Development of a counterselectable seamless mutagenesis system in lactic acid bacteria

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Abstract

Background: Lactic acid bacteria (LAB) are receiving more attention to act as cell factories for the production of high-value metabolites. However, the molecular tools for genetic modifying these strains are mainly vector-based double-crossover strategies, which are laborious and inefficient. To address this problem, several counterselectable markers have been developed, while few of them could be used in the wild-type host cells without pretreatment.

Results: The pheS gene encoding phenylalanyl-tRNA synthetase alpha subunit was identified in Lactococcus lactis NZ9000 genome. When mutant pheS gene (pheS*) under the control of the Lc. lactis NZ9000 i-lactate dehydrogenase promoter (Pldh) was expressed from a plasmid, the resulted PheS* with an A312G substitution rendered cells sensitive to the phenylalanine analog p-chloro-phenylalanine (p-Cl-Phe). This result suggested pheS* was suitable to be used as a counterselectable marker in Lc. lactis. However, the expression level of pheS* from a chromosomal copy was too low to confer p-Cl-Phe sensitivity. Therefore, a strategy of cascading promoters was attempted for strengthening the expression level of pheS*. Expectedly, a cassette 5Pldh-pheS* with five tandem repetitive promoters Pldh resulted in a sensitivity to 15 mM p-Cl-Phe. Subsequently, a counterselectable seamless mutagenesis system PheS*/pG灾host9 based on a temperature-sensitive plasmid pG灾host9 harboring a 5Pldh-pheS* cassette was developed in Lc. lactis. We also demonstrated the possibility of applying pheS* to be a counterselectable marker in Lactobacillus casei BL23.

Conclusions: As reported in E. coli, pheS* as a counterselectable marker has been demonstrated to be functional in targeted gene(s) deletion in Lc. lactis as well as in L. casei. Moreover, the efficiency and timesaving counterselectable seamless mutagenesis system PheS*/pG灾host9 could be used in the wild-type host cells without pretreatment.

Keywords: Lactic acid bacteria, Temperature-sensitive plasmid, Seamless mutagenesis, Counterselectable marker, pheS

Background

Lactic acid bacteria (LAB) are important microorganisms used as starter cultures in the dairy fermented processes [1, 2]. Due to their generally recognized as safe status, some LAB strains have been used as cell factories or vaccine delivery vehicles for the heterogeneous production of specific compounds [3, 4] or pharmaceutical molecules [5–8]. Also, since the wealth of genomic data being delivered by massively parallel sequencing, interests in development of high-efficiency genome engineering tools for rerouting natural metabolic pathways to produce high valuable end products were increasing [9, 10]. Considering the importance of the recombineering system in LAB, significant efforts have been recently concentrated on the exploitation of gene targeting techniques in LAB to accelerate genome engineering or gene functional analysis, as the recently reported single-stranded DNA recombineering (SSDR) system and double-stranded DNA recombineering (DSDR) system [11–13].

The SSDR system was established in Lactobacillus reuteri and Lactococcus lactis and could generate precision genome mutations without leaving any other foreign
DNA [11]. The major limitation is the ability to achieve efficiencies that would allow the modification of any sites in the genome and easily recover the mutants without selection [14]. To address this problem, enhanced SSDR has been achieved with the assistant of the type-II clustered regularly interspaced short palindromic repeats locus from Streptococcus pyogenes in L. reuteri and >99.99% of non-recombinants could be eliminated without antibiotic selection [12]. But it could not be used for gene(s) deletion or insertion in other LAB because it has not proved the functional application of type-II CRISPR–Cas system in LAB except for L. reuteri. The DSDR technique was established in L. plantarum which was involved in the efficiently generation of gene(s) deletion or insertion [13]. However, this genetic system was not functional in other LAB and still left a 

Seamless mutagenesis refers to targeted mutagenesis without any other micro-change, such as the presence of the selectable marker used to screen mutants or a 

