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Developing a CRISPR-assisted base-editing system for genome engineering of *Pseudomonas chlororaphis*

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Summary

Pseudomonas chlororaphis **is a non-pathogenic, plant growth-promoting rhizobacterium that secretes phenazine compounds with broad-spectrum antibiotic activity. Currently available genome-editing methods for *P. chlororaphis* are based on homologous recombination (HR)-dependent allelic exchange, which requires both exogenous DNA repair proteins (e.g. λ-Red–like systems) and endogenous functions (e.g. RecA) for HR and/or providing donor DNA templates. In general, these procedures are time-consuming, laborious and inefficient. Here, we established a CRISPR-assisted base-editing (CBE) system based on the fusion of a rat cytidine deaminase (rAPOBEC1), enhanced-specificity Cas9 nickase (eSpCas9pp^{D10A}) and uracil DNA glycosylase inhibitor (UGI). This CBE system converts C:G into T:A without DNA strands breaks or any donor DNA template. By engineering a premature *STOP* codon in target spacers, the *hmgA* and *phzO* genes of *P. chlororaphis* were successfully interrupted at high efficiency. The *phzO*-inactivated strain obtained by base editing exhibited identical phenotypic features as compared with a mutant obtained by HR-based allelic exchange. The use of this CBE system was extended to other *P. chlororaphis* strains (subspecies LX24 and HT66) and also to *P. fluorescens* 10586, with an equally high editing efficiency. The wide applicability of this CBE method will accelerate bacterial physiology research and metabolic engineering of non-traditional bacterial hosts.**

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Introduction

Pseudomonas chlororaphis is a safe plant growth-promoting rhizobacterium that produces and secretes phenazine compounds with broad-spectrum antibiotic activity (Liu *et al.*, 2016; Yue *et al.*, 2020; Raio and Puopolo, 2021). Phenazine-1-carboxylic acid (PCA) – an important bioactive substance secreted by *P. chlororaphis* (Biessy and Filion, 2018) – has been successfully developed and registered as *Shenqinmycin* by the Ministry of Agriculture of China, due to its high efficiency in preventing rice sheath blight and the 'take-all' wheat disease (Song *et al.*, 2020; Yue *et al.*, 2020). The potential of *P. chlororaphis* to produce PCA and other value-added compounds, however, has not been fully exploited. *P. chlororaphis* is a non-traditional microbial host (Weimer *et al.*, 2020; Bitzenhofer *et al.*, 2021), which requires effective tools for quick genome manipulations towards providing an in-depth understanding of cell physiology and also for the construction of engineered strains (Liu *et al.*, 2015; Gurdo *et al.*, 2022; Ke *et al.*, 2022). Some genome-editing approaches have been developed for *P. chlororaphis* and related species. In this sense, a series of molecular tools, for example transposon mutagenesis, insertional inactivation, counter-selection markers [including *sacB*; Liu *et al.* (2019)] and suicide vectors, have been applied for introducing mutations in *P. chlororaphis* (Huang *et al.*, 2011; Wu *et al.*, 2019). However, these traditional methods are based on homologous recombination (HR)-dependent allelic exchange, which requires heterologous repair proteins and providing donor DNA templates. The application of

such methodologies relies on sequence-dependent single and double crossover events that happen naturally, but with a very low frequency. As such, these low-throughput approaches are time-consuming, laborious and inefficient to produce mutants.

In the past few years, the development of clustered regularly interspaced short palindromic repeat (CRISPR)-assisted genome-editing systems has greatly promoted metabolic engineering of different organisms, including mammalian cells (Ran *et al*., 2013), *Escherichia coli* (Jiang *et al*., 2015), *Corynebacterium glutamicum* (Cho *et al*., 2017), *Bacillus subtilis* (Westbrook *et al*., 2016), *Streptomyces* (Zhang *et al*., 2017; Tong *et al*., 2019), *P. putida* (Batianis *et al*., 2020; Volke *et al*., 2020a,b; 2021; Wirth *et al*., 2020) and yeast (Kang *et al*., 2016). In the most commonly used CRISPR/Cas9 systems, the Cas9 DNA nuclease and a single guide RNA (sgRNA) forms a complex (Jiang *et al*., 2013; Adli, 2018). The programmable 20-nucleotide (nt) sequence of the sgRNA combines the sequence of the target locus with an adjacent protospacer adjacent motif (PAM, 5'-*N*GG-3' for *Streptococcus pyogenes* Cas9) through base pairing (Anders *et al*., 2014; Cho *et al*., 2017). Then, the Cas9 DNA nuclease cleaves the target locus, resulting in a double-stranded break (DSB) in the chromosome. The DSB is repaired either by homology directed repair (HDR) in bacteria (Sung and Klein, 2006) or non-homologous end joining (NHEJ) in eukaryotes (Guirouilh-Barbat *et al*., 2004). In bacteria, λ-Red recombination systems and HDR templates are usually provided to assist homologous repair (Pyne *et al*., 2015). Based on these observations, it would seem that CRISPR/Cas9 systems are promising tools for genome engineering of *P. chlororaphis*. However, our previous attempts to introduce a CRISPR/Cas9 module into *P. chlororaphis* proved to be very difficult (a > 90% reduction in viability was observed upon transforming plasmids with a wild-type Cas9 gene into the cells; unpublished). This result is probably connected to toxicity of the Cas9 nuclease, low transformation efficiency or poor genome stability of the recombinant strain. Similar detrimental effects have been reported for several microbial species (Yao *et al*., 2018; Zhang and Voigt, 2018). Additionally, adopting non-traditional *Pseudomonas* species (e.g. *P. chlororaphis*) as hosts calls for the use of broad-host-range plasmids that can be used in different isolates and subspecies (thus increasing portability), an aspect that has received relatively little attention in the literature.

The CRISPR-assisted base editors (CBE) developed recently for mammalian cells opened a new avenue for genome editing in a variety of organisms (Komor *et al*., 2016). Typical CBE systems contain the engineered fusions of the defective Cas9 (carrying the D10A mutation), the rat cytidine deaminase APOBEC1 (rAPOBEC1) and a uracil glycosylase inhibitor (UGI; Banno *et al*., 2018). Guided by a $Cas9^{D10A}$/sgRNA complex, rAPOBEC1 catalyses the transformation of a C:G base pair in a U:G heteroduplex, which can be converted to a T:A upon DNA replication (Banno *et al*., 2018; Kim, 2018). By targeting the CAA, CAG or CGA codons in the coding (non-template) strand or the TGG codon in the non-coding (template) strand, the CBE is capable of inactivating a target gene by introducing a premature *STOP* codon (Chen *et al*., 2018; Sun *et al*., 2020). This tool can efficiently and rapidly generate mutations without DSBs. Additionally, since no foreign DNA template are needed, CBE-based modifications may circumvent GMO regulations in several countries and benefit industrial applications (Kim, 2018; Wang *et al*., 2018). Other base-editing tools for *Pseudomonas* have been developed recently, for example a highly-efficient adenine editor (Abdullah *et al*., 2022).

