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*pheS**, an effective host-genotype-independent counter-selectable marker for marker-free chromosome deletion in *Bacillus amyloliquefaciens*

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Abstract Aside from applications in the production of commercial enzymes and metabolites, *Bacillus amyloliquefaciens* is also an important group of plant growth-promoting rhizobacteria that supports plant growth and suppresses phytopathogens. A host-genotype-independent counter-selectable marker would enable rapid genetic manipulation and metabolic engineering, accelerating the study of *B. amyloliquefaciens* and its development as both a microbial cell factory and plant growth-promoting rhizobacteria. Here, a host-genotype-independent counter-selectable marker *pheS** was constructed through a point mutation of the gene *pheS*, which encodes the α-subunit of phenylalanyl-tRNA synthetase in *Bacillus subtilis* strain 168. In the presence of 5 mM *p*-chloro-phenylalanine, 100 % of *B. amyloliquefaciens* strain SQR9 cells carrying *pheS** were killed, whereas the wild-type strain SQR9 showed resistance to *p*-chloro-phenylalanine. A simple *pheS** and overlap-PCR-based strategy was developed to create the marker-free deletion of the *amyE* gene as well as a 37-kb *bmy* cluster in *B. amyloliquefaciens* SQR9. The effectiveness of *pheS** as a counter-selectable marker in *B. amyloliquefaciens* was further confirmed through the deletion of *amyE* genes in strains *B. amyloliquefaciens* FZB42 and NJN-6. In addition, the potential use of *pheS** in other *Bacillus* species was preliminarily assessed. The expression of PheS* in *B. subtilis* strain 168 and *B. cereus* strain ATCC 14579 caused pronounced sensitivity of both hosts to *p*-chloro-phenylalanine, indicating that *pheS** could be used as a counter-selectable marker (CSM) in these strains.

Keywords *B. amyloliquefaciens* · Counter-selectable marker · *pheS** · Unmarked genetic manipulation

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Introduction

Bacillus amyloliquefaciens is a species of Gram-positive aerobic endospore-forming bacteria used to produce various commercially important enzymes and metabolites (Zhang et al. 2011b). It is also a group of plant growth-promoting rhizobacteria (PGPR) that are capable of promoting plant growth and suppressing soil pathogens through the secretion of various secondary metabolites (Xu et al. 2013). *B. amyloliquefaciens* such as strains FZB42 (Chen et al. 2007) and SQR9 (Li et al. 2014) have been used commercially as biofertilisers and biocontrol agents in agriculture (Chen et al. 2007; Chowdhury et al. 2015; Huang et al. 2015; Li et al. 2014; Xu et al. 2013). As more genome sequences of *B. amyloliquefaciens* are released, including strains FZB42 (Chen et al. 2009), SQR9 (Zhang et al. 2015), LL3 (Geng et al. 2011), DSM7 (Rückert et al. 2011), NJN-6 (Yuan et al. 2015), CAUB946 (Blom et al. 2012) and YAUB9601-Y2 (He et al. 2012), simple and rapid genetic manipulation tools are

needed to gain insight into gene function and improve these strains via genetic engineering.

Marker-free gene deletion is considered an ideal genetic manipulation with the advantage of no polar effect, guaranteeing safety in case of gene flow by evicting the resistance marker gene. Site-specific recombination systems and counter-selectable markers (CSMs) are generally employed to realize this marker-free manipulation. Site-specific recombination systems, such as Cre/*lox* (Yan et al. 2008) derived from phage P1 and Flp/*FRT* (Hoang et al. 1998) first discovered in yeast, allow the excision of a selection marker by catalysing reciprocal site-specific recombination between two *lox* or *FRT* sites. However, the *lox* or *FRT* site will be left at the replaced locus. Thus, subsequent rounds of manipulations in the same strain may result in the recombination between the *lox* or *FRT* sites. In contrast with site-specific recombination systems, CSM-based marker-free manipulation depends upon the host's endogenous recombination systems. Under defined growth conditions, a CSM leads to the death of the cells harbouring it. Mutants losing both CSM and the resistance marker will survive and be easily selected (Ueki et al. 1996; Wu and Kaiser 1996; Zhang et al. 2006). Wu et al. (2015) used the Cre/*lox* system to replace the native promoter of the bacilysin operon with the constitutive promoters P_{repB} and P_{spac} in strain FZB42, leaving no resistance marker. Zhang et al. (2014) combined the CSM *upp*, encoding uracil phosphoribosyl-transferase (UPRTase) and a temperature-sensitive plasmid to successfully remove the 47-kb *bae* cluster from the genome of *B. amyloliquefaciens* LL3. However, it was necessary to delete the *upp* gene of the host strain prior to use of *upp* as a CSM for marker-free manipulation. Thus, a host-genotype-independent CSM is more desirable to perform marker-free manipulation in *B. amyloliquefaciens*.

A host-genotype-independent CSM *pheS* first developed in *Escherichia coli* (Kast 1994) has been adapted for use as a CSM in several bacteria (Barrett et al. 2008; Carr et al. 2015; Kast and Hennecke 1991; Kristich et al. 2007; Xie et al. 2011; Zhou et al. 2015). The *pheS* gene encodes the highly conserved phenylalanyl-transfer (RNA) tRNA synthetase α-subunit, and a PheS protein containing an A294G substitution can aminoacylate phenylalanine analogues such as *p*-chloro-phenylalanine (*p*-Cl-Phe) (Kast and Hennecke 1991). Presumably, the incorporation of *p*-Cl-Phe into cellular proteins causes cell death. Therefore, by expressing this point mutant of *pheS*, it was feasible to perform negative selection in the presence of *p*-Cl-Phe (Kast 1994). The advantage of this CSM was its versatile utility in a wild-type host, omitting any pre-mutation. To our knowledge, the strategy has not been developed in *Bacillus* species, and no effective and host-genotype-independent CSM has been employed in *B. amyloliquefaciens*. In this work, we demonstrate that a mutant of *pheS* (*pheS**) from *B. subtilis* could be used as a highly effective CSM in three *B. amyloliquefaciens* strains. A *pheS** and overlap-PCR-based method was employed to perform an unmarked deletion of the target gene or large-scale DNA fragments in these strains. Additionally, the potential application of *pheS** to other *Bacillus* species was preliminarily evaluated.

