of *V. natriegens* as a novel chassis for diverse molecular biology and biotechnology  
15 applications.

16

## 17 **METHODS**

18 Methods and any associated references are available in the online version of the paper.

19

## 20 **ACKNOWLEDGEMENTS**

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25

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## 16 **FIGURE LEGENDS**

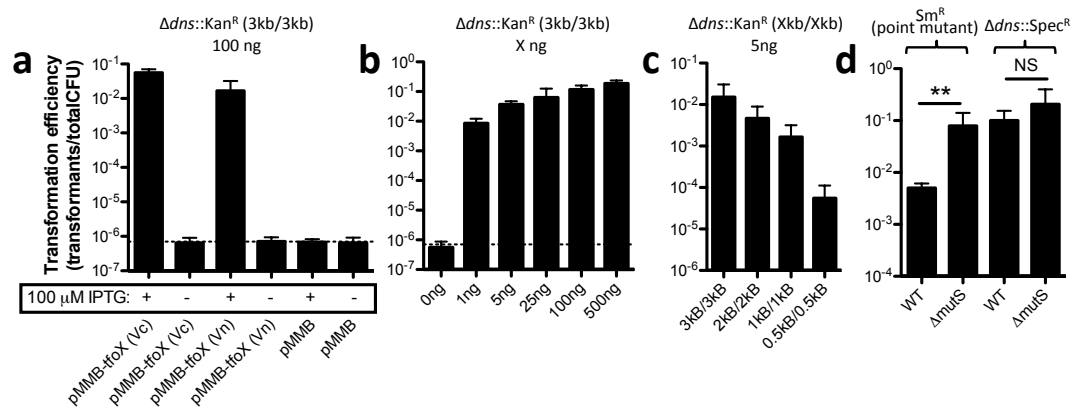
17 **Fig. 1** – Natural transformation of *V. natriegens*. (**a-d**) Transformation assays of *V.*  
18 *natriegens*. (**a**) *V. natriegens* strains containing a pMMB empty vector or pMMB with the  
19 *tfoX* gene from either *V. natriegens* (Vn) or *V. cholerae* (Vc) were transformed with 100 ng  
20 of a  $\Delta dns::Kan^R$  tDNA containing 3 kb flanks of homology on both sides of the mutation (i.e.  
21 3 kb/3 kb). Transformation assay of *V. natriegens* pMMB-*tfoX* (Vc) with (**b**) the indicated  
22 concentration of  $\Delta dns::Kan^R$  (3 kb/3 kb) tDNA or (**c**) 5 ng of  $\Delta dns::Kan^R$  tDNA containing  
23 the indicated amount of homology on each side of the mutation. (**d**) Transformation assay  
24 in the indicated strain backgrounds with 5 ng of *rpsL* K43R  $Sm^R$  (3 kb/3 kb) or  $\Delta dns::Spec^R$   
25 (3 kb/3 kb) tDNA as indicated. All strains in **d** harbor  $\Delta dns::Kan^R$  mutations and pMMB-*tfoX*  
26 (Vc). All data are shown as the mean  $\pm$  SD and are the result of at least 4 independent  
27 biological replicates. \*\* =  $p < 0.01$ .

28

29 **Fig. 2** – MuGENT rapidly enhances PHB production in *V. natriegens*. (**a**) Schematic of  
30 MuGENT. The selected product is indicated by a red box, while multiple unselected genome  
31 edits are depicted by distinct gray shapes. Since there is no selection for genome edits *in cis*,

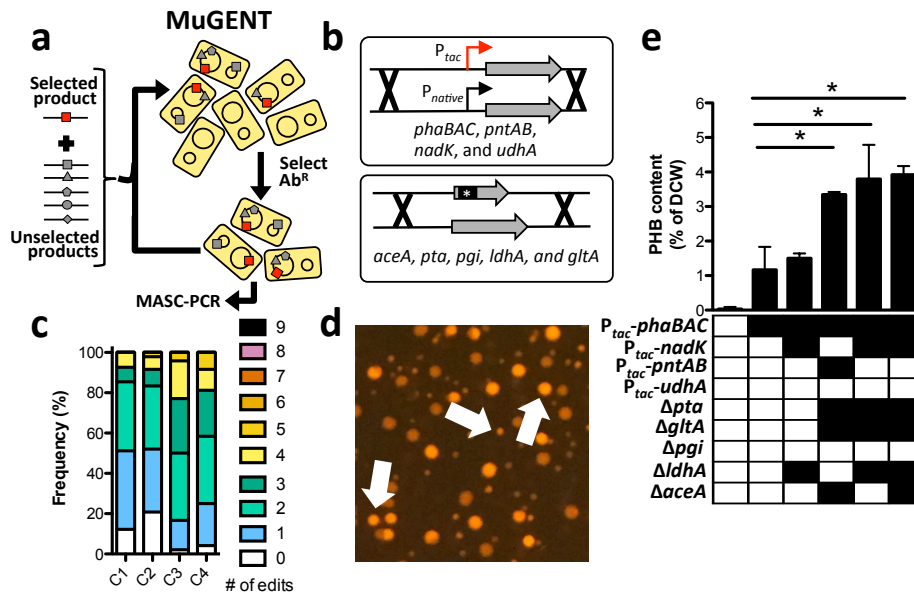
1 output mutants can have any number and combination of the unselected genome edits. **(b)**  
2 The indicated targets were subjected to either a promoter swap (top) or inactivation by  
3 replacing ~500bp of the 5' end of each gene with a short sequence to introduce a  
4 premature stop codon. **(c)** Distribution of the 9 genome edits in a population of cells  
5 following four cycles of MuGENT. **(d)** Representative image of the mutant pool generated in  
6 **c** plated on Nile red containing plates, which stain PHB granules. White arrows indicate  
7 colonies with increased fluorescence intensity. **(e)** PHB content of select MuGENT  
8 optimized strains is shown as the % of dry cell weight (DCW). The genotype of each mutant  
9 is shown below each bar where a filled box indicates the presence of the genome edit  
10 indicated on the left. Data are shown as the mean  $\pm$  SD and are from at least 2 independent  
11 biological replicates. \* =  $p < 0.05$ .