In this study, we have developed a CBE tool specifically tailored for *P. chlororaphis*, which efficiently catalysed C:G to T:A changes in the target window sequence. The CBE was used to inactivate the *hmgA* and *phzO* genes of *P. chlororaphis* (encoding homogentisate 1,2-dioxygenase and PCA monooxygenase respectively) by introducing premature *STOP* codons in the coding sequences with an efficiency close to 100%. Moreover, a convenient and efficient plasmid curing method, based on the addition of readily-available, low-cost chemicals to the culture medium, was established to facilitate genome engineering efforts. Additionally, the use of this base-editing tool was extended into other *P. chlororaphis* strains (subspecies LX24 and HT66) and *P. fluorescens* 10586 with an equally high base-editing efficiency. As such, our study offers a powerful and versatile CBE tool for genome editing of non-traditional *Pseudomonas* species, and paves the way for developing CRISPR-assisted editing methodologies for microorganisms that lack efficient genetic manipulation toolkits.

Results

Design and construction of a plasmid-based CBE system for Pseudomonas *species*

Traditional CRISPR/Cas9 technologies are widely used for gene editing in many organisms, but they are still ineffective in some hosts, for example *P. chlororaphis*. Therefore, we sought to establish a base-editing system as an alternative tool for genome engineering of this species, since these systems generally adopt Cas9 variants with low toxicity and do not lead to DSBs. Hence, a base-editing system (borne by plasmid pBRC1, Table S1) was designed and constructed as indicated in Fig. 1. In this tool, the cytidine deaminase (rat APOBEC1) was fused to the *N*-terminus of an

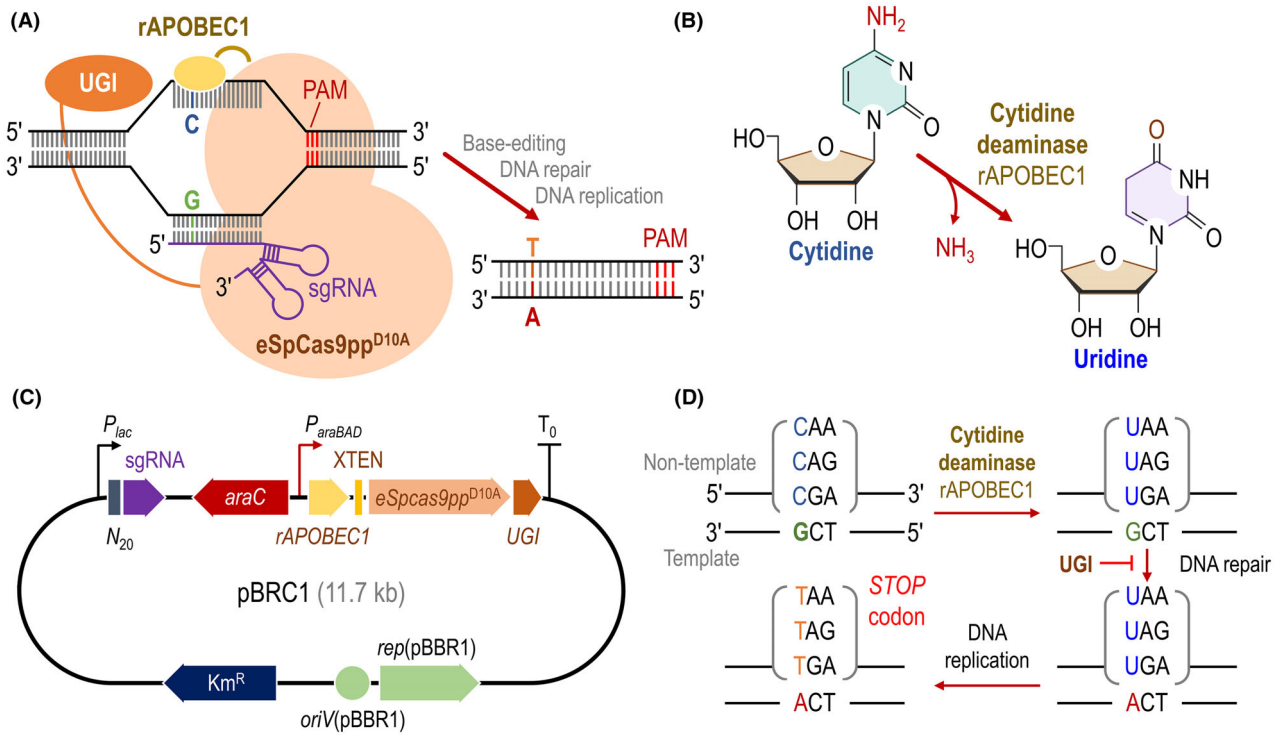

Fig. 1. Design of a CRISPR-assisted base-editing system tailored for *Pseudomonas chlororaphis*.
A. Schematic illustration of the plasmid-borne base-editing system. The tool consists of an enhanced specificity Cas9 nickase (eSpCas9pp^{D10A}), fused to a cytidine deaminase (rAPOBEC1, which catalyses the C-to-T conversion), and the uracil DNA glycosylase inhibitor (UGI). The base-editing machinery is directed to the target DNA by a single guide RNA (sgRNA). *PAM*, protospacer adjacent motif.
B. Biochemical reaction catalysed by the cytidine deaminase rAPOBEC1.
C. Structure of plasmid pBRC1, containing the construct that encodes the SpCas9pp^{D10A}, rAPOBEC1 and UGI fusion. This module is under transcriptional control by the L-arabinose-inducible AraC/P_{araBAD} system, whereas the sgRNA is constitutively expressed from a P_{lac} promoter. XTEN, short peptide linker sequence with no specific structure; N_{20}, specific 20-nt sequence targeting the gene to be edited; and Km^R, kanamycin resistance.
D. Strategy followed for gene inactivation by introducing premature *STOP* codons (TAA, TAG and TGA) in target genes using the base-editing system. UGI inhibits uracil base excision on the non-template DNA strand, so that DNA repair occurs on the template strand.