Materials and methods

Bacterial strains, media, growth conditions and phenotypic characterisation

The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium consisted of (g/L) peptide (10), yeast extract (5) and NaCl (5). Minimal medium-glucose-yeast (MGY) medium contained (g/L) glucose (5), yeast extract (4), NH_4NO_3 (1), NaCl (0.5), K_2HPO_4 (1.5), KH_2PO_4 (0.5) and $MgSO_4$ (0.2). MGY-Cl medium was an MGY-based medium supplemented with 5 mM *p*-Cl-Phe (Sigma, lot no. SHBC0245V, Nanjing, China), which was added to the media prior to autoclaving at 115 °C. Solid medium was obtained by adding 15 g/L agar to the liquid medium. All strains were grown at 37 °C. Unless otherwise indicated, the final concentrations of antibiotics were as follows (mg/L): ampicillin (Amp), 100; chloramphenicol (Cm), 5; and erythromycin (Em), 200 for *E. coli* and 5 for *Bacillus*. Amylase activity was detected by growing *B. amyloliquefaciens* overnight on LB plates with 1 % starch and then staining the plate with iodine.

DNA manipulation techniques

Oligonucleotide synthesis (Supplementary Table S1, Online Resource) and DNA sequencing were performed by Sangon Biotech Co., Ltd. (Shanghai, China). The isolation and manipulation of recombinant DNA were performed using standard techniques. All enzymes were commercial preparations. Phusion DNA high-fidelity polymerase was purchased from NEB (Shanghai, China). *B. amyloliquefaciens* transformation was performed through the artificial induction of genetic competence (Chen et al. 2016). The transformation of other *Bacillus* was carried out according to published protocols (Anagnostopoulos and Spizizen 1961; Brown and Carlton 1980; Peng et al. 2009; Turgeon et al. 2006; Waschkau et al. 2008; Wemhoff and Meinhardt 2013).

Fusion of multiple DNA fragments by overlap PCR

Fusion of multiple DNA fragments by overlap PCR was carried out as described by Shevchuk et al. (2004). In brief, overlaps of approximately 40 nucleotides were introduced between each of 2 fragments through primers. The fragments

Table 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Plasmids		
pMD19-T	Ap^R, TA clone vector	TaKaRa (Dalian, China)
pTPC	pMD19-T harbouring the PC cassette	This work
pNW33N	Cm^R, *E. coli-Bacillus* shuttle vector	BGSC
pNZT1	Em^R, temperature-sensitive *E. coli-Bacillus* shuttle vector	Zakataeva et al. (2010)
pNZT1-*pheS**	pNZT1 with P_{bc}-*pheS** cassette inserted in the multicloning sites *Sal*I and *Pst*I	This work
Strains		
E. coli Top10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)7697 *galU galK endA1 nupG*	TransGene Biotech (Beijing, China)
B. amyloliquefaciens		
SQR9	Wild type	CGMCC 5808 Shao et al. (2015)
SQR9A	Strain SQR9 with PC cassette inserted upstream of the *amyE* gene, Cm^R	This work
SQR9AS	Strain SQR9 derivate, Δ*amyE*	This work
SQR9B	Strain SQR9 with PC cassette inserted upstream of the *bmy* gene, Cm^R	This work
SQR9BS	Strain SQR9 derivate, Δ*bmy*	This work
FZB42	Wild type	BGSC 10A6
FAB42A	Strain FZB42 with PC cassette inserted upstream of the *amyE* gene, Cm^R	This work
FZB42AS	Strain FZB42 derivate, Δ*amyE*	This work
NJN-6	Wild type	CGMCC 3183
NJN-6A	Strain NJN-6 with PC cassette inserted upstream of the *amyE* gene, Cm^R	This work
NJN-6AS	Strain NJN-6 derivate, Δ*amyE*	This work
Other *Bacillus* species		
B. subtilis 168	*trpC2*	BGSC 1A1
B. subtilis 168A	Strain 168 with PC cassette inserted upstream of the *amyE* gene, Cm^R	This work
B. subtilis 168AS	Strain 168 derivate, Δ*amyE*	This work
B. licheniformis ATCC 14580	Wild type	ATCC
B. pumilus NJLZ-8	Wild type	CGMCC 3182
B. thuringiensis DSM 2046	Wild type	DSMZ
B. cereus ATCC 14579	Wild type	ATCC
B. megaterium MS941	Mutant of DSM 319, Δ*nprM*	Mobitec (Göttingen, Germany) (Malten et al. 2005)

Ap^R ampicillin resistance, *Cm^R* chloramphenicol resistance, *Em^R* erythromycin resistance, *BGSC Bacillus* Genetic Stock Center, *CGMCC* China General Microbiology Culture Collection Center, *ATCC* American Type Culture Collection, *DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen

were amplified and gel purified. The reaction mixture of step A contained 9.5 μL of water, 4 μL of Phusion buffer (×5), 2 μL of deoxynucleoside triphosphate (dNTP) mix (2.5 mM each), 4 μL of gel-purified fragments (approximately 100 ng each) and 0.5 μL of Phusion DNA polymerase. The cycling parameters were an initial denaturation at 98 °C for 3 min and subsequent steps of 98 °C for 15 s, annealing at 55 °C for 10 s and extension at 72 °C for 3 min for 15 cycles total. The reaction mixture of step B contained 32.5 μL of water, 10 μL of Phusion buffer, 4 μL of dNTP mix, 1 μL of forward and reverse primers (20 mM) specific for the expected fragment, 1 μL of the unpurified PCR product from step A and 0.5 μL of Phusion DNA polymerase. The cycling parameters were an initial denaturation at 98 °C for 3 min and subsequent

steps of 98 °C for 10 s, annealing at 58 °C for 10 s and extension at 72 °C for 3 min for 30 cycles total. The resulting product was purified using an AxyPrep DNA Gel Purification and Extraction Kit (Axygen, Hangchow, China), and the purified product was directly transformed into the strains.