12



**Fig. 1** – Natural transformation of *V. natriegens*. (a-d) Transformation assays of *V. natriegens*. (a) *V. natriegens* strains containing a pMMB empty vector or pMMB with the *tfoX* gene from either *V. natriegens* (Vn) or *V. cholerae* (Vc) were transformed with 100 ng of a  $\Delta dns::Kan^R$  tDNA containing 3 kb flanks of homology on both sides of the mutation (i.e. 3 kb/3 kb). Transformation assay of *V. natriegens* pMMB-*tfoX* (Vc) with (b) the indicated concentration of  $\Delta dns::Kan^R$  (3 kb/3 kb) tDNA or (c) 5 ng of  $\Delta dns::Kan^R$  tDNA containing the indicated amount of homology on each side of the mutation. (d) Transformation assay in the indicated strain backgrounds with 5 ng of *rpsL* K43R  $Sm^R$  (3 kb/3 kb) or  $\Delta dns::Spec^R$  (3 kb/3 kb) tDNA as indicated. All strains in d harbor  $\Delta dns::Kan^R$  mutations and pMMB-*tfoX* (Vc). All data are shown as the mean  $\pm$  SD and are the result of at least 4 independent biological replicates. \*\* =  $p < 0.01$ .





**Fig. 2 – MuGENT rapidly enhances PHB production in *V. natriegens*.** (a) Schematic of MuGENT. The selected product is indicated by a red box, while multiple unselected genome edits are depicted by distinct gray shapes. Since there is no selection for genome edits *in cis*, output mutants can have any number and combination of the unselected genome edits. (b) The indicated targets were subjected to either a promoter swap (top) or inactivation by replacing ~500bp of the 5' end of each gene with a short sequence to introduce a premature stop codon. (c) Distribution of the 9 genome edits in a population of cells following four cycles of MuGENT. (d) Representative image of the mutant pool generated in c plated on Nile red containing plates, which stain PHB granules. White arrows indicate colonies with increased fluorescence intensity. (e) PHB content of select MuGENT optimized strains is shown as the % of dry cell weight (DCW). The genotype of each mutant is shown below each bar where a filled box indicates the presence of the genome edit indicated on the left. Data are shown as the mean  $\pm$  SD and are from at least 2 independent biological replicates. \* =  $p < 0.05$ .

## SUPPLEMENTARY ONLINE INFORMATION

### ONLINE METHODS

#### *Bacterial strains and culture conditions*

The parent *V. natriegens* strain used throughout this study was a spontaneous rifampicin-resistant derivative of ATCC14048<sup>1</sup>. For a list of all strains used / generated in this study, see **Table S1**. Strains were routinely grown in LB+v2 salts (LBv2)<sup>2</sup>, which is LB Miller broth (BD) supplemented with 200 mM NaCl, 23.14 mM MgCl<sub>2</sub>, and 4.2 mM KCl. LBv2 was supplemented with 100 μM IPTG, 50 μg/mL kanamycin (Kan), 200 μg/mL spectinomycin (Spec), 100 μg/mL rifampicin (Rif), 100 μg/mL streptomycin (Sm), or 100 μg/mL carbenicillin (Carb) as appropriate.

#### *Generation of mutant strains and constructs*

Mutant constructs were generated by splicing-by-overlap extension (SOE) PCR exactly as previously described<sup>3</sup>. Briefly, for three-piece mutant constructs (i.e. for constructs where a gene of interest is replaced with an Ab<sup>R</sup> cassette or where the native promoter is swapped for a P<sub>tac</sub> promoter) segments were designated UP, MIDDLE, and DOWN and correspond to: (1) UP = the upstream region of homology amplified with F1 and R1 primers, (2) DOWN = the downstream region of homology amplified with F2 and R2 primers, and (3) MIDDLE = the Ab<sup>R</sup> marker or promoter swap fragment. For two-piece mutant constructs (i.e. for constructs where ~501 bp of the 5' end of a gene is replaced with a stop codon), the mutation of interest is incorporated into the R1 and F2 primers used to amplify the upstream and downstream regions of homology, respectively. Gel purified segments were then mixed in equal ratios and used as template for a SOE PCR reaction with the F1 and R2 primers. All mutant constructs were made using Phusion polymerase. These were introduced into the *V. natriegens* genome via natural transformation as described below. All primers used to generate mutant constructs are listed in **Table S2**.