eSpCas9pp^{D10A} nickase by means of an XTEN linker, and the uracil glycosylase inhibitor (UGI) was fused to the *C*-terminus of eSpCas9pp^{D10A} (Fig. 1A). In this system, the cytidine deaminase rAPOBEC1 catalyses the conversion of a cytidine into uridine *via* deamination (Fig. 1B). The broad-host-range pBBR1 vector (Kovach *et al.*, 1995), containing the universal pBBR1 replicon that works efficiently in *P. chlororaphis* (Jin *et al.*, 2016; Liu *et al.*, 2016), was used as the backbone. All functional components of the CBE were combined into a single plasmid, termed pBRC1 (Fig. 1C). In this base-editing plasmid, the transcription of the sgRNA is driven by the constitutive P_{lac} promoter, while the expression of the fusion protein consisting of the deaminase, eSpCas9pp^{D10A} nickase and UGI is controlled by the L-arabinose-inducible AraC/P_{araBAD} expression system (Fig. 1C). As shown in Fig. 1D, the non-edited DNA strand is first targeted by the eSpCas9pp^{D10A} nickase and generates a nick, and then the nicked strand is repaired using the edited strand as a template (Komor *et al.*, 2016). UGI inhibits the excision of uracil bases caused by cytidine deaminase and thereby helps fixing the mutation (Banno *et al.*, 2018). To use the pBRC1 plasmid for base editing of target genes, specific 20-nt spacers should be inserted into the sgRNA cassette (Fig. 1B). Upon transforming the corresponding pBRC1 plasmid carrying such sgRNA into *P. chlororaphis* by electroporation, the CBE system mediates the conversion of target C residues to Ts with high efficiency. By specifically targeting CGA, CAG or CAA in the non-template DNA strand of the target genes, a premature *STOP* codon can be introduced to inactivate the corresponding loci (Fig. 1D).

Assessing the base-editing efficiency of plasmid pBRC1 in P. chlororaphis *GP72*

To test whether the pBRC1 system was an effective base-editing tool, the first gene of the homogentisate pathway, *hmgA* (encoding homogentisate 1,2-dioxygenase), was

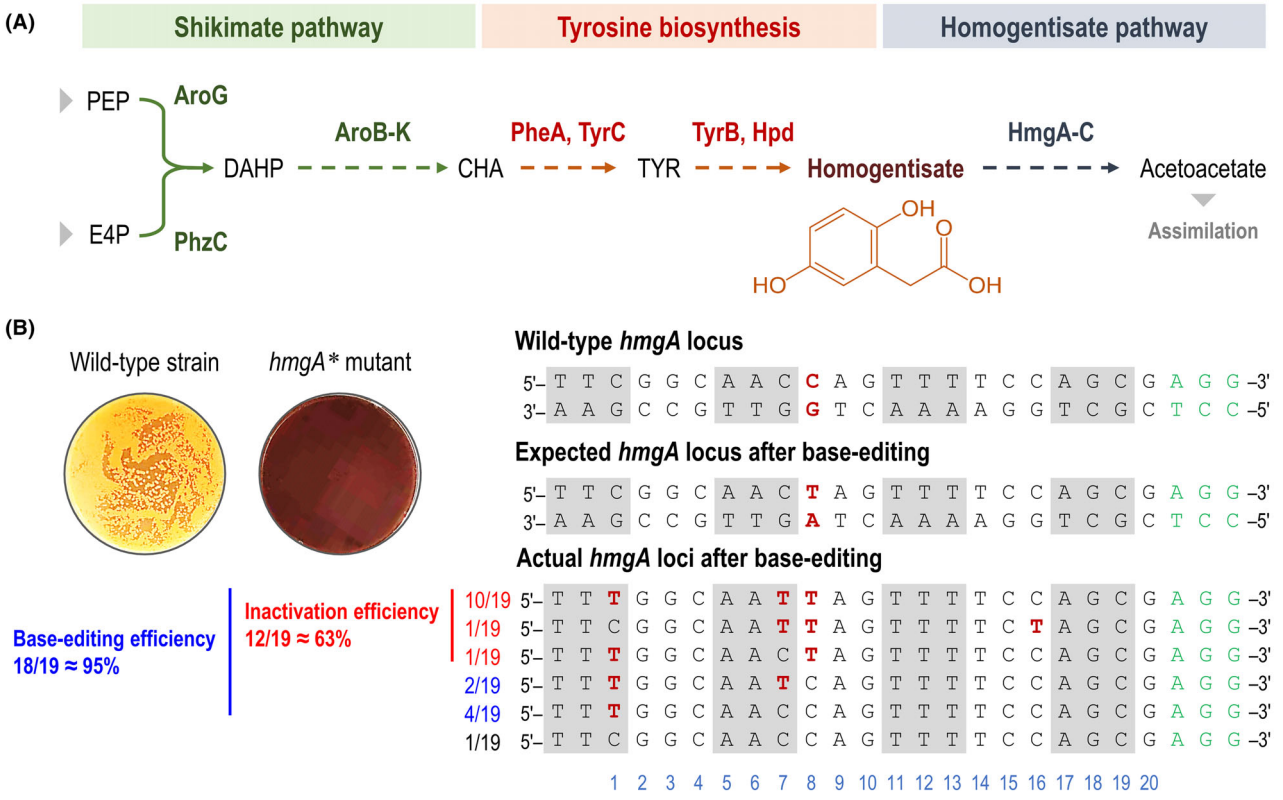

Fig. 2. Inactivation of the *hmgA* gene of *P. chlororaphis* GP72 by CRISPR-assisted base editing.
A. Overview of the homogentisate pathway in *P. chlororaphis* GP72. The three main metabolic blocks involved in biosynthesis and degradation of homogentisate are identified with different colour shadings. Grey arrowheads indicate further biochemical steps, both upstream and downstream. PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; CHA, chorismate and TYR, L-tyrosine.
B. Phenotypes of *hmgA* inactivation mutants (indicated with an asterisk, *) and alignment of the targeted 20-nt sequence in *hmgA* mutants obtained by base editing. The wild-type *hmgA* locus is shown as the double-stranded DNA sequence in the *P. chlororaphis* GP72 chromosome. The three bases indicated with grey boxes represent codons encoding amino acids, and the three bases highlighted in green identify the nearest PAM sequence (i.e. *N*GG). The C residue in the non-template strand, shown in red, is a candidate site for engineering a *STOP* codon (i.e. CAG→TAG). Actual *hmgA* loci after base editing illustrate the sequencing results of selected *hmgA** strains, and bases labelled in red in each line pinpoint the occurrence of a C → T mutation. Numbers on the left indicate the occurrence of different types of mutations relative to the number of individual clones selected for sequencing; red, mutations leading to the intended premature *STOP* codon; blue, mutations leading to non-terminating codons; and black, no mutation. The light blue numbers below the sequence alignment represent the position of the N_{20} region targeted in the *hmgA* gene. Inactivation efficiency (in red, bold face) was defined as the proportion of mutants with premature *STOP* codons relative to the number of all clones selected for sequencing. Base-editing efficiency (in blue, bold face) was calculated as the proportion of strains with any type of mutation relative to the number of sequenced clones.