Seamless mutagenesis strategy usually appropriate for mutating the protein coding region in which any extraneous sequence introduced could interfere with protein expression. So far, several seamless mutagenesis methods based on homologous double-crossover have been successfully achieved in LAB, but the most widely used seamless mutagenesis strategy was based on a temperature-sensitive plasmid such as pG^+^host9 [16] or pG^+^host5 [17]. The merit of these plasmids is that both the non-replicate temperature at 37 °C and the replicate temperature at 28 °C are the adaptive temperature for the growth of most LAB. With plasmid pG^+^host9 [16], several chromosomal deletion derivatives of Lc. lactis and Streptococcus thermophilus were obtained in our laboratory [18–20]. However, these vectors, while powerful, suffer from a relatively low rate of recombination events and require labor-intensive screening procedures to distinguish clones with the desired seamless mutants. Therefore, improving the efficiency of this seamless mutagenesis system for fast analysis the function of gene(s) in LAB is very instant.

In recent years, a two-step selection/counterselection strategy has been demonstrated to be functional in improving the efficiency of method for fast generating seamless mutagenesis in the genome, which is normally consist of a positive selectable marker (usually an antibiotic resistance gene) and a counterselectable cassette. Counterselectable markers, including the genes upp [21–23] and oroP [24], have been characterized and functionally analyzed. However, the counterselectable marker upp could not be made in wild-type LAB strains without pre-treatment while oroP has not been widely used for other LAB strains [21–24].

Recently, gene pheS encoding phenylalanyl-tRNA synthetase alpha subunit has been demonstrated to function as a host strain-independent counterselectable marker in Thermus thermophilus, Bacteroides sp., Escherichia coli and Streptococcus mutans [25–28], but has yet not been used in the model strain Lc. lactis. In E. coli, only an A294G substitution in the protein PheS altered the specificity of the phenylalanyl-tRNA synthetase which resulted in the sensitivity to phenylalanine analogs such as p-chloro-phenylalanine (p-Cl-Phe) [27]. In this study, we identified a conserved alanine residue in the PheS protein, and demonstrated that the dominant-negative mutant protein PheS* with an A312G amino acid substitution rendered cells sensitive to 15 mM p-Cl-Phe in Lc. lactis NZ9000 and 10 mM p-Cl-Phe in L. casei BL23. To employ this conditional lethal gene pheS^* as a negative selectable marker, a high-efficiency seamless mutagenesis system PheS^*/pG^+^host9 based on a temperature-sensitive plasmid pG^+^host9 carrying a Sp^+^pheS^* cassette was constructed in Lc. lactis NZ9000. The aim of this study is to explore the potential of using pheS^* as a counterselectable marker for rapidly screening mutants for targeted gene analysis or genome engineering in LAB.

Methods

Plasmids, bacterial strains, and growth conditions

The plasmids and bacterial strains used in this study are shown in Table 1. E. coli DH5α was used as the host for cloning procedures and grown aerobically in Luria–Bertani (LB) medium at 37 °C. Unless otherwise specified, Lc. lactis and L. casei were grown statically at 30 °C in M17 (Oxoid) broth supplemented with 0.5% glucose (GM17) and at 37 °C in MRS (Oxoid) broth, respectively. For counterselection, semi-defined M9 plates [29] supplemented with 0.4% glucose, namely GM9 plates, were added with 15 mM p-Cl-Phe (Sigma) for Lc. lactis and 10 mM p-Cl-Phe (Sigma) for L. casei. If required, antibiotics were added as follows: 10 µg/ml erythromycin or 5 µg/ml chloramphenicol for Lc. lactis, 5 µg/ml erythromycin for L. casei, 10 µg/ml chloramphenicol, 100 µg/ml ampicillin and 300 µg/ml erythromycin for E. coli DH5α.

Reagents and enzymes

All enzymes used in this study were purchased from TaKaRa. Restriction enzymes and T4 DNA ligases were used according to standard procedures. PCR amplicons for cloning purposes were generated by 2× PrimeSTAR max premix, and PCR reactions for screening purposes were performed with rTaq DNA polymerase. All oligonucleotides used in this study are listed in Table 2.
Bioinformatic analysis
A multiple-sequence alignment was performed using Clustal X, version 2.0 [30] and ESPript 3.0 [31]. The amino acid sequences of PheS proteins from six LAB strains were aligned with the amino acid sequences of PheS proteins from E. coli [27] and E. faecalis [32].