Construction of a positive and negative selection cassette *P_{bc}-pheS*-cat*

The *P_{bc}-pheS*-cat* (PC) cassette (Fig. 1a) was constructed in three steps using overlap PCR. First, the site-directed mutant of *pheS** (GCA309GGC) was generated by overlap extension

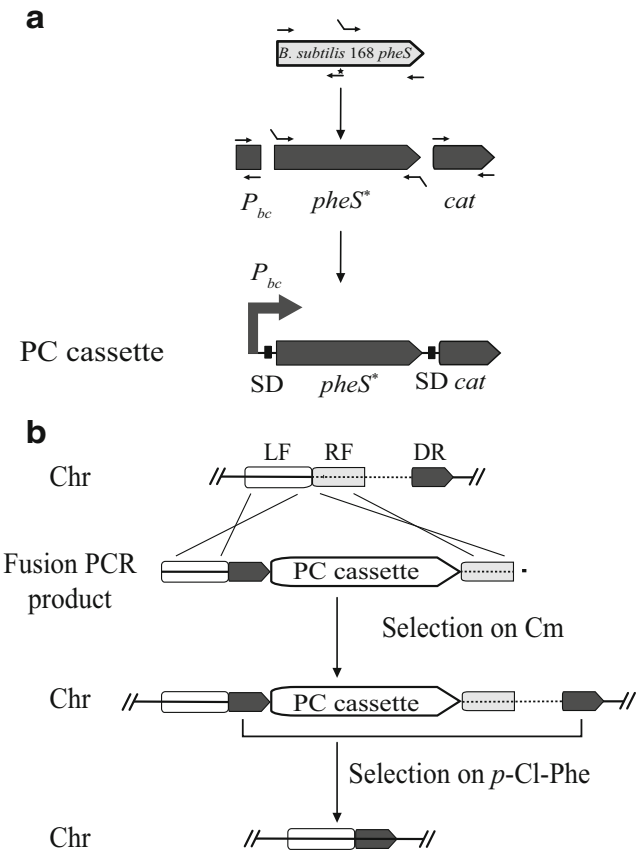

Fig. 1 Graphic representation of the construction of the PC cassette (**a**) and the strategy of PC cassette and overlap PCR-based marker-free DNA fragment deletion in *B. amyloliquefaciens* (**b**). **a** Primer binding sites are denoted by *small arrows*, whereas primers containing overlapping sequences are denoted by *bent arrows*. Overlap PCR was used to introduce a GCA-to-GGC point mutation into codon 309. **b** To delete a target fragment (*dotted line*), an approximately 500-bp fragment just downstream of the target region is used as a direct repeat sequence (DR) and is placed ahead of the PC cassette. Then, transformants with an insertion of the fragment containing the DR and PC cassette just upstream of the target region are selected on Cm. Finally, recombination between two DR sequences excises both the PC cassette and the target fragment, and the resulting mutant is selected on 5 mM *p*-Cl-Phe. *P_{bc}*, the promoter of the uracil phosphatase gene from *B. cereus*. *SD* Shine-Dalgarno sequence, *cat* chloramphenicol resistance gene, *LF* left flanking region, *RF* right flanking region, *Chr* chromosome

with primer pairs *pheS*F/*pheS*mR and *pheS*m-F/*pheS*-R using the *pheS* gene of *B. subtilis* 168 as a template. The GCA309GGC mutation was introduced by the primer *pheS*m-F. Next, as intracellular PheS* should be maintained at a level high enough to induce *p*-Cl-Phe sensitivity, the strong promoter *P_{bc}* (http://parts.igem.org/Part:BBa_K090504), which drives the transcription of the uracil phosphatase gene in *B. cereus*, was amplified from *B. cereus* strain American Type Culture Collection (ATCC) 14579 using the primer pair *P_{bc}*-F/*P_{bc}*-R and was assembled to the 5′ end of *pheS** using primers *P_{bc}*-F/*pheS*-R to generate *P_{bc}-pheS**. The Shine-Dalgarno (SD) sequence (aaggagg) was introduced 8-bp upstream of the start codon of *pheS** using primer *P_{bc}*-R. Finally, the *cat* gene, together with its putative SD sequence, was amplified from plasmid pNW33N and fused to the 3′ end of the *P_{bc}-pheS** with primers *cm*-F, *cm*-R, *pheS**-F and *pheS**-R. Thus, *pheS** and *cat* were arranged into an operon under the control of promoter *P_{bc}*. This 1.9-kb synthetic positive and negative selection cassette was named PC and cloned into the vector pMD19-T (TaKaRa) to yield the plasmid pTPC.

Assessment of the *p*-Cl-phe resistance of different *Bacillus* strains

Overnight-cultured *B. amyloliquefaciens* strains SQR9, FZB42 and NJN-6 and other *Bacillus* species (*B. subtilis* 168, *B. licheniformis* ATCC 14580, *B. pumilus* NJLZ-8, *B. thuringiensis* DSM 2046, *B. cereus* ATCC 14579 and *B. megaterium* MS941) were diluted 100-fold into freshly prepared LB broth and shaken at 37 °C. When the optical density at 600 nm (OD_{600}) reached 1.0, 100 μL of 10^{-4}-fold dilutions was plated on MGY medium containing different concentrations (0, 5, 10, 20 mM) of *p*-Cl-Phe and incubated overnight at 37 °C.

Assessment of the *p*-Cl-phe sensitivity of different *Bacillus* strains containing *pheS**

The strains containing *pheS** were grown in LB broth supplemented with corresponding antibiotics to OD_{600} 1.0, and 100-μL aliquots of 10^{0}- and 10^{-1}-fold dilutions were spread on MGY plate containing the corresponding antibiotics and different concentrations of *p*-Cl-Phe and the plates were incubated overnight at 37 °C.