selected as a target for inactivation (Arias-Barrau *et al.*, 2004). Mutations in *hmgA* disrupt the ring-cleavage reaction on homogentisate, resulting in the accumulation of this metabolic intermediate (Fig. 2A). Homogentisate can be spontaneously oxidized into a dark brown product in contact with air (Fig. 2B), and we employed *hmgA* as a reporter gene to assess the efficiency of the base-editing tool in *P. chlororaphis* GP72. We designed a specific 20-nt sequence targeting *hmgA* (Fig. 2B), that harbours potentially editable C(s) in the editable window, which has been reported to lie within positions 4 and 8 in mammalian cells (Komor *et al.*, 2016). The plasmid pBRC1-*hmgA*, carrying the specific 20-nt spacer designed for *hmgA* targeting, was electroporated into *P. chlororaphis* GP72. Then, the mixture was suspended in 100 μL of LB medium and spread onto LB plates (containing L-arabinose) to facilitate the detection of brownish *P. chlororaphis* colonies, indicative of positive base-editing events. Indeed, the wild-type strain formed light yellow colonies, while the *hmgA* mutants had a dark brown phenotype, qualitatively indicating an impaired HmgA function in these clones (Fig. 2B). Next, 19 randomly picked colonies (the total number of transformants typically obtained in these experiments was about 60–70; Fig. S1) were selected for colony PCR of the *hmgA* locus followed by DNA sequencing with the primers specified in Table S2. The sequencing results identified 18 colonies carrying the intended C-to-T mutation in the target 20-nt sequence,

yielding an overall editing efficiency for *hmgA* of ca. 95%. The C residue at position 8 of the *hmgA* spacer was mutated to T with an efficiency of ca. 63%, and this mutation introduced a premature *STOP* codon within the 20-nt frame of the gene, resulting in *hmgA* inactivation. In addition, to assess the potential non-specific mutation effects of plasmid pBRC1, the top 7 similar spacers for *hmgA* were identified in the genome of *P. chlororaphis* GP72 (Table S3). Then, all similar sequences as the spacer used for *hmgA* gene modifications were amplified and sequenced. The sequencing results show that none of all similar spacers were mutated, indicating a low-to-undetectable background of non-intended mutations (Table S3).

Establishing a method for rapid plasmid curing

Sodium dodecyl sulfate (SDS) is an agent commonly used to disrupt cell membrane integrity, and it is known to interfere with normal plasmid replication and distribution of plasmid DNA to the offspring cells (Buckner *et al.*, 2018). SDS has been used to help eliminating bacterial plasmids in laboratories or clinical setups, for example for *Enterococcus faecalis* (Keyhani *et al.*, 2006), *Klebsiella pneumoniae* (El-Mansi *et al.*, 2000) and *P. aeruginosa* (Raja and Selvam, 2009). Moreover, the effect of SDS addition is supposed to be boosted by the presence of divalent metallic cations (e.g. Ca^{2+}) that can mediate further cell membrane destabilization. We tested if this principle can be extended to *Pseudomonas* species to mediate plasmid curing after base-editing procedures. This aspect is particularly important considering the relative stability reported for plasmids bearing the origin of vegetative replication *oriV*(pBBR1) even in the absence of selective pressure (Jahn *et al.*, 2016). To this end, a single colony of *P. chlororaphis* GP72 harbouring the desired inactivation of *hmgA* and still carrying the base-editing plasmid pBRC1-*hmgA* was inoculated in fresh LB medium with 0.2% (w/v) SDS and $CaCl_2$ (i.e. equal concentrations of the detergent and Ca^{2+}). When cell growth was evident [the optical density measured at 600 nm (OD_{600}) reached ca. 0.1–0.2], the culture was diluted 10^4-fold using fresh LB medium likewise added with 0.2% (w/v) SDS and $CaCl_2$. Then, 100 μL aliquots of the diluted culture were spread onto LB medium plates without antibiotics. Next, isolated colonies grown on antibiotic-free LB plates were re-inoculated on separate LB plates containing either ampicillin (Amp, to which *P. chlororaphis* GP72 is naturally resistant) or kanamycin (Km). Strains capable of growing on LB plates with Amp but unable to grow in the presence of Km were selected (Fig. 3A), and tested again in liquid LB medium with either Amp or Km to confirm the phenotype observed in the plates. Under these conditions, we found that 22 colonies out of 48 had lost the plasmid. Based on these results, the impact of varying both the incubation time and the reagents (i.e. SDS and Ca^{2+}) concentration on plasmid curing was investigated. As shown in Fig. 3B and C, the optimal incubation time of strains carrying base-editing plasmids to facilitate plasmid loss was 15 h (55% of colonies were plasmid-free) and the optimal SDS and $CaCl_2$ concentrations were found to be 0.2% (w/v), above which toxicity issues were too significant for practical applications. Hence, these optimized conditions were kept for further CBE experiments.

Testing the CBE system in different chromosome loci in P. chlororaphis *GP72*

After demonstrating the functionality of CBE-based gene modification on the *hmgA* gene, we further expanded the system to modify the *phzO* locus in *P. chlororaphis* GP72. The *phzO* gene encodes a monooxygenase that catalyses the conversion of PCA to 2-hydroxyphenazine (2-OH-PHZ) (Delaney *et al.*, 2001). Eliminating the PhzO activity should lead to strains that, in theory, can only synthesize PCA (Huang *et al.*, 2011; Liu *et al.*, 2016). Four specific 20-nt spacers were designed to target *phzO*, containing potentially editable C(s) in the editing window, to explore the effect of premature *STOP* codons at different positions of the gene (Fig. 4A). In addition, and due to the industrial relevance of PCA biosynthesis, these modifications in *phzO* were adopted to study the use of plasmid pBRC1 as a gene-editing tool for metabolic engineering purposes. The selected sequences were inserted in the sgRNA cassette of plasmid pBRC1, resulting in plasmids pBRC1-*phzO1*, pBRC1-*phzO2*, pBRC1-*phzO3* and pBRC1-*phzO4* (Table S1 in the Supporting Information). The four constructs were transferred into *P. chlororaphis* GP72, and single colonies were scored for modifications in *phzO* by colony PCR and sequencing. Interestingly, all plasmids led to the intended C-to-T modifications in the target locus (Fig. 4B–E), yet plasmids pBRC1-*phzO1* and pBRC1-*phzO4* mediated the introduction of premature, in-frame *STOP* codons in loci 1 and 4 with 100% efficiency (Fig. 4B and E). Plasmids pBRC1-*phzO2* and pBRC1-*phzO3*, in contrast, caused in-frame, non-terminating mutations in loci 1 and 3 (Fig. 4C and D). In all, the high efficiency observed in *phzO* illustrates the potential of the pBRC1-based CBE system for base editing in *P. chlororaphis*.