Construction of the counterselectable system PheS*/pG+host9 in L. lactis
The counterselectable P_{ldh}-pheS* cassette was constructed using an overlap extension PCR strategy. The constitutive promoter region of the L-lactate dehydrogenase gene (ldh) (accession number: NC_017949) in L. lactis NZ9000 [33] was amplified by PCR using primer pair ldhF1 and ldhR1. The pheS* gene was generated as two fragments by PCR using the L. lactis NZ9000 chromosomal DNA as a template with the primer pairs pheSF and siteR, siteF and pheSR, respectively. The point mutation responsible for p-Cl-Phe sensitivity was introduced by the primers siteF and siteR annealing internal to the wild-type pheS gene. There are overlapping regions among the three amplicons, which allowed an overlap extension PCR step using primers ldhF1 and pheSR to create P_{ldh}-pheS* cassette. The generated 1270 bp P_{ldh}-pheS* cassette was digested with EcoRI and BglII and ligated to the compatible sites of L. lactis/E. coli shuttle vector pSec:Leiss:Nuc [34], creating pleiss-P-pheS*.

To investigate the feasibility of the counterselectable P_{ldh}-pheS* cassette, the plasmid pleiss-P-pheS* was introduced into the competent cells of L. lactis NZ9000 by electroporation [35]. The recombinant strain L. lactis NZ9000/pleiss-P-pheS* was incubated in GM17 with 5 µg/ml chloramphenicol. Overnight cultures were ten-fold serially diluted, and 5 µl of diluted solution were pipetted onto air dried GM9 plates containing 0, 5, 10, 15 mM p-Cl-Phe, the cell survival was measured.

Construction of plasmids pleiss-nP-gfp
To demonstrate whether cascading promoters could increase the gfp gene expression, a series of plasmids pleiss-nP-gfp carrying promoter clusters nP_{ldh}-gfp were constructed as follows: the gfp gene was PCR amplified using primers gfpF and gfpR from plasmid pOgfp [18]. The promoter P_{ldh} and gfp gene were fused by an overlap extension PCR using primers ldhF2 and gfpR. The resulting product P_{ldh}-gfp was digested with BglII and EcoRI and ligated into the corresponding sites of pSec:Leiss:Nuc [34] to create plasmid pleiss-P-gfp. The promoter P_{ldh} was generated by PCR with primers ldhF2 and ldhR2, and the PCR product was digested with BglII and BamHI and inserted into the BglII site of pleiss-P-gfp to generate pleiss-2P-gfp. The same procedure was carried out to construct the plasmid pleiss-nP-gfp (n: the copy number of P_{ldh} in the promoter clusters nP_{ldh}-gfp). Then, the above plasmids pleiss-nP-gfp were introduced into L. lactis NZ9000.

Fluorescence assay
Recombinant strains harboring the pleiss-nP-gfp were grown aerobically in 5 ml GM17 broth containing 5 µg/ml chloramphenicol. Overnight cultures were ten-fold serially diluted, and 5 µl of diluted solution were pipetted onto air dried GM9 plates containing 0, 5, 10, 15 mM p-Cl-Phe, the cell survival was measured.
ml chloramphenicol at 30 °C. Samples for measurement were taken out after 12 h and harvested by centrifugation at 10,000 × g for 3 min. After being resuspended twice with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4), 200 μl of bacterial suspension was transferred into a 96-well plate in which OD600 and fluorescence were read with excitation at 485 nm and emission at 528 nm using a Multi-Detection Microplate Reader, Synergy HT (BioTek). For each sample, three repetitions were performed with PBS buffer as a blank.