Deletion of *amyE* gene in *B. amyloliquefaciens* strain SQR9

The left flanking (LF) region (~800 bp), direct repeat (DR) sequence (~500 bp) and right flanking (RF) region (~800 bp) were amplified from strain SQR9 using the primer pairs 9aLF-F/9aLF-R, 9aDR-F/9aDR-R and 9aRF-F/9aRF-R, respectively. The PC cassette was amplified with the primer pair 9aPC-F/9aPC-R using pTPC as a template. These four fragments

were fused using overlap PCR in the order of LF, DR, PC cassette and RF. The resulting 4.0-kb *amyE* deletion amplicon (PCR product) was directly transformed into strain SQR9, and the transformants were selected on LB plates containing Cm. Cm^R colonies were cultivated to an OD_{600} of 1.0 without Cm, and a 100-μL aliquot of a 10-fold dilution of the cultures (approximately 10^5 cells) was plated on MGY-Cl medium. Mutants growing on MGY-Cl were further confirmed by PCR, DNA sequencing and amylase activity analysis.

Deletion of the 37-kb *bmy* cluster in *B. amyloliquefaciens* strain SQR9

Deletion of the *bmy* cluster was carried out using the same strategy as described for *amyE* deletion. The LF, DR, PC cassette and RF fragments were amplified using the primer pairs 9bLF-F/9bLF-R, 9bDR-F/9bDR-R, 9bPC-F/9bPC-R and 9bRF-F/9bRF-R, respectively. The four fragments were fused to generate the 4.0-kb *bmy* deletion amplicon, which was subsequently transformed into *B. amyloliquefaciens* SQR9.

Deletion of *amyE* gene in *B. subtilis* strain 168

Deletion of *amyE* in the *B. subtilis* strain 168 was also performed according to the method used for the deletion of *amyE* in the *B. amyloliquefaciens* strain SQR9. LF, DR, PC cassette and RF fragments were amplified using the primer pairs 8aLF-F/8aLF-R, 8aDR-F/8aDR-R, 8aPC-F/8aPC-R and 8aRF-F/8aRF-R, respectively. The four fragments were fused to generate the 4.0-kb PCR product, which was directly transformed to *B. subtilis* strain 168.

Construction of the vector pNZT1-*pheS**

The fragment containing the P_{bc}-*pheS** cassette was amplified with the primer pair Salpbes-F/Pstpbes-R using the PC cassette as a template, digested by *Sal*I and *Pst*I sites and inserted into the corresponding sites of the temperature-sensitive vector pNZT1 (Zakataeva et al. 2010). This produced a vector pNZT1-*pheS**, which is able to replicate in both *E. coli* and *Bacillus* spp. at or below 30 °C.

Results

p-Cl-phe resistance of wild-type *B. amyloliquefaciens* strains

To determine whether *p*-Cl-Phe can be used in counter-selection with its marker *pheS** in *B. amyloliquefaciens*, the resistance of three wild-type strains to different concentrations of *p*-Cl-Phe was tested as described in the "Materials and methods" section. Strains with survival rates of >80, 50–80 and <50 % were defined as strongly, moderately and weakly resistant to *p*-Cl-Phe, respectively. As shown in Fig. 2, all three tested wild-type strains of *B. amyloliquefaciens* (SQR9, FZB42 and NJN-6) showed strong resistance to 5 mM *p*-Cl-Phe, with survival rates of 97, 95 and 85 %, respectively. In the presence of 10 mM *p*-Cl-Phe, the *B. amyloliquefaciens* strain FZB42 showed strong resistance, with a survival rate of 87 %, whereas strains *B. amyloliquefaciens* SQR9 and NJN-6 showed moderate resistance, with survival rates of 58 and 79 %, respectively. In the presence of 20 mM *p*-Cl-Phe, strain *B. amyloliquefaciens* FZB42 still showed strong resistance and a survival rate of 80 %, whereas the strains *B. amyloliquefaciens* SQR9 and NJN-6 showed weak resistance, with survival rates of 12 and 1 %, respectively. These results lay the foundation for *pheS** application in these strains.

Generation of a positive and negative selection cassette PC

In previous work, efficient negative selection systems have been developed based on a point-mutant *pheS** gene encoding an A294G substitution in *E. coli* PheS (Kast 1994), A314G substitution in *Streptococcus mutans* PheS (Xie et al. 2011) or A312G substitution in *Enterococcus faecalis* PheS (Kristich et al. 2007). To identify the amino acid residue for mutagenesis, a multiple sequence alignment of the PheS proteins from a variety of species was performed by Clustal W2 (Larkin et al. 2007). As shown in Fig. 3, PheS from *Bacillus* species have a valine at position 294, and the relevant amino acid position of the alanine is 309, which therefore could be substituted (A309G) to create *p*-Cl-Phe sensitivity. Moreover, the *pheS* of *B. subtilis* shares 82 % nucleic acid sequence identity and 92 % amino acid sequence identity with the *pheS* of *B. amyloliquefaciens*, suggesting that they probably can replace each other. To reduce undesirable homologous recombination at the wild-type copy of the *pheS* locus in *B. amyloliquefaciens* during allelic replacement, the *pheS* of *B. subtilis* was engineered and used as a CSM in *B. amyloliquefaciens*. Codon 309 of *B. subtilis pheS* was changed from GCA to GGC (A309G) through overlap PCR to generate *pheS**. Then, *pheS** and *cat* (chloramphenicol resistant gene) were assembled into an operon under the control of promoter P_{bc}, as described in the "Materials and methods" section, to give the PC cassette.