Next, plasmid-free *P. chlororaphis* GP72 strains Δ*phzO*1* and Δ*phzO*4* were obtained by applying the plasmid-curing protocol developed herein. These genome-edited strains were tested for PCA production in shaken-flask cultures. The key fermentation parameters were compared to those of the wild-type strain (Fig. 5A) and a Δ*phzO* deletion mutant obtained by HR-dependent allelic exchange (Fig. 5B). All

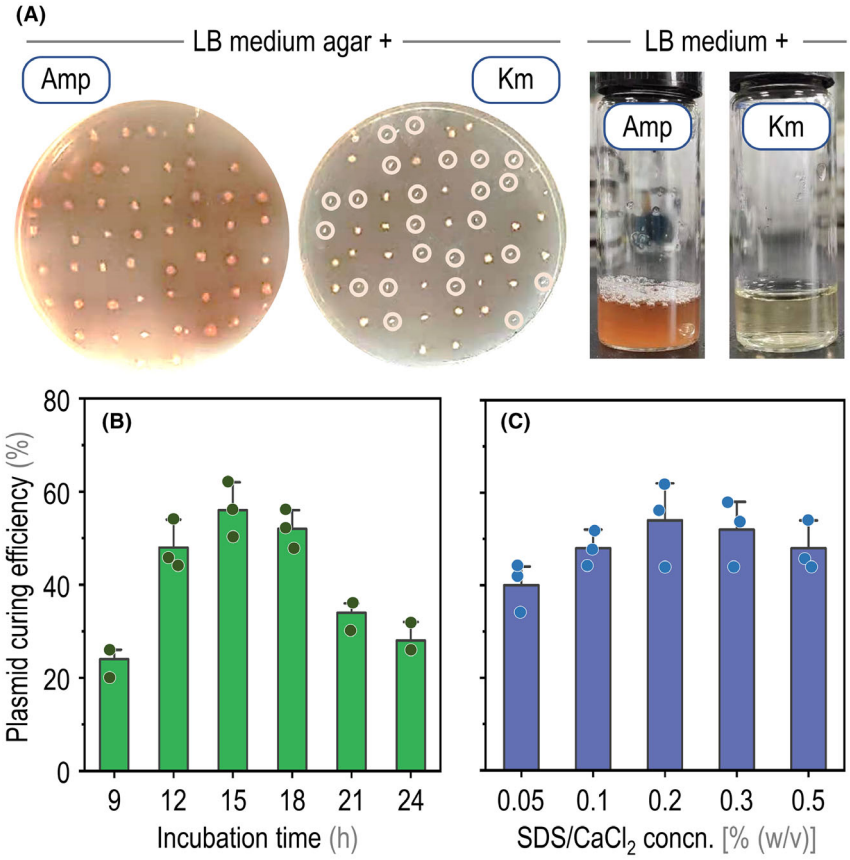

Fig. 3. Quick curing of plasmid pBRC1-*hmgA* from *P. chlororaphis* GP72 recombinants after base-editing.
A. Growth phenotype of individual clones on LB medium plates, added with either ampicillin (Amp) or kanamycin (Km), upon treatment of the cultures with 0.2% (w/v) SDS and $CaCl_2$. *P. chlororaphis* GP72 is endowed with natural resistance to Amp but not to Km, which facilitates the screening of plasmid-positive and plasmid-negative clones. Km-sensitive colonies, which had lost plasmid pBRC1-*hmgA*, are identified with circles. B. Km-sensitivity phenotype of selected clones, confirmed by growing the isolates in LB medium added with the same antibiotics.
C. Effect of extended incubation times in LB medium containing 0.2% (w/v) SDS and $CaCl_2$ on plasmid curing efficiency (represented as a percentage of the total bacterial population).
D. Effect of increasing SDS and $CaCl_2$ concentrations (concn.) on plasmid curing efficiency (percentage of the total bacterial population). Note that the concentration of the two additives was varied in the same magnitude across experiments. Data are shown as average values of three biological replicates (with individual data points imposed over the bars), and error bars represent standard deviations.

strains grew with similar kinetics and reached comparable cell densities over the course of the experiment. The fermentation profiles showed that, unlike the wild-type strain, the Δ*phzO1** and Δ*phzO4** strains only synthesized PCA (i.e. no 2-OH-PHZ was detected in these cultures, Fig. 5C and D). In these experiments, wild-type *P. chlororaphis* GP72 produced 4.3 mg l^{-1} 2-OH-PHZ after 72 h (Fig. 5A), while all *phzO* mutant strains lost their ability to synthesize the by-product. Moreover, the PCA titre in cultures of the base-editing strains was essentially the same as that of strain GP72 Δ*phzO* (peaking at ca. 32 mg l^{-1}; Fig. 5B–D).

Expansion of the CBE system to other P. chlororaphis *subspecies and* P. fluorescens

The *P. chlororaphis* group of species includes *P. chlororaphis* subsp. *aureofaciens*, *P. chlororaphis* subsp. *aurantiaca* and *P. chlororaphis* subsp. *chlororaphis* (Peix *et al.*, 2007). To explore the scope of genome modifications in other subspecies of this bacterium, we applied the plasmid pBRC1 for base editing in *P. chlororaphis* subsp. *aurantiaca* LX24 and *P. chlororaphis* subsp. *chlororaphis* HT66. The specific 20-nt spacer that targets *hmgA* (originally designed for *P. chlororaphis* subsp. *aureofaciens* GP72; Fig. 2) was applied for manipulations in both *P. chlororaphis* subsp. *aurantiaca* LX24 and *P. chlororaphis* subsp. *chlororaphis* HT66. Hence, plasmid pBRC1-*hmgA* was transformed into *P. chlororaphis* subsp. *aurantiaca* LX24 and subsp. *chlororaphis* HT66 and, as shown in Fig. 6A, the colour of the resulting transformants was dark brown. This phenotype was clearly different from that of the wild-type strains (Fig. S2), indicating that *hmgA* had been successfully inactivated in all strains. We also extended to use of the