**Construction of counterselectable cassettes nP<sub>ldh</sub>-pheS* and plasmids pG<sup>+</sup>-nP-pheS**

To increase the expression of PheS* protein, a series of counterselectable cassettes, namely nP<sub>ldh</sub>-pheS* were constructed as follows: the P<sub>ldh</sub>-pheS* cassette was PCR amplified using primer pair ldhF3 and pheSR2 from plasmid pleiss-P-pheS*. The resulting DNA fragment was digested with SalI and EcoRI and ligated into the corresponding sites of pUC19 to create plasmid pUC-P-pheS*. The promoter P<sub>ldh</sub> was generated by PCR with primers ldhF3 and ldhR3, and the PCR product was digested with XhoI and SalI and inserted into the SalI site of pUC-P-pheS* to generate pUC-2P-pheS. The same procedure was carried out to construct the plasmid pUC-nP-pheS (n: the copy number of P<sub>ldh</sub> in the nP<sub>ldh</sub>-pheS* cassettes). To develop a counterselectable system in Lc. lactis, the above plasmids pUC-nP-pheS* were digested with SalI and EcoRI, and the generating nP<sub>ldh</sub>-pheS* cassettes (Fig. 4a) were ligated to the SalI and EcoRI sites of pG<sup>+</sup>host9 to yield plasmid pG<sup>+</sup>-nP-pheS*.

**Table 2 Oligonucleotide primers used in this study**

| Primer  | Sequence (5′-3′)<sup>a</sup> | Restriction site |
|---------|------------------------------|------------------|
| aldB-uF | AGGGTACCGCGGAAAGGCTAGTAAACCTCC | KpnI |
| aldB-uR | CTGACATGATATTTCTCCTGATTTTCTAT | XhoI |
| aldB-dR | CCCGGTACAAGCCTGACAGATGCGCTGTCGTCGAGC | XhoI |
| aldB-dF | GAAAGGAAGAATATATCGTACAAGTTTCTGATTTTAT | BglII |
| siteR   | ACCAAACCCGAAATACAAAGAAG | EcoRI |
| siteF   | CTGTTTATCTGTTTTTGCACTGACATGCAGTACATC | EcoRI |
| ldhF1   | CCGGAATTCTCACTACCCGTTTTCACATGGAAC | XhoI |
| ldhF2   | GACAGATCTATCTTACCATACATGGAAC | BglII |
| ldhF3   | AGACGTCGACATCTTACCATACATGGAAC | SalI |
| ldhR1   | TTTTACTCCCTTCTGACATTATTAT | EcoRI |
| ldhR2   | CGGGCATCTTTTATCTTCTGTTCAGTTTTAT | BamHII |
| ldhR3   | CGCGTCGAGTTTTTCTCCTTTCGATTTTTAT | XhoI |
| upF     | AGGGTACGATGTCAATAGTTGTCGAAAA | KpnI |
| upR     | CAGTTTATCTGTTTTCACATGGAAC | SalI |
| downF   | TGATACCTTACGAGAAAAGGATGGAATTGCACACACAGTTG | SalI |
| downR   | CGCGTCGAGCTCTGAGTTAAAGGATGGAATTGC | XhoI |
| testF   | TTAAGGAAATGATTTGACAGGAG | KpnI |
| testR   | AACACCTCATGCTCCTTGTAG | SalI |
| BL-pheSF | AAAGATCTATGATCCTCCTAATCCCACCTTGAAC | BglII |
| BL-siteR | ACCAAACCCGCGGAAACGTC | EcoRI |
| BL-siteF | GACGTTTACGGCGGTTTTTGCCCTCTGTTCATGGAAC | XhoI |
| BL-pheSR | GATTTCGAAGTAACCCCTCCTGAGTTGCGAATGGTCGAC | PstI |
| gfpF    | ATAAAAAATCGAAAAGGAGATAAAAAGATATGAGCAAGAAAAGGAG | EcoRI |
| gfpR    | CGCGGATCCCTTTAGGAGATACCTGGTCGAC | EcoRI |