PC cassette and PCR-based chromosome marker-free deletion strategy in *B. amyloliquefaciens*

As shown in Fig. 1b, this strategy depends on three key strategies. First, an approximately 500-bp fragment just downstream of the target region to be deleted is used as a DR sequence and added ahead of the PC cassette. Next, two

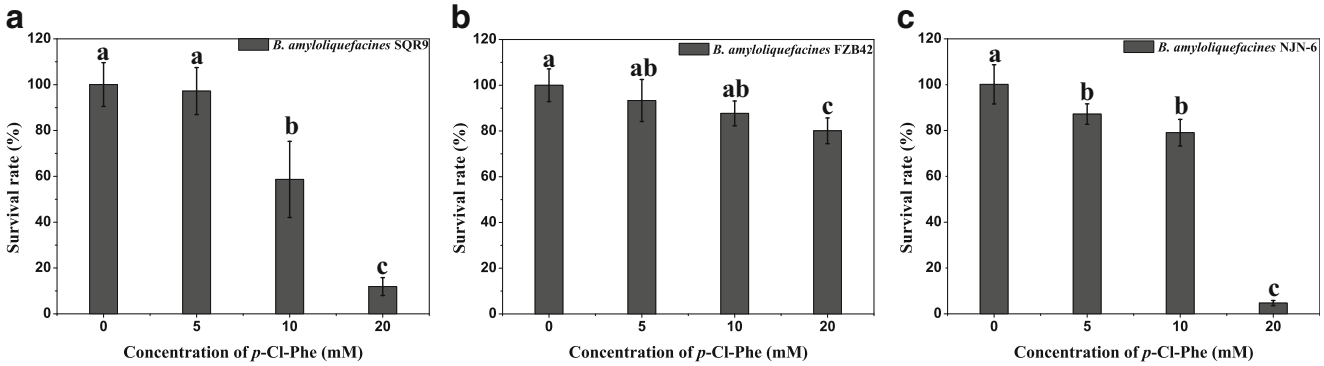

Fig. 2 *p*-Cl-Phe resistance of *B. amyloliquefaciens*. *B. amyloliquefaciens* strains SQR9, FZB42 and NJN-6 were grown to an OD_{600} of 1, diluted 10^4-fold and plated on MGY medium containing different concentrations of *p*-Cl-Phe. The *different letters above bars* indicate significant differences ($P < 0.05$). Data represent means ± standard deviation (SD) ($n = 3$). The survival rate of each strain on MGY plate was defined as 100 %

flanking regions (LF, RF) (~800 bp on each side), DR and a PC cassette were fused by overlap PCR and transformed into *B. amyloliquefaciens* strains. Thus, the DR and PC cassette are inserted just upstream of the target region via a double-crossover recombination event, which is selected by Cm. Notably, no target region (or only a very short region) was deleted in this step, ensuring the successful insertion of the DR and PC cassette. Last, the target region together with the PC cassette is excised via a single recombination between the two DR sequences, as selected by *p*-Cl-Phe. The elongated DR sequences will lead to the efficient eviction of the fragment between them, even if the fragment is large.

Knockout of the *amyE* gene in *B. amyloliquefaciens* SQR9

As a proof of concept, we used this strategy to delete the *amyE* gene in strain SQR9. The *amyE* deletion amplicon containing the LF, DR, PC cassette and RF was assembled by PCR and transformed into strain SQR9. The DR and PC cassette were inserted upstream of the *amyE* gene, generating the mutant Cm^R strain SQR9A. The transformation efficiency was approximately 2.5×10^2 CFU/μg DNA. To investigate the effectiveness of *pheS**, approximately 10^6 cells of mutant strain SQR9A were spread on an MGY plate supplemented with Cm and varying concentrations of *p*-Cl-Phe. As shown in Fig. 4a, 100 % of the cells of mutant strain SQR9A were killed in the presence of 5 mM *p*-Cl-Phe, demonstrating that *pheS** can act as an effective CSM in strain SQR9. The mutant strain SQR9A was cultured in LB broth without Cm, and approximately 10^5 cells were plated on an MGY-Cl plate to select the cells that had lost the PC cassette together with the *amyE* gene. Approximately 400 colonies were obtained per plate. PCR and amylase activity analyses (Fig. 4b, d) showed that all 50 randomly chosen clones had undergone the expected deletion. The final mutant was further verified by DNA sequencing (data not shown) and named as SQR9AS.

Deletion of the 37-kb *bmy* cluster in *B. amyloliquefaciens* SQR9

To demonstrate the capacity of this strategy to remove large fragments, the 37-kb *bmy* cluster that is responsible for the synthesis of bacillomycin D (Zhang et al. 2015) was targeted for deletion in strain SQR9. As described in the "Materials and

Escherichia coli	WLEVLGCGMVHPNVLRNVGIDPEVYSGF**A**FGMGMERLTMLRYGVTDLRSFFENDLRF	322	WP_047601123.1
Veillonella atypica	WLEILGCGMVHPRVLELNGYDPNKVKGF**A**FGMGVERIAMLLYGIGDLRLFFEDDIRF	336	WP_005383539.1
Streptococcus mutans	WIEILGAGMVHPSVLEMSGVNSEEYSGF**A**FGLGQERMAMLRYGINDIRGFYQGDSRF	342	WP_002262925.1
Burkholderia pseudomallei	WLEISGSGQVHPTVIRNMGLDPERYIGF**A**FGSGLERLTMLRYGVQDLRLFFENDLRF	335	WP_004534778.1
Enterococcus faecalis	WIEILGAGMVHPDVLQMSGIDPTEYSGF**A**FGLGPDRVAMLRYGVNDIRNFYQNDLRF	340	WP_010825138.1
B. subtilis	WIEILGAGMVHPNVLKMAGFDPKEYQGF**A**FGMGVERIAMLKYGIDDIRHFYTNDVRF	337	WP_041851070.1
B. amyloliquefaciens	WIEILGAGMVHPNVLKMAGFNPEEYQGF**A**FGMGVERIAMLKYGIEDIRHFYTNDVRF	337	WP_061573713.1
B. licheniformis	WIEILGAGMVHPNVLEMAGFDSKQYQGF**A**FGMGVERIAMLKYGIDDIRHFYTNDVRF	337	WP_061566014.1
B. pumilus	WIEILGAGMVHPNVLKMSGFDPETYQGF**A**FGMGVERIAMLKYGIDDIRHFYTNDIRF	337	WP_012010834.1
B. thuringiensis	WIEILGAGMVHPNVLEMAGYDSKEYQGF**A**FGMGAERIAMLKYGVDDIRHFYTNDVRF	337	WP_044790621.1
B. cereus	WIEILGAGMVHPNVLEMAGYDSKEYQGF**A**FGMGAERIAMLKYGVDDIRHFYTNDVRF	337	WP_002015457.1
B. megaterium	WIEILGAGMVHPNVLEMAGYDSTKYRGF**A**FGIGVERIAMLKHGVDDIRHFYTNDVRF	337	WP_013085175.1