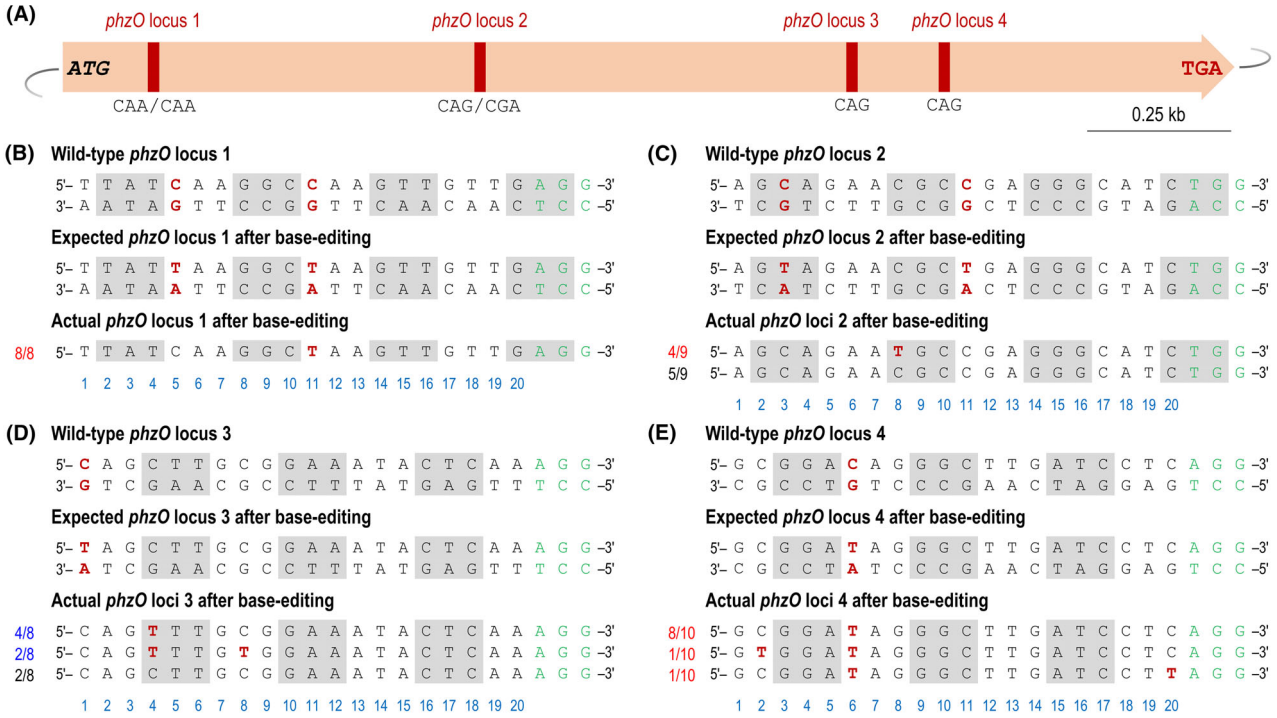

Fig. 4. Inactivation of the *phzO* locus in *P. chlororaphis* GP72 by CRISPR-assisted base editing.
A. Physicial map indicating the position of the four loci targeted in the *phzO* gene, shown relative to the ATG and *STOP* codon.
B–E. Sequence alignment of the *phzO* loci 1, 2, 3 and 4 in individual mutants obtained by base editing. The wild-type *phzO* loci are shown as the double-stranded DNA sequence in the *P. chlororaphis* GP72 chromosome. The three bases indicated with grey boxes represent codons encoding amino acids, and the three bases highlighted in green identify the nearest PAM sequence (i.e. *N*GG). The C residue in the non-template strand, shown in red, is a candidate site for engineering a *STOP* codon (i.e. CAA/CAA→TAA/TAA, CAG/CGA→TAG/TGA, CAG→TAG and CAG→TAG). Actual *phzO* loci after base editing illustrate the sequencing results of selected *phzO** strains, and bases labelled in red in each line pinpoint the occurrence of a C → T mutation. Numbers on the left indicate the occurrence of different types of mutations relative to the number of individual clones selected for sequencing; red, mutations leading to the intended premature *STOP* codon; blue, mutations leading to non-terminating codons; and black, no mutation. The light blue numbers below the sequence alignment represent the position of the N_{20} region targeted in the *phzO* gene.

CBE system to *P. fluorescens* 10586. In this case, we repeated the same procedure as indicated for *P. chlororaphis*, but targeting the *crc* and *rpoN* genes (selected as an example of two regulatory functions, as opposed to the metabolic-related genes targeted thus far). In these experiments, the base-editing efficiency for the *crc* and *rpoN* loci of *P. fluorescens* 10586 was 100% and 58%, respectively (Fig. 6B and C). Taken together, these observations support the notion that the CBE system developed in this study can be harnessed for manipulating other *Pseudomonas* species and not only for metabolic engineering purposes, but also for gene regulation studies.

Discussion

P. chlororaphis is a non-pathogenic host ideally for the synthesis of the biopesticide PCA (Song *et al.*, 2020). Since traditional genetic tools in *P. chlororaphis* involve laborious, time-consuming and inefficient protocols, constructing mutants of this species has been remarkably difficult thus far. CRISPR/Cas9-based genome editing technologies have not been developed in *P. chlororaphis* (and other species), likely due to the known toxicity of Cas9 proteins (Zhang and Voigt, 2018) or some other unknown factors. CRISPR-assisted base-editing systems adopt a catalytically-inactive version of Cas9 that does not lead to DSBs, thus the burden mediated by the nuclease is greatly reduced (Cho *et al.*, 2018). Moreover, CBE systems do not require DNA repair templates and/or heterologous repair enzymes, so they constitute a convenient and efficient tool for gene manipulation.

In this work, we developed a CRISPR-assisted base-editing system in *P. chlororaphis* by the fusion the $Cas9^{D10A}$ nickase, a rat cytidine deaminase (rAPOBEC1) and UGI. C-to-T substitutions were detected at target sites of *hmgA* and *phzO*, and most of these site mutations were concentrated at positions 3–8, which was consistent with the editable window previously reported

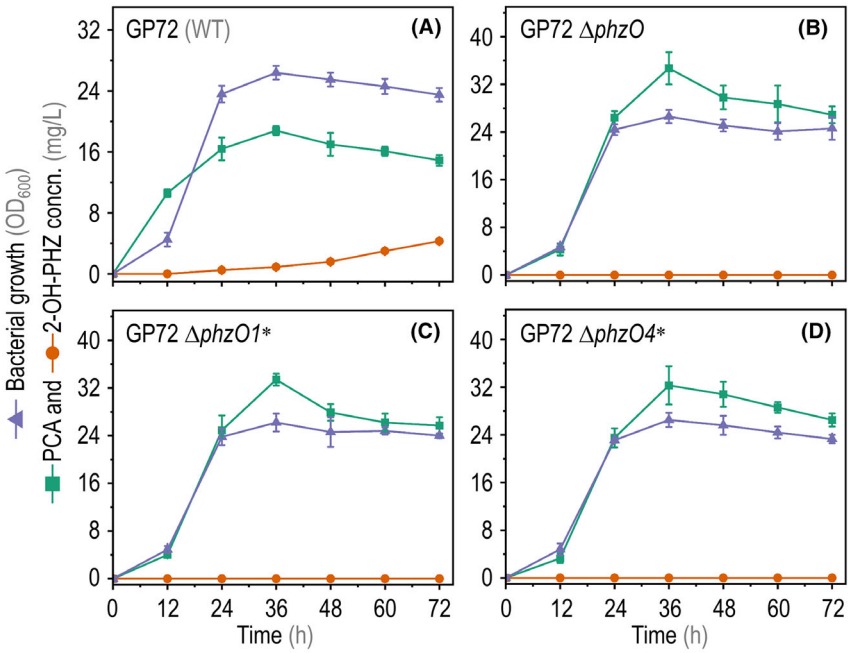

Fig. 5. Fermentation parameters of wild-type (WT) and mutant *P. chlororaphis* strains. Bacterial growth (estimated as the optical density at 600 nm, OD_{600}), phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ) titres in shaken-flask cultures of (A) the WT strain GP72, (B) a clean, in-frame Δ*phzO* deletion strain and (C-D) two mutant strains constructed by using the base-editing system (in loci 1 and 4, Δ*phzO*1* and Δ*phzO*4*). Data are shown as average values from three biological replicates, and error bars represent standard deviations.