#### *Natural transformation / MuGENT assays*

Strains harboring pMMB-*tfoX* (*Vn tfoX* or *Vc tfoX*) were induced to competence by growing overnight (12-18 hours) in LBv2+100 μg/mL carbenicillin+100 μM IPTG in a rollerdrum at 30°C. Then, ~10<sup>8</sup> CFUs of this overnight culture (~3.5 μL) were diluted directly into 350 μL of instant ocean medium (28 g/L; Aquarium Systems Inc.) supplemented with 100 μM IPTG. Transforming DNA (tDNA) was then added as indicated, and reactions were incubated statically at 30°C for 5 hours. Next, 1 mL of LBv2 was added and reactions were outgrown at 30°C with shaking (250 rpm) for ~1-2 hrs. Then, reactions were plated for quantitative culture onto media to select for integration of tDNA (i.e. LB+drug = transformants) and onto nonselective media (i.e. plain LB = total viable counts). Transformation efficiency is shown as: transformants / total viable counts.

For MuGENT, transformation assays were conducted exactly as described above. Unless otherwise specified, ~50 ng of the selected product was incubated with cells along with ~200 ng of each unselected product. After outgrowth, 1/10<sup>th</sup> of the reaction was removed and plated for MASC-PCR analysis (described below). If multiple cycles of MuGENT were performed, the rest of the reaction was grown overnight in LBv2 supplemented with 100 μM IPTG, 100 μg/mL carbenicillin (to maintain pMMB-*tfoX*), and the antibiotic to select for

integration of the selected product. The following day, the population was then subjected to another round of MuGENT as described above using a selected product containing a different Ab<sup>R</sup> marker to maintain coselection at each cycle.

Integration of genome edits was detected via MASC-PCR exactly as previously described<sup>4,5</sup>. Briefly, colonies were boiled in 50  $\mu$ L of sterile water, vortexed, and then 2  $\mu$ L were used as template in a 25  $\mu$ L PCR reaction. PCR was conducted with Taq polymerase (SydLabs) using a modified 5X Taq buffer: 200 mM Tris pH 8.8, 100 mM KCl, 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 30 mM MgSO<sub>4</sub>, and 1% Triton X-100. The total primer used in each MASC-PCR reaction (regardless of the number of multiplexed products being detected) was 1200 nM (i.e. for detection of 4 multiplexed genome edits, 300 nM of each genome edit-specific primer pair was used). The cycling conditions used were: 95°C 3 min; 26  $\times$  [95°C 40s, 58°C 30s, 72°C 3 min]; 72°C 3 min; 12°C hold. Reactions were then run on 2% agarose gels and imaged with GelGreen dye according to manufacturer's instructions (Biotium). For a list of all primers used for MASC-PCR see **Table S2**.

#### *Alcian blue stained gels*

To prepare cell lysates,  $\sim 10^9$  cells of the indicated *V. natriegens* strains were pelleted and then resuspended in 180  $\mu$ L of Buffer ATL (Qiagen). Then, 20  $\mu$ L of a 20 mg/mL proteinase K stock solution was added to each reaction and incubated at 56°C for 20 mins. Samples were then boiled in 2X SDS PAGE sample buffer and separated on 4-12% SDS PAGE gels. Gels were then stained with 0.1% Alcian Blue 8GX in 40% ethanol/3% acetic acid as previously described<sup>6</sup>. The gel was then destained in a 40% ethanol/3% acetic acid and imaged on a Biorad ChemiDoc MP Imaging system.

#### *Whole genome sequencing*

Genomic DNA was prepped from strains and sequencing libraries were prepped via homopolymer-tail mediated ligation exactly as previously described<sup>7</sup>. Single-end 50 bp reads were collected on the Illumina platform. Then, data was analyzed for small indels and single nucleotide variants using CLC Genomics Workbench exactly as previously described<sup>8,9</sup>.

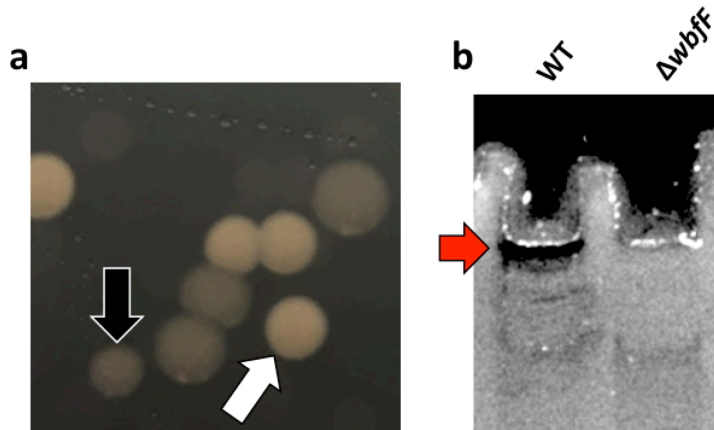
#### *Qualitative and quantitative assessment of PHB production*