Fig. 3 Multiple sequence alignment of the C-terminal region of PheS from different strains. C-terminal region of strains *E. coli* K-12, *Veillonella atypica* OK5, *Streptococcus mutans* UA159, *Burkholderia pseudomallei* K42, *Enterococcus faecalis* OG1, *B. subtilis* 168, *B. amyloliquefaciens* strains FZB42, *B. licheniformis* ATCC 14580, *B. pumilus* NJLZ-8, *B. thuringiensis* DSM 2046, *B. cereus* ATCC 14579 and *B. megaterium* MS941 were aligned using Clustal W2. The *arrow* indicates the conserved alanine residue that can be subjected to mutagenesis to create *p*-Cl-Phe sensitivity. The *stars* refer to the conserved amino acids

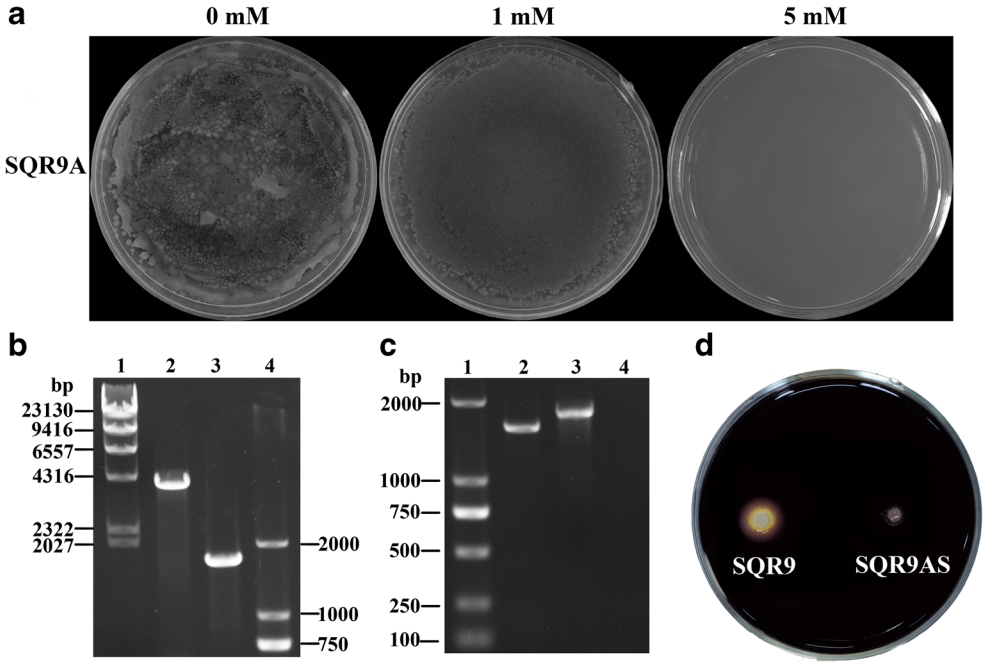

Fig. 4 Confirmation of the PC cassette-based DNA fragment deletion in *B. amyloliquefaciens* SQR9. **a** Conditional growth inhibition mediated by PC cassette in *B. amyloliquefaciens* SQR9A. The strain SQR9A carrying the PC cassette was cultivated to an OD_{600} of 1 in LB broth containing Cm, and 100-μL aliquots without dilution were plated on MGY plate supplemented with Cm and the indicated concentration of *p*-Cl-Phe. The plates were incubated at 37 °C overnight. **b** *Lanes 1 and 4* DNA marker. *Lanes 2 and 3* show the amplicons of the *amyE* region in strain SQR9 (*lane 2*) and strain SQR9AS (*lane 3*) using outside oligonucleotides 9a–VF/9a–VR as primers. **c** *Lane 1* DNA marker. *Lane 2* shows the length of *bmy* region in strain SQR9BS using outside oligonucleotides 9b–VF/9b–VR as primers. *Lanes 3 and 4* show a 1.8-kb fragment from *bmy* cluster in strain SQR9 and strain SQR9BS using the primer pair 9b–MF/9b–MR. **d** Strains SQR9 and SQR9AS were grown on LB plates with 1 % starch overnight and stained with iodine to detect the α-amylase activity, which was indicated by the transparent plaque

Methods" section, the *bmy* deletion amplicon was generated by PCR and introduced into strain SQR9. The transformation efficiency was approximately 2×10^2 CFU/μg DNA. The Cm^R clones carrying the PC cassette were designated SQR9B. Subsequently, approximately 10^5 cells of mutant strain SQR9B were spread on an MGY-Cl plate to select the cells that had lost the PC cassette together with the *bmy* cluster. Approximately 40 colonies were obtained from each plate, which is less than in the *amyE* deletion. PCR analysis (Fig. 4c) showed that all 50 randomly selected clones were correct. The final mutant was further checked by DNA sequencing (data not shown) and designated SQR9BS. Thus, this strategy performed very well in deleting a large chromosomal fragment in strain SQR9.

Application of the *pheS** in other *B. amyloliquefaciens* strains

To determine whether the application of *pheS** can be extended to other *B. amyloliquefaciens* strains, we further tested the effectiveness of *pheS** in *B. amyloliquefaciens* strains FZB42 and NJN-6. The *amyE* genes of both strains were successfully deleted using the strategy mentioned above (Fig. 1b). The deletion amplicons were assembled by PCR and transferred into strains FZB42 and NJN-6, generating mutant strains FZB42A and NJN-6A, which harboured the PC cassette. The transformation efficiencies of this step for strain FZB42 and NJN-6 were approximately 3×10^2 and 0.3×10^2 CFU/μg DNA, respectively. When 10^5 cells were spread on an MGY plate containing *p*-Cl-Phe and Cm, mutant FZB42A showed pronounced sensitivity to 10 mM *p*-Cl-Phe, and mutant NJN-6A exhibited pronounced sensitivity to 5 mM *p*-Cl-Phe (Supplementary Figs. S1 and S2, Online Resource).