in mammalian cells (Komor *et al.*, 2016). Moreover, it was also observed that this system has a catalytic preference for different motifs similar to that of mammals, that is T:C ≥ C:C ≥ A:C > G:C (Komor *et al.*, 2016). After demonstrating the effectiveness of this system, we established a plasmid-curing strategy based on changes in cell permeability. SDS and $CaCl_2$ compromise the integrity of cell membranes and impair normal plasmid replication (Buckner *et al.*, 2018), thus the offspring cells usually contain no plasmid DNA. This vector-curing strategy is independent of the introduction of new genes, which would increase the CBE plasmid size. This situation, in turn, leads to higher transformation efficiencies than what would be expected for large plasmid constructs, which are often difficult to introduce in bacterial cells by electroporation (Sheng *et al.*, 1995). Furthermore, the engineered strains Δ*phzO1** and Δ*phzO4** obtained by base editing exhibited similar phenotypes as compared to strain Δ*phzO*, previously obtained by traditional, HR-based methods for gene deletion (Fig. 5). This result indicates that the base-editing system can be used in studies dealing with (fundamental) cell physiology, metabolic engineering and synthetic biology of *P. chlororaphis*. Moreover, other *Pseudomonas* species can be likewise targeted with this genome engineering toolkit. *P. fluorescens* 10586, for instance, is a bacterial strain that can synthesize the drug mupirocin (Whatling *et al.*, 1995), and the genetic manipulation of this species has been described as relatively inefficient and time-consuming (Di Gioia *et al.*, 2011). The application of our base-editing system also showed efficient editing efficiency, which will greatly promote metabolic engineering efforts for enhanced synthesis of mupirocin (Gao *et al.*, 2014).

The whole base-editing process of *P. chlororaphis*, including electroporation, genome modification, identification of mutants and plasmid curing, took only 5 days. Moreover, the gene inactivation efficiency can reach up to 100%, and other site-directed mutagenesis can be also achieved by using this method. The efficiency of traditional strategies based on HR-dependent suicide plasmids is generally < 10%, and the shortest time required for these manipulations is around 10 days (Liu *et al.*, 2016). The efficiency of insertional inactivation and site-specific recombination, on the other hand, is typically < 30%, with ca. 8 days as the shortest time required for this procedure (Huang *et al.*, 2011). Taken together, our results indicate that the base-editing system developed in this work represents a convenient and efficient tool for gene modification in *P. chlororaphis* and other *Pseudomonas* species. As such, the base-editing plasmid pBRC1 established here is expected to accelerate metabolic engineering efforts of *P. chlororaphis* for enhancing the production of PCA and other value-added metabolites in the future. These applications notwithstanding, this toolbox can also be deployed onto other

Fig. 6. Applying the CRISPR-assisted base-editing system for gene inactivation in different *P. chlororaphis* strains and *P. fluorescens*.
A. Representative LB medium plates seeded with *P. chlororaphis* subsp. *aureofaciens* GP72, *P. chlororaphis* subsp. *aurantiaca* LX24 and *P. chlororaphis* subsp. *chlororaphis* HT66 upon inactivation of the *hmgA* gene mediated by base editing.
B. Sequence alignment of the *crc* locus in *P. fluorescens* 10586 mutants obtained by base editing.
C. Sequence alignment of the *rpoN* locus in *P. fluorescens* 10586 mutants obtained by base editing. The wild-type *crc* and *rpoN* loci are shown as the double-stranded DNA sequence in the *P. fluorescens* 10586 chromosome. The three bases indicated with grey boxes represent codons encoding amino acids, and the three bases highlighted in green identify the nearest PAM sequence (i.e. *N*GG). The C residue in the non-template strand, shown in red, is a candidate site for engineering a *STOP* codon (i.e. CAG→TAG and CAA→TAA). Actual *crc/rpoN* loci after base editing illustrates the sequencing results of selected strains, and bases labelled in red in each line pinpoint the occurrence of a C → T mutation. Numbers on the left indicate the occurrence of different types of mutations relative to the number of individual clones selected for sequencing; red, mutations leading to the intended premature *STOP* codon; blue, mutations leading to non-terminating codons; and black, no mutation. The light blue numbers below the sequence alignment represent the position of the N_{20} region targeted in the *crc/rpoN* genes.

Pseudomonas species, especially towards engineering complex phenotypes (Calero *et al.*, 2020).

Experimental procedures

Plasmids, primers, bacterial strains and growth conditions

The bacterial strains, plasmids and oligonucleotide primers used in this study are listed in Tables S1 and S2 in the Supporting Information respectively. *Escherichia coli* DH5α were used as the host for plasmid cloning and maintenance. *E. coli* strains were cultured at 37°C in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl) containing 50 μg ml^{-1} kanamycin. *P. chlororaphis* GP72, LX24 and HT66, and *P. fluorescens* 10586 and their derivatives used in base-editing experiments were cultured in LB medium at 28°C in the presence of 100 μg ml^{-1} ampicillin. Previously-published protocols were adopted for general molecular biology procedures, routinely cultivation of *E. coli* and

Pseudomonas and quantitative physiology experiments (Ruiz *et al*., 2006; Nikel *et al*., 2009, 2016, 2021; Green and Sambrook, 2012; Sánchez-Pascuala *et al*., 2019; Volke *et al*., 2020a,b). In shaken-flask fermentation experiments, *P. chlororaphis* GP72 and its derivatives were cultured in the semi-synthetic KB medium (20 g l^{-1} tryptone, 18.9 g l^{-1} glycerol, 0.514 g l^{-1} K_2HPO_4 and 0.732 g l^{-1} $MgSO_4$) at 28°C in the presence of 100 μg ml^{-1} ampicillin. Other culture conditions were as described previously by Huang *et al*. (2011).