PHB was qualitatively assessed in MuGENT edited populations of *V. natriegens* by plating onto Nile red containing medium with excess glucose as a carbon source and 100  $\mu$ M IPTG to induce P<sub>tac</sub>-containing genome edits = recipe per L: 28 g instant ocean, 2.5 g tryptone, 1 g yeast extract, 20 g glucose, 15 g agar, and 1 mg Nile red. Fluorescence of colonies was detected using a PrepOne Sapphire LED blue light base (475 nm  $\pm$  30 nm) and amber filter (530 nm long pass) (Embi Tec).

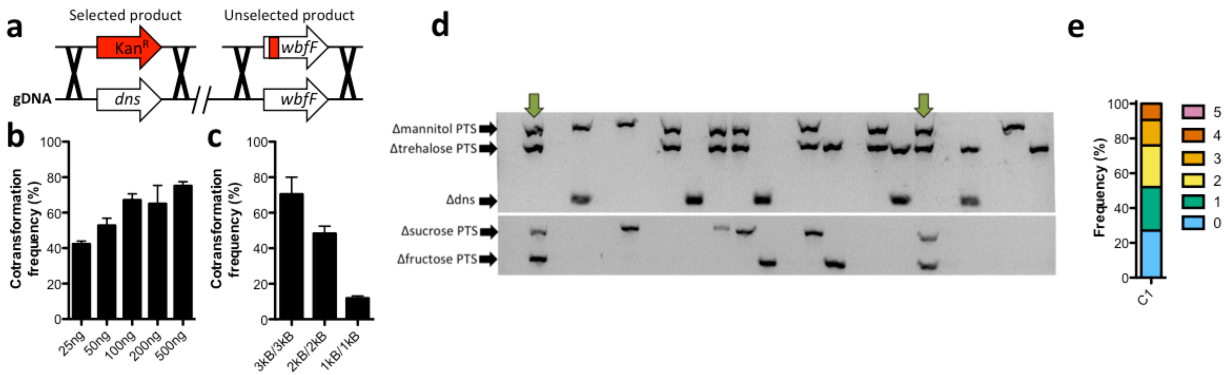
For quantitative assessment of PHB levels, the indicated strains were grown overnight in M9 minimal medium (BD) supplemented with 2 mM MgSO<sub>4</sub>, 100  $\mu$ M CaCl<sub>2</sub>, 200 mM NaCl, 30  $\mu$ M FeSO<sub>4</sub>, 100  $\mu$ M IPTG, 1% tryptone, and 2% glucose. Approximately  $8 \times 10^9$  cells were then pelleted, resuspended with 50  $\mu$ L water and transferred to pre-weighed glass screw-cap tubes. Cell suspensions were dried for 5 h at 80°C and then the tubes were weighed again to determine dry cell weights. PHB was then hydrolyzed and extracted as crotonic

acid by boiling the dried cells in 1 ml of pure sulfuric acid. Extracts were chilled on ice and diluted with 4 ml ice-cold water. Aliquots were further diluted 10-fold with water, centrifuged, filtered, and then crotonic acid was quantified by HPLC as described<sup>10</sup>.

## SUPPLEMENTARY FIGURES



**Fig. S1** – *V. natriegens* produces a *WbF*-dependent capsular polysaccharide. Colony morphologies of WT (white arrow) and  $\Delta wbfF$  (black arrow) strains, which demonstrate the phenotypes screened for in cotransformation assays. Cell lysates of the indicated strains were run on a 4-12% SDS PAGE gel and stained with the carbohydrate stain Alcian blue. The presence of a high molecular weight polysaccharide in the WT is indicated by a red arrow.



**Fig. S2 – Cotransformation is highly efficient in *V. natriegens*.** (A) Cotransformation was tested using a  $\Delta$ *dns*::*Kan<sup>R</sup>* (3 kb/3 kb) selected product and an unselected product that deleted ~500 bp of the 5' end of *wbff* gene. Cotransformation assays were performed using 50ng of the  $\Delta$ *dns*::*Kan<sup>R</sup>* (3 kb/3 kb) selected product and (B) the indicated amount of the  $\Delta$ *wbff* (3kb/3kb) unselected product or (C) 200 ng of  $\Delta$ *wbff* unselected products containing the indicated length of homology on each side of the mutation. (D and E) MuGENT was performed with 5 unselected genome edits. The selected product was  $\Delta$ *wbff*::*Kan<sup>R</sup>*, while the unselected products targeted four carbohydrate transporters and *dns* for inactivation by replacing ~500 bp of the 5' end of each gene with a premature stop codon. (D) A representative MASC-PCR gel of 24 colonies from the edited population. The targets of each genome edit are indicated on the left and the presence of a band indicates integration of the indicated genome edit. Strains containing 4 genome edits are indicated by the green arrows. (E) Distribution of genome edits in the population determined by MASC-PCR analysis of 48 random mutants.