Approximately 10^5 cells of mutant FZB42A or NJN-6A were plated on an MGY-Cl plate containing 5 mM *p*-Cl-Phe to select the cells that had lost the PC cassette together with the *amyE* gene. Approximately 400 colonies were obtained per plate, and 50 clones were randomly chosen for each strain. Of these, 100 % had undergone the expected deletion (Supplementary Figs. S3 and S4, Online Resource). Notably, although the strains FZB42 and NJN-6 harbouring *pheS** are less sensitive to *p*-Cl-Phe than strain SQR9 (Fig. 4a), *pheS**-based counter-selection was equally efficient (100 %) in these three strains. The final *amyE*-deletion mutant strains verified by DNA sequencing were designated FZB42AS and NJN-6AS, respectively. The effectiveness of *pheS** was thus validated in three *B. amyloliquefaciens* strains, so it is reasonable to expect that *pheS** could be used as a CSM in other *B. amyloliquefaciens* strains.

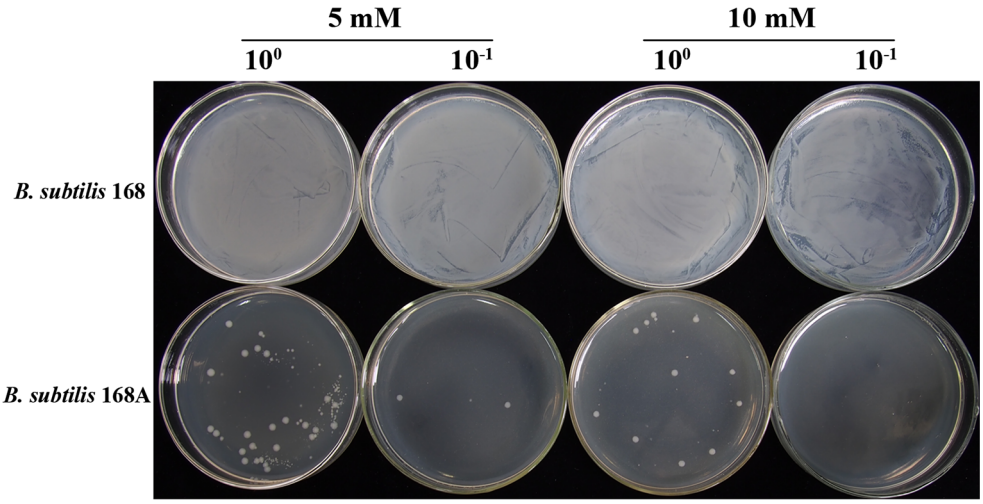

Fig. 5 *p*-Cl-Phe sensitivity of *B. subtilis* strains 168 and 168A. The strains 168 and 168A carrying the PC cassette were cultivated to an OD_{600} of 1 in LB broth, and 100-μL aliquots of each dilution were plated on MGY plate supplemented with indicated concentration of *p*-Cl-Phe. To prevent the loss of PC cassette, Cm was added in the cultivation of strain 168A. The plates were incubated at 37 °C overnight

Evaluation of the potential use of *pheS** in other *Bacillus* species

(i) *p*-Cl-Phe resistance of other *Bacillus* species

To evaluate the potential use of *pheS** in other *Bacillus* species, the *p*-Cl-Phe resistance of *Bacillus* species, including *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. thuringiensis*, *B. cereus* and *B. megaterium*, was determined on MGY medium containing different concentrations of *p*-Cl-Phe. As shown in Supplementary Fig. S5, Online Resource, all tested strains of *B. subtilis* 168, *B. licheniformis* ATCC 14580, *B. pumilus* NJLZ-8, *B. thuringiensis* DSM 2046, *B. cereus* ATCC 14579 and *B. megaterium* MS941 showed strong resistance to 5 mM *p*-Cl-Phe, with survival rates of 82, 96, 93, 95, 93 and 89 %, respectively. In the presence of 10 mM *p*-Cl-Phe, the strains *B. licheniformis* ATCC 14580, *B. pumilus* NJLZ-8, *B. thuringiensis* DSM 2046 and *B. cereus* ATCC 14579 showed strong resistance, with survival rates of 87, 91, 86 and 90 %, respectively, whereas strains *B. subtilis* 168 and *B. megaterium* MS941 showed weak resistance, with survival rates of 43 and 24 %, respectively. In the presence of 20 mM *p*-Cl-Phe, the strains *B. licheniformis* ATCC 14580, *B. pumilus* NJLZ-8 and *B. cereus* ATCC 14579 still showed strong resistance, with survival rates of 83, 88 and 85 %, respectively, whereas *B. thuringiensis* strain DSM 2046 showed moderate resistance and a survival rate of 69 %. The strains *B. subtilis* 168 and *B. megaterium* MS941 showed weak resistance (both with survival rates of 1 %).

(ii) PC cassette and PCR-based marker-free deletion of *amyE* gene in *B. subtilis* 168

The PC cassette was first inserted at the *amyE* locus following the strategy shown in Fig. 1b, generating mutant 168A. When approximately 10^6 cells were spread on an MGY plate supplemented with *p*-Cl-Phe and Cm, mutant 168A exhibited prominent sensitivity to 5 mM *p*-Cl-Phe (Fig. 5). Next, mutant 168A was grown to OD_{600}~1.0 in LB broth without Cm, and approximately 10^5 cells of mutant