* The restriction sites in the primer sequences are underlined
as follows. Upstream with 1000 bp in size (amplified with 
primers upF and upR) and downstream with 1011 bp 
amplified with primers downF and downR) homology 
arms were PCR amplified from the genomic DNA of Lc. lactis 
NZ9000 and spliced by an overlap extension PCR 
using primers upF and downR; Subsequently, the fused 
fragment was digested by KpnI and XhoI and inserted 
into the corresponding sites of the temperature-sensitive 
vectos pG+host9 [16] and pG+-nP-pheS*, resulting the 
plasmids pG+UD and pG+UD-nP-pheS*, respectively. 
The plasmid pG+UD and pG+UD-nP-pheS* were intro-
duced into Lc. lactis NZ9000 to perform double-crossover 
hologous recombination as described previously 
[16]. Briefly, the recombinants were grown at 28 °C until 
OD600 0.8–1.0, then transferred to 37 °C for 2 h to allow 
the single-crossover integrants growth. Appropriate 
cultures were plated onto GM17 medium with 5 µg/ml 
erthyromycin at 37 °C. Subsequently, the single-cross-
over integrants were cultured in GM17 medium with-
out erythromycin at 28 °C for excision of the vector by a 
second crossover process. The cultures were then plated 
on GM9 plates containing 15 mM p-Cl-Phe at 37 °C. 
The single-crossover integrants and double-crossover 
mutations were both verified utilizing primer pair testF 
and testR.

To further confirm the function of pG+-5P-pheS*, the 
aldB gene encoding α-acetolactate decarboxylase was 
knocked out from the Lc. lactis NZ9000 genome using 
the above protocols. Primer pairs of aldB-uF/aldB-uR 
and aldB-dF/aldB-dR were utilized for amplifying the 
upstream and downstream homology arms, and fused 
by an overlap extension PCR. The resultant ~2.0 kb frag-
ment was digested and inserted into the KpnI and XhoI 
sites of the vector pG+UD-pheS*. Subsequently, the 
yielding plasmid pG+UD2-5P-pheS* was transferred into Lc. 
laeis NZ9000 to perform the double-crossover homologous 
recombination as described above. The mutant Lc. lactis 
daA was verified by PCR with the primer pair aldB-testF 
and aldB-testR, and the mutant genotype was also con-
firmed by sequence analysis (Biosune Company, Shang-
hai, China).

Extending this counterselectable marker pheS* to other 
LAB
To extend this counterselectable marker pheS* to other 
LAB, L. casei was selected as a host. The mutant gene 
pheS* was generated as two fragments by an overlap 
extension PCR using the L. casei BL23 [36] genomic DNA 
as a template with the primers BL-pheSF and BL-siteR, 
BL-pheSR and BL-siteF, respectively. The point mutation 
responsible for p-Cl-Phe sensitivity was PCR amplified by 
the primer pair BL-siteF and BL-siteR annealing internal 
to pheS gene. The generated pheS* was digested with PstI 
and BglII and ligated to the compatible sites of pTRKH2 
[37], creating pTRKH2-pheS*.

To investigate the feasibility of the counterselectable 
marker pheS*, the plasmid pTRKH2-pheS* was intro-
duced into L. casei BL23 by electroporation [13]. The 
recombinant L. casei BL23/pTRKH2-pheS* was incubated 
in MRS with 5 µg/ml erythromycin. Overnight 
cultures were streaked onto a GM9 plate containing 
10 mM p-Cl-Phe, the cell survival was measured.

Results

Bioinformatic analysis of PheS protein in selected LAB 
species
Previously, it was reported that only a point mutant 
pheS* gene encoding an A294G substitution in E. coli PheS [27] 
or an A312G substitution in Enterococcus faecalis PheS 
[32] resulted in the obviously sensitivity to the phenyla-
lane analog p-Cl-Phe. Hence, to identify the amino acid 
residue for site-directed mutagenesis, the amino acid 
sequences of PheS from Lc. lactis, L. casei, L. plantarum, 
L. brevis, L. rhamnosus and S. thermophilus were aligned 
with the amino acid sequences from E. coli and E. faecali-
is by Clustal X version 2.0 [30] and ESPript 3.0 [31]. As 
shown in Fig. 1, the amino acid residues A312 in Lc. lactis 
NZ9000, A312 in L. casei, A312 in L. plantarum, A312 in 
L. brevis, A312 in L. rhamnosus and A314 in S. thermophi-
lius are strictly conserved compared to the residue 
A294 in E. coli [27] and A312 in E. faecalis [32], indicat-
ing that this alanine residue of PheS protein was highly 
conserved in LAB.