## SUPPLEMENTARY TABLES

**Table S1 – Strains used in this study**

Strain name	Genotype and antibiotic resistances	Description	Reference / (strain#)
WT	Rif <sup>R</sup>	Spontaneous Rif <sup>R</sup> derivative <i>V. natriegens</i> ATCC14048 that is the parent isolate for all strains used in this study.	This study (SAD1304)
pMMB-tfoX (Vc)	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup>	SAD1304 containing pMMB- <i>tfoX</i> (Vc), a vector containing the <i>tfoX</i> gene from <i>V. cholerae</i> (VC1153) under the control of an IPTG-inducible P <sub>tac</sub> promoter. Vector is derived from pMMB67EH and has a Carb <sup>R</sup> gene for selection.	This study (SAD1306)
pMMB-tfoX (Vn)	pMMB- <i>tfoX</i> (Vn) Carb <sup>R</sup>	SAD1304 containing pMMB- <i>tfoX</i> (Vn), a vector containing the <i>tfoX</i> gene from <i>V. natriegens</i> (BA890_05980) under the control of an IPTG-inducible P <sub>tac</sub> promoter. Vector is derived from pMMB67EH and has a Carb <sup>R</sup> gene for selection.	This study (TND0322 / SAD1495)
pMMB	pMMB empty vector Carb <sup>R</sup>	SAD1304 containing the pMMB67EH empty vector	This study (TND0321 / SAD1496)
WT (Fig. 1D)	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup> , $\Delta$ <i>dns</i> ::Kan <sup>R</sup>	SAD1306 with $\Delta$ <i>dns</i> ::Kan <sup>R</sup> ( $\Delta$ BA890_12415)	This study (SAD1313)
$\Delta$ <i>mutS</i>	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup> , $\Delta$ <i>dns</i> ::Kan <sup>R</sup> , $\Delta$ <i>mutS</i>	Generated by cotransformation into SAD1306 with $\Delta$ <i>dns</i> ::Kan <sup>R</sup> and a product to delete ~500bp of the 5' end of the <i>mutS</i> gene (BA890_12150).	This study (TND0362 / SAD1497)
$\Delta$ <i>wbfF</i>	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup> , $\Delta$ <i>wbfF</i> ::Kan <sup>R</sup>	Introduced a $\Delta$ <i>wbfF</i> ::Kan <sup>R</sup> mutation ( $\Delta$ BA890_01135) into the SAD1306 strain background.	This study (CAH509 / SAD1498)
MuGENT quadruple mutant	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup> , $\Delta$ <i>wbfF</i> ::Kan <sup>R</sup> , $\Delta$ BA890_01815 (mannitol transporter), $\Delta$ BA890_19540 (sucrose transporter), $\Delta$ BA890_16410 (fructose transporter), $\Delta$ <i>dns</i>	MuGENT into SAD1306 strain with 5 unselected genome edits. This quadruple mutant was whole genome sequenced and no off target mutations were identified.	This study (TND0338 / SAD1499)
Fig. 2E, second bar	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup> , $\Delta$ <i>dns</i> ::Kan <sup>R</sup> , P <sub>tac</sub> - <i>phaBAC</i>	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (TND0364 / SAD1500)
Fig. 2E, third bar	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup> , $\Delta$ <i>dns</i> ::Kan <sup>R</sup> , P <sub>tac</sub> - <i>phaBAC</i> , P <sub>tac</sub> - <i>nadK</i> , $\Delta$ <i>ldhA</i>	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (SAD1501)
Fig. 2E, fourth bar	pMMB- <i>tfoX</i> (Vc) Carb <sup>R</sup> , $\Delta$ <i>dns</i> ::Kan <sup>R</sup> , P <sub>tac</sub> - <i>phaBAC</i> , P <sub>tac</sub> -	MuGENT into SAD1306 to enhance PHB production. The	This study (SAD1502)



	<i>pntAB, Δpta, ΔgltA, ΔaceA</i>	strain contains the genome edits indicated.	
Fig. 2E, fifth bar	pMMB-tfoX (Vc) Carb <sup>R</sup> , <i>Δdns::Spec<sup>R</sup>, P<sub>tac</sub>-phaBAC, P<sub>tac</sub>-nadK, Δpta, ΔgltA, ΔldhA</i>	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (SAD1503)
Fig. 2E, sixth bar	pMMB-tfoX (Vc) Carb <sup>R</sup> , <i>Δdns::Spec<sup>R</sup>, P<sub>tac</sub>-phaBAC, P<sub>tac</sub>-nadK, Δpta, ΔgltA, ΔldhA, ΔaceA</i>	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (SAD1504)