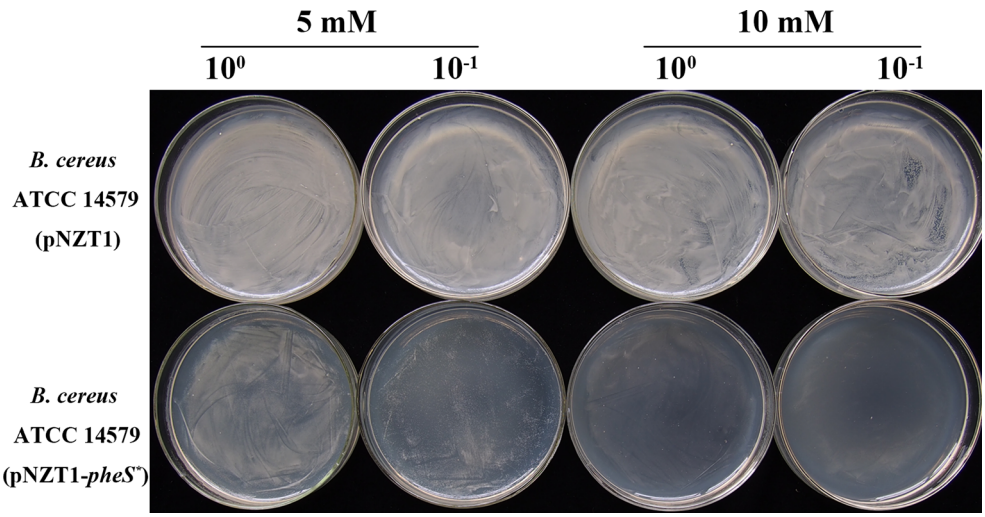

Fig. 6 *p*-Cl-Phe sensitivity of *B. cereus* strains ATCC 14579 (pNZT1) and ATCC 14579 (pNZT1-*pheS**). The strains ATCC 14579 (pNZT1) and ATCC 14579 (pNZT1-*pheS**) were cultivated to an OD_{600} of 1 in LB broth containing Em, and 100-μL aliquots of each dilution were plated on MGY plate supplemented with Em and indicated concentration of *p*-Cl-Phe. The plates were incubated at 30 °C overnight

168A were plated on an MGY plate containing 5 mM *p*-Cl-Phe. Approximately 150 clones were obtained per plate. All of the 50 randomly tested clones lost the PC cassette through recombination between the two DR sequences (Supplementary Fig. S6, Online Resource), and the final mutant was designated 168AS.

(iii) Expression of PheS* in *B. cereus*

To investigate whether *pheS** could be used as a CSM in *B. cereus* ATCC 14579, P_{bc}-*pheS** was ligated to vector pNZT1, generating the plasmid pNZT1-*pheS**. Plasmids pNZT1 and pNZT1-*pheS** were transformed to *B. cereus* ATCC 14579. The strain carrying pNZT1-*pheS** was sensitive to 5 mM *p*-Cl-Phe on MGY plates (Fig. 6), indicating that *pheS** could be an effective CSM in *B. cereus*.

Discussion

In this study, a host-genotype-independent CSM *pheS** was generated for *B. amyloliquefaciens*. The *pheS** was demonstrated to be extremely effective through unmarked gene knockout or large-fragment deletion in three important *B. amyloliquefaciens* strains. The *pheS* gene encodes the α-subunits of the Phe-tRNA synthetase. Kast and Hennecke (1991) found that replacement of the Ala^{294} of PheS with a smaller Gly residue renders *E. coli* sensitive to *p*-Cl-Phe and demonstrated that this site-directed mutant of *pheS* could act as an effective CSM in *E. coli*. However, to extend this strategy to other bacteria, at least three points should be noted. First, the wild-type host cell is resistant to *p*-Cl-Phe. For example, the majority of wild-type sphingomonads failed to grow under addition of 0.1 mM *p*-Cl-Phe, so *pheS* cannot be used as a CSM for these strains (Kaczmarczyk et al. 2012). On the other hand, host cells harbouring mutant *pheS* must be sensitive to *p*-Cl-Phe, enabling efficient negative selection. Finally, undesirable homologous recombination at the wild-type copy of the *pheS* locus should be reduced during gene knockout, which can be accomplished by introducing a series of silent mutations into the mutant *pheS*.

In this work, the *p*-Cl-Phe resistance of *B. amyloliquefaciens* was assessed, and all three strains tested showed strong resistance to 5 mM *p*-Cl-Phe. To reduce the undesirable homologous recombination at the wild-type copy of the *pheS* locus in *B. amyloliquefaciens*, the *pheS* of *B. subtilis* with 82 % nucleic acid sequence identity to *B. amyloliquefaciens* was mutated and used as a CSM in *B. amyloliquefaciens*. A strong promoter P_{bc} was used to drive *pheS**, generating high intracellular levels of PheS* to create *p*-Cl-Phe sensitivity. Fortunately, P_{bc}-*pheS** rendered the *B. amyloliquefaciens* strains that harboured it pronounced sensitivity to *p*-Cl-Phe.

Many *Bacillus* species are of considerable interest to industry and agriculture. Both gene function research and strain improvement require effective CSM to enable efficient unmarked genetic manipulation in these species. Although several CSMs have been developed for *Bacillus* species (Brans et al. 2004; Liu et al. 2008; Wemhoff and Meinhardt 2013; Zhang et al. 2011a; Zhang et al. 2006), only *mazF* was demonstrated as a host-genotype-independent CSM in *B. subtilis*. Other CSMs require pre-mutation of the host; for instance, to use the *upp* gene as a CSM in *B. subtilis* (Fabret et al. 2002) and *B. amyloliquefaciens* (Zhang et al. 2014), the native *upp* must first be deleted. Before employing *blaI* (Brans et al. 2004) as a CSM, the endogenous P_{lysA} promoter of *B. subtilis* should be replaced with the P_{blaP} promoter. The *B. subtilis* chromosomal *araR* locus must be replaced with a promoterless neomycin-resistance gene (*neo*) fused to the *ara* promoter prior to the use of *araR* as a CSM (Liu et al. 2008). The *pheS** generated in this work was demonstrated as an effective host-genotype-independent CSM in *B. amyloliquefaciens* as well as in *B. subtilis* 168 and *B. cereus* ATCC 14579, which will facilitate functional genome research and industrial application of these *Bacillus* spp. In addition, plasmids pTPC and pNZT1-*pheS** will be available from BGSC (http://www.bgsc.org) under the accession numbers ECE355 and ECE356, respectively.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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