To verify the above putative result, we chose the amino 
acid residues A312 in Lc. lactis NZ9000 PheS for site-
directed mutagenesis. The pheS gene (accession number: 
NC_009004) encoding phenylalanyl-tRNA synthetase 
alpha subunit was identified from the genome of Lc. lactis 
NZ9000. It was 1038 bp in size and composed of 
345 amino acid residues. After precision mutation GCT 
to GGT by an overlap extension PCR strategy in codon 
312 of the pheS gene, an A312G point mutation was intro-
duced into the PheS protein, resulting a dominant-
negative mutant protein PheS*. Moreover, we also tested 
the potential of A312G point mutation of PheS for coun-
terselection in L. casei BL23.

Functional analysis of the counterselectable marker 
Pldh-pheS* in Lc. lactis
To test the feasibility of the gene pheS* as a counterse-
lectable marker in Lc. lactis, it was driven constitutively 
by a strong promoter of l-lactate dehydrogenase gene 
(ldh) in Lc. lactis NZ9000. Subsequently, this resultant 
Pldh-pheS* cassette was inserted into the Lc. lactis/ E. coli
Fig. 1 A multiple-sequence alignment of PheS from a variety of distantly related species. Full length sequences of PheS were determined using Clustal X. The secondary structure of PheS in *E. coli* (PDB code: 3PCO) is shown at the top of each set of sequence. The conserved alanine residues mutated to generate p-Cl-Phe sensitivity were boxed with a thick line and indicated with a pentagram. α-helix, β-sheet, π-helix, 310-helix, TT β-turn.
shuttle vector pSec:Leiss:Nuc, yielding the recombinant plasmid pleiss-P-pheS*. The schedule of construction of pleiss-P-pheS* was shown in Fig. 2.

After introduction of the plasmid pleiss-P-pheS* into \emph{Lc. lactis} NZ9000, the sensitivity of the recombinant \emph{Lc. lactis} NZ9000/pleiss-P-pheS* was measured on the GM9 plates containing 0, 5, 10, 15 mM phenylalanine analog \textit{p}-Cl-Phe, respectively. As shown in Fig. 3, \emph{Lc. lactis} NZ9000/pleiss-P-pheS* grew well on the GM9 plate without \textit{p}-Cl-Phe, while the growth was completely inhibited in the presence of 15 mM \textit{p}-Cl-Phe. But, unlike the \emph{Lc. lactis} NZ9000/pleiss-P-pheS*, the growth of \emph{Lc. lactis} NZ9000 equipped with the plasmid pSec:Leiss:Nuc as a control was not inhibited under the equivalent concentrations of \textit{p}-Cl-Phe, indicating that the dominant-negative mutant protein PheS* is functional as a stringent counterselectable marker in the presence of 15 mM \textit{p}-Cl-Phe.

### Creation of a counterselectable cassette \textit{SP}_{\textit{ldh}}-\textit{pheS*} in \emph{Lc. lactis}

The temperature sensitive plasmid pG\textsuperscript{+}host9 was widely used for gene(s) deletion and insertion in LAB. Here, a counterselectable system PheS*/pG\textsuperscript{+}host9 was constructed based on a pG\textsuperscript{+}host9 carrying the fragment \textit{P}_{\textit{ldh}}-\textit{pheS*} from the plasmid pleiss-P-pheS*, and yielding pG\textsuperscript{+}-P-pheS*. To investigate the feasibility of this vector for gene deletion, upstream and downstream homology arms of the 709 bp fragment of galactose operon were spliced and inserted into pG\textsuperscript{+}host9 and pG\textsuperscript{+}-P-pheS*, resulting pG\textsuperscript{+}UD and pG\textsuperscript{+}UD-P-pheS*. The single-crossover integrants \emph{Lc. lactis} IG0 and \emph{Lc. lactis} IG1 were both pipetted onto GM9 plates containing 15 mM \textit{p}-Cl-Phe, respectively. Unfortunately, \emph{Lc. lactis} IG1 was not completely inhibited by \textit{p}-Cl-Phe. We supposed that this unexpected phenomenon might resulted from the low expression of PheS* protein from a chromosomal copy.