**Table S2 – Primers used in this study**

Primer Name	Primer Sequence (5'→3')*	Description
<b>Primers for Mutant constructs</b>		
ABD123	ATTCCGGGGATCCGTCGAC	Amplify MIDDLE Ab <sup>R</sup> (Kan <sup>R</sup> , Spec <sup>R</sup> , or Tm <sup>R</sup> cassettes) F
ABD124	TGTAGGCTGGAGCTGCTTC	Amplify MIDDLE Ab <sup>R</sup> (Kan <sup>R</sup> , Spec <sup>R</sup> , or Tm <sup>R</sup> cassettes) R
BBC1264	CTAACATGGCTAAGCACCTG	<i>Δdns</i> F1 (3kb)
BBC1605	GCACTTCTTCGCGAATTCGC	<i>Δdns</i> F1 (2kb)
BBC1607	AGTGATTGGGTCACCTATTGG	<i>Δdns</i> F1 (1kb)
BBC1609	AATGAGATTCGCCTTAACCC	<i>Δdns</i> F1 (0.5kb)
BBC1265	gtcgacggatccccggaatAGAGAACAGGTATTTTCATAGTTAAAGTC	<i>Δdns</i> R1
BBC1266	gaagcagctccagcctacaTAATCCTCACCAATCGCGAC	<i>Δdns</i> F2
BBC1610	TCGAGCTTTACGCCACAACG	<i>Δdns</i> R1 (0.5kb)
BBC1608	ACACCTTGGTCGAGGTGAAG	<i>Δdns</i> R1 (1kb)
BBC1606	ATAACGCAGTAGAAAAGTATCCAC	<i>Δdns</i> R1 (2kb)
BBC1267	ACTGGTAAGCCATAACGACC	<i>Δdns</i> R1 (3kb)
DOG0246	AGGCTCGTGTTGCATGTGAG	<i>Δdns</i> 501bp F1
DOG0247	gctaattcagtttaagcgccatCATAGTTAAAGTCTTTAAAAAGTATGACTT	<i>Δdns</i> 501bp R1
DOG0248	atggccgcttaaactgaattagcATCGCTCGTACCTATCTTTATATG	<i>Δdns</i> 501bp F2
DOG0249	TAAGGTGTCTCAAATCTCAATCTAGG	<i>Δdns</i> 501bp R2
BBC1255	TGAGAAATCTTTGCATCACATC	<i>rpsL</i> K43R (Sm <sup>R</sup> ) F1
BBC1256	GAAGTGCTGAGTTAGGTTTTcTAGGTGTAGTAGTGTAACAC	<i>rpsL</i> K43R (Sm <sup>R</sup> ) R1
BBC1257	GTGTTTACACTACTACACCTAgAAAACCTAACTCAGCACTTC	<i>rpsL</i> K43R (Sm <sup>R</sup> ) F2
BBC1258	GTAGTGACGAGTTGGAGTG	<i>rpsL</i> K43R (Sm <sup>R</sup> ) R2
BBC1552	GAATGCATGAATACGTTGTTCC	<i>ΔmutS</i> 501bp F1
BBC1553	gctaattcagtttaagcggcCACAGGTAAGTCTTTTGTATTTC	<i>ΔmutS</i> 501bp R1
BBC1554	GTGgccgcttaaactgaattagcCGCACCGCACCGTGAG	<i>ΔmutS</i> 501bp F2
BBC1555	GAGTATCAGCAACACAGTAACC	<i>ΔmutS</i> 501bp R2
BBC1347	TAGCAACTGTTTTAGCGCTG	<i>ΔwbfF</i> F1
BBC1348	gtcgacggatccccggaatCTTTTATCATCATACTCATTATTAAAG	<i>ΔwbfF</i> R1
BBC1349	gaagcagctccagcctacaTGATGTATAAGCGTCATTTATTTCG	<i>ΔwbfF</i> F2
BBC1350	GTTCTGTTCGATAAGTATTGATC	<i>ΔwbfF</i> R2
DOG0353	AATGTCGGCCTTCTGATTAG	<i>ΔwbfF</i> 501bp F1 (3kb)