Construction of base-editing plasmids

Plasmid pBBR1MCS-2 [Addgene ID 85168; Kovach *et al*. (1995)] was used as the scaffold for the construction of the base-editing plasmids reported in this study. A DNA cassette encoding the cytidine deaminase from rat [rAPOBEC1; Komor *et al*. (2016)], an enhanced specificity Cas9 nickase [eSpCas9pp^{D10A}, carrying the point mutations K848A, K1003A, R1060A and D10A; Sun *et al*. (2020)], a XTEN linker [a short connector sequence; Komor *et al*. (2016)] and the uracil DNA glycosylase inhibitor (UGI) was amplified from plasmid pSEVA2BE [a derivative of standard vector pSEVA644; Sun *et al*. (2020)] and assembled in plasmid pBRC1 (deposited in Addgene, ID 183064). All functional DNA sequences used in the construction of plasmids are presented in the Supporting Information. A specific 20-nt sequence targeting *hmgA*, which harbours potentially editable C(s) residues within the editing window, was introduced in oligonucleotide *hmgA*-F. The guidelines indicated by Komor *et al*. (2016) were followed to design the sgRNAs used in this work. The fragments obtained by PCR were ligated into vector pBBR1MCS-2 (previously digested with EcoRI and XbaI) and the mixture was transformed into *E. coli* DH5α, resulting in plasmid pBRC1-*hmgA*. The screening of transformants was carried out by colony PCR with primers seq-F(sgRNA) and seq-R (sgRNA), and the amplification products were used for DNA sequencing (Shanghai PersonalBio Technology Co.; Xuhui District, Shanghai, China). The construction of plasmids pBRC1-*phzO*1, pBRC1-*phzO*2, pBRC1-*phzO*3, pBRC1-*phzO*4, pBRC1-*crc* and pBRC1-*rpoN* followed the same procedure described for plasmid pBRC1-*hmgA*. In this case, different forward primers (i.e. *phzO*1-F, *phzO*2-F, *phzO*3-F, *phzO*4-F, *crc*-F and *rpoN*-F) and the corresponding reverse primers (R) were used for constructing base-editing plasmids.

Electroporation and screening of mutant strains

For the preparation of electrocompetent cells, a 20-ml overnight culture of *P. chlororaphis* or *P. fluorescens*, grown in LB medium in a 50-ml Falcon tube, was centrifuged at 5,000 × *g* for 10 min at 4°C. The cell pellet was washed by resuspension in 10 ml of 10% (v/v) glycerol, followed by centrifugation at 5000 × *g* for 10 min. The supernatant was carefully discarded, and the washing step was repeated twice. In the final step, the cell pellet was resuspended in 0.1 ml of 10% (v/v) glycerol. A 0.1-ml aliquot of the resulting suspension was mixed with 300 ng of each plasmid and transferred to a 0.1-cm gap electroporation cuvette. A single 2.5-kV electric pulse was applied for up to 6 ms in an electroporator (Bio-Rad; Hercules, CA, USA), followed by immediate addition of 1 ml of LB medium to the cuvette. Cells were recovered by incubating the suspension at 28°C with rotational agitation for 2 h. The recovered cells were spread onto LB agar plates supplemented with 50 μg ml^{-1} kanamycin, 100 μg ml^{-1} ampicillin and 6 g l^{-1} L-arabinose and incubated at 28°C until the appearance of visible colonies (about 36 h). Primers seq-F(*hmgA*) and seq-R(*hmgA*) were used to amplify the target *hmgA* gene from isolated colonies, and the PCR products were purified for DNA sequencing to confirm the desired mutation events. The similarity within spacer sites in *hmgA* was assessed by comparing with the genome sequence of strain GP72 [National Center for Biotechnology Information (NCBI) database; GenBank assembly accession GCA_000237045.2] using BLAST (Ladunga, 2017). Primers F(M1-3) and R(M1-3) were used to amplify the similar spacer sites M1, M2 and M3 of *hmgA* for DNA sequencing. Primers F(4) and R(4) were used to amplify the similar spacer site M4 of *hmgA*. Likewise, primers F(5-6) and R(5-6), and F(7) and R(7), were used to amplify the similar spacer sites M5, M6 and M7, respectively, of *hmgA* for DNA sequencing. The base editing of *phzO* locus 1, locus 2, locus 3 and locus 4, and *crc* and *rpoN*, followed the same procedure as described for *hmgA*.

Quantification of biomass formation and PCA and 2-hydroxyphenazine (2-OH-PHZ) concentrations

P. chlororaphis cultures were centrifuged at 12,000 × *g* for 2 min, washed twice with distilled water, and the resulting bacterial suspension was diluted to fit the linear range of the spectrophotometer. The absorbance at 600 nm (OD_{600}) of this suspension was measured in an ultraviolet-visible spectrophotometer. *P. chlororaphis* fermentation broth aliquots (400 μL) were acidified to pH = 2.0 with 6 M HCl and mixed with 3,600 μl of ethyl acetate under vigorous shaking. The upper layer organic phase (400 μL) was evaporated at room temperature, and the residue was resuspended in 1 ml of acetonitrile and filtered through a 0.22-μm membrane for PCA and 2-OH-PHZ determinations using high-performance liquid chromatography (HPLC; Agilent 1260) with XDB-C18

reversed-phase column (4.6 mm × 250 mm, Agilent, Santa Clara, CA, USA). The mobile phase consisted of acetonitrile and 0.1% (w/v) formic acid in water at a flow rate of 1 ml min^{-1}, and the detection wavelength was set at 254 nm.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

S.J.Y. performed the experimental work and wrote the manuscript. P.H., S.L. and Y.Y.C. assisted in experimental work and analysed the data. H.B.H., P.I.N., W.W. and X.H.Z. analysed the data and proofread the manuscript. All the research work was performed under the supervision of H.B.H. who designed and coordinated the experiments, with further input and guidance from P.I.N. All authors have read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Visualization of base-editing events in *P. chlororaphis* GP72. The left plate shows *P. chlororaphis* GP72 transformed with the empty pBBR1-MCS2 vector, and the right plate was seeded with *P. chlororaphis* GP72 transformed with the base-editing plasmid pBRC1-*hmgA*. In both cases, LB medium plates were photographed after 36 h of incubation at 28°C.

Fig. S2. Phenotype of wild-type subspecies of *P. chlororaphis*. LB medium plates were seeded (from left to right) with *P. chlororaphis* subsp. *aureofaciens* GP72, *P. chlororaphis* subsp. *aurantiaca* LX24 and *P. chlororaphis* subsp. *chlororaphis* HT66. In all cases, LB medium plates were photographed after 36 h of incubation at 28°C.

Table S1. Strains and plasmids used in this study.

Table S2. Oligonucleotides used in this study.

Table S3. Sequence of spacers targeting *hmgA* and its top seven similar spacer sites.