BBC1612	TAAACTTTATCAGCGACGTCAG	$\Delta wbfF$ 501bp F1 (2kb)
BBC1614	TTCAGGAACGATGTGACAG	$\Delta wbfF$ 501bp F1 (1kb)
DOG0354	gctaattcagtttaagcgccatTATCATCATACTCATTCATTAAG TTTTAA	$\Delta wbfF$ 501bp R1
DOG0355	atggccgcttaaactgaattagcACTAATAACGTCAGTGTATACGTA AAC	$\Delta wbfF$ 501bp F2
BBC1615	CCACGCAATGTAGTCATCAATC	$\Delta wbfF$ 501bp R2 (1kb)
BBC1613	GGATACGCAGCATACCTTG	$\Delta wbfF$ 501bp R2 (2kb)
BBC1611	TTAATTGTGCCTGAGCAAGC	$\Delta wbfF$ 501bp R2 (3kb)
DOG0271	AAGTAGTGATGATCCGAAGCG	$\Delta BA890\_01815$ 501bp (mannitol transporter) F1
DOG0272	gctaattcagtttaagcgccatCATAACAATCCCCGTTTCGATG	$\Delta BA890\_01815$ 501bp (mannitol transporter) R1
DOG0273	atggccgcttaaactgaattagcCTTGTATCAGCGCACCTTCTAC	$\Delta BA890\_01815$ 501bp (mannitol transporter) F2
DOG0274	ATCGTGGTAAATATCGTCAGGTAG	$\Delta BA890\_01815$ 501bp (mannitol transporter) R2
DOG0266	ATCTCGGCTTGTCTACACCAG	$\Delta BA890\_19540$ (sucrose transporter) F1
DOG0267	gctaattcagtttaagcgccatCATTCACACCCCGATTGG	$\Delta BA890\_19540$ (sucrose transporter) R1
DOG0268	atggccgcttaaactgaattagcTATTTACCTGTTTTATTGGCGTTTT TC	$\Delta BA890\_19540$ (sucrose transporter) F2
DOG0269	TGAACTGAATCCTCGCAGG	$\Delta BA890\_19540$ (sucrose transporter) R2
DOG0256	ATGCTCGTCATCCATGGGAC	$\Delta BA890\_16410$ (fructose transporter) F1
DOG0257	gctaattcagtttaagcgccatCATACTGATAACCTTCTGTTCCTT AG	$\Delta BA890\_16410$ (fructose transporter) R1
DOG0258	atggccgcttaaactgaattagcACCGCGCAAGAGATCGAAG	$\Delta BA890\_16410$ (fructose transporter) F2
DOG0259	TTGGGTGCTTTGCTTCTCG	$\Delta BA890\_16410$ (fructose transporter) R2
DOG0261	ATCTGAACTTAGGATACTCACATC	$\Delta BA890\_03375$ (trehalose transporter) F1
DOG0262	gctaattcagtttaagcgccatCATAACTTTGCCACCTGTATG	$\Delta BA890\_03375$ (trehalose transporter) R1
DOG0263	atggccgcttaaactgaattagcTTCTTCTGCCTGTTGGC	$\Delta BA890\_03375$ (trehalose transporter) F2
DOG0264	AGTCAGATGGCGATTGATGTG	$\Delta BA890\_03375$ (trehalose transporter) R2
ABD840	TTAATTGCGTTGCGCTCACTGCCGACTCCCGTTCTGGATA ATGTTTTTGC	Amplify MIDDLE $P_{tac}$ construct F
ABD625	CTGATGAATCCCCTAATGATTTTGG	Amplify MIDDLE $P_{tac}$ construct R
BBC1536	GTAACGAACGTGTCATCAGTG	$P_{tac}$ - <i>phaBAC</i> F1
BBC1540	CGGGCAGTGAGCGCAACGCAATTAATGCAAGCGCACTAAT ATGAC	$P_{tac}$ - <i>phaBAC</i> R1
BBC1541	CAAAATCATTAGGGGATTCATCAGAAAAGATGGAGTCGTC AATGAATAAAG	$P_{tac}$ - <i>phaBAC</i> F2
BBC1577	CGACATCTTCACCAACACG	$P_{tac}$ - <i>phaBAC</i> R2
BBC1621	TCTGGAGAGTATGTTGGCC	$P_{tac}$ - <i>pntAB</i> F1
BBC1622	cgggcagtgagcgcaacgaattaaCCTTGTATACATATCAATTA TTAGTCCC	$P_{tac}$ - <i>pntAB</i> R1

BBC1623	caaatcattaggggattcatcagAggaggTTGCGTTTTGCAAATCGG TGTAC	<i>P<sub>tac</sub>-pntAB</i> F2
BBC1624	AGACTACGCCAAACTATACAGC	<i>P<sub>tac</sub>-pntAB</i> R2
BBC1616	CTTCTTCGTCTTCAAACGACG	<i>P<sub>tac</sub>-nadK</i> F1
BBC1617	cgggcagtgagcgaacgaattaaGCATTAAGAGGCTTGAATCA GG	<i>P<sub>tac</sub>-nadK</i> R1
BBC1618	caaatcattaggggattcatcagaggaggTAAATGCTATGAAAAATCC ATGTAACG	<i>P<sub>tac</sub>-nadK</i> F2
BBC1619	CTGCGCTGATAATAACAAGC	<i>P<sub>tac</sub>-nadK</i> R2
BBC1626	CACAAATAGCGAAGCTAACTG	<i>P<sub>tac</sub>-udhA</i> F1
BBC1627	cgggcagtgagcgaacgaattaaTATTTGCTTAAACATTGCTTA GC	<i>P<sub>tac</sub>-udhA</i> R1
BBC1628	caaatcattaggggattcatcagAggaggTACATCATGGCGCATGT AAATC	<i>P<sub>tac</sub>-udhA</i> F2
BBC1629	GTGAAAGTATTTTCGCTTTTCG	<i>P<sub>tac</sub>-udhA</i> R2
BBC1636	GACAAGTCAGAAAGTCCAGTCAC	$\Delta$ <i>pta</i> 501bp F1
BBC1637	gctaattcagtttaagcggccatAGACATTCGTAGAGTACCTTTGC	$\Delta$ <i>pta</i> 501bp R1
BBC1638	atggccgcttaaactgaattagcGTTATCATCAACAAGCTAAACGCA C	$\Delta$ <i>pta</i> 501bp F2
BBC1639	GATATCAACGAGTTTGCATCTG	$\Delta$ <i>pta</i> 501bp R2
BBC1646	GCTAACATCAATGCGTATGCC	$\Delta$ <i>pgi</i> 501bp F1
BBC1647	gctaattcagtttaagcggccatCAACATGGTCTTTATCCCGATG	$\Delta$ <i>pgi</i> 501bp R1
BBC1648	atggccgcttaaactgaattagcGCACTGGCACCATACAAAAAC	$\Delta$ <i>pgi</i> 501bp F2
BBC1649	CCTTTCTCAGACACTATCGACAC	$\Delta$ <i>pgi</i> 501bp R2
BBC1641	AGCCTTCTTCTACATCAAGTGTG	$\Delta$ <i>gltA</i> 501bp F1
BBC1642	gctaattcagtttaagcggccatATCCGCCATAACAATCTCCTTTG	$\Delta$ <i>gltA</i> 501bp R1
BBC1643	atggccgcttaaactgaattagcACACTGGCGGCAATGTGTTAC	$\Delta$ <i>gltA</i> 501bp F2
BBC1644	CAAGAGTACTACGAAGAGCTG	$\Delta$ <i>gltA</i> 501bp R2
BBC1651	CTTGTAACACTGCCGCTAAGAG	$\Delta$ <i>ldhA</i> 501bp F1
BBC1652	gctaattcagtttaagcggccatCATGGTTCTCTCTCGAAATCATTG	$\Delta$ <i>ldhA</i> 501bp R1
BBC1653	atggccgcttaaactgaattagcATGGAAATCTTTGCCATGATCC	$\Delta$ <i>ldhA</i> 501bp F2
BBC1654	AGTGTGTTACTTATTTGGAGGATG	$\Delta$ <i>ldhA</i> 501bp R2
BBC1631	TGAACTGCTGGCGAAAGGAC	$\Delta$ <i>aceA</i> 501bp F1
BBC1632	GCTAATTCAGTTTAAAGCGGCCATTGGTCTATCCCTCTTTAT AATTTGC	$\Delta$ <i>aceA</i> 501bp R1
BBC1633	ATGGCCGCTTAAACTGAATTAGCCTAAATGCTTACGAACTG ATGAAATC	$\Delta$ <i>aceA</i> 501bp F2
BBC1634	CGATTGAAGCTTGAAGAACAAGC	$\Delta$ <i>aceA</i> 501bp R2
<b>Primers for MASC-PCR</b>		
ABD969	ATGGCCGCTTAAACTGAATTAGC	Universal F primer for all $\Delta$ 501 bp genome edits
DOG0250	TGGTTGCCTTGTACTTTGGC	R detect for $\Delta$ <i>dns</i> 501 bp (152bp product)
BBC1556	AGTGATCGAGAACAGCGG	R detect for $\Delta$ <i>mutS</i> 501bp (402bp product)
DOG0356	ATAGCTACCGGTTTCAAGG	R detect for $\Delta$ <i>wbfF</i> 501bp (165bp product)
DOG0275	AGTGACGTGGATGTTTCAGAC	R detect for $\Delta$ BA890_01815 501bp (mannitol transporter) (750bp product)
DOG0270	AACCCAGTGATACCAGATGG	R detect for $\Delta$ BA890_19540 (sucrose transporter) (650bp product)
DOG0260	TATTCATCAGTGCAGCGGC	R detect for $\Delta$ BA890_16410 (fructose

		transporter) (352 bp product)
DOG0265	TCTTGCATTAAGTAAATCCACG	R detect for $\Delta$ BA890_03375 (trehalose transporter) (500 bp product)
BBC435	ACACTCTTTGGGGGCCAAAATCATTAGGGGATTCATCAG	Universal F primer to detect all $P_{tac}$ genome edits
BBC1551	GGTAAACCCCTTGCTGTAAACC	R detect for $P_{tac}$ - <i>phaBAC</i> (170bp product)
BBC1625	CTTGAGCTCGAGAGATACG	R detect for $P_{tac}$ - <i>pntAB</i> (400bp product)
BBC1620	GATAAAATTCGTGCGGCTC	R detect for $P_{tac}$ - <i>nadK</i> (260bp product)
BBC1630	AGATAATGATATGACGAGGGTC	R detect for $P_{tac}$ - <i>udhA</i> (550bp product)
BBC1640	CGAATTGGAGAAGTGTGAAG	R detect for $\Delta$ <i>pta</i> (140bp product)
BBC1650	AACCCAGTCCCAGAATTCAAAC	R detect for $\Delta$ <i>pgi</i> (300bp product)
BBC1645	GATGTTGACGCTTTTGTTCG	R detect for $\Delta$ <i>gltA</i> (200bp product)
BBC1655	GGCTTCTACGTTATTTAGTGTC	R detect for $\Delta$ <i>ldhA</i> (450bp product)
BBC1656	TGTTGTGAATACCCGCTAGAG	R detect for $\Delta$ <i>aceA</i> (600bp product)

\*Lower case nucleotides specify overlap regions for SOE PCR

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