**Fig. 2** Construction of a vector for detecting the PheS*/pG\textsuperscript{+}host9 counterselectable system. The wild-type \textit{pheS} was changed to \textit{pheS*} using an overlap extension PCR to introduce a point mutation of GCT to GGT. \textit{P}_{\textit{ldh}}: the promoter region of the \textit{\iota}-lactate dehydrogenase gene (\textit{ldh}) (accession number: NC\textsubscript{0}017949) from \emph{Lc. lactis} NZ9000
To increase the expression level of *pheS*<sup>+</sup>, we firstly tested whether cascading promoters could be functional in *Lc. lactis*. A series of plasmids pleiss-nP-gfp carrying promoter clusters nP<sub>ldh</sub>-gfp were constructed and introduced into the *Lc. lactis* NZ9000. To optimize the constructed nP<sub>ldh</sub>-gfp promoter clusters, fluorescence intensity of each construct radiated from the green fluorescence protein after 12 h of aerobic incubation was determined. By analyzing the cell growth and relevant fluorescence of each recombinant strain, we found that the more copies of the *Pldh* promoter were present in the expression cassette, the higher the specific fluorescence was (Fig. 4a). This result indicated that cascading promoters could improve the expression level of gfp gene.

Subsequently, various copies of the *Pldh* were driven the expression of dominant-negative mutant protein *PheS*<sup>+</sup> (Fig. 4b). As shown in Fig. 4c, the increase of the *Pldh* copies in the nP<sub>ldh</sub>-phe<sup>S+</sup> cassette inserted into the chromosomal locus of the integrants resulted in the enhanced sensitivity to 15 mM p-CI-Phe. When the *pheS*<sup>+</sup> gene expressed from five copies of the *Pldh*, the growth of *Lc. lactis* IG5 was substantially inhibited. Thus, we chose the 5P<sub>ldh</sub>-phe<sup>S+</sup> cassette as a negative selectable marker for development of a counterselectable seamless mutagenesis system PheS*/pG<sup>+</sup>+host9 in *Lc. lactis*NZ9000 to perform seamless gene deletion.

To further determine whether the counterselectable system PheS*/pG<sup>+</sup>+host9 would be feasible for genome engineering, *aldB* gene which encodes for α-acetolactate decarboxylase catalyzing α-acetolactate to acetoin in the diacetyl biosynthesis in *Lc. lactis* was deleted by this system (Fig. 5a). Twenty-one colonies were selected randomly and detected by PCR amplification. The double-crossover events also occurred in 100%, and six out of them were the expected mutants (Fig. 6). This result indicated that the efficiency of screening double-crossover mutants was significantly improved compared with using pG<sup>+</sup>+host9 alone in our laboratory previously [18].

### Potential of the counterselectable marker *pheS*<sup>+</sup> in other LAB

To test the feasibility of the gene *pheS*<sup>+</sup> as a counterselection marker in other LAB, the strain *L. casei* BL23 was selected as a host. After insertion of *pheS*<sup>+</sup> into pTRKH2 [37], the obtained plasmid pTRKH2-phe<sup>S+</sup> was introduced into *L. casei* BL23. Subsequently the sensitivity of the recombinant *L. casei* BL23/pTRKH2-phe<sup>S+</sup> to p-CI-Phe was measured on the GM9 plates containing 10 mM p-CI-Phe. As shown in Fig. 7, the recombinant *L. casei* BL23/pTRKH2 grew well on the GM9 plate containing 10 mM p-CI-Phe, while the growth of *L. casei* BL23/
pTRKH2-pheS* was obviously inhibited, indicating that the conditional-lethal mutant gene pheS* has the potential as a counterselectable marker in *L. casei* and other LAB.

**Discussion**

In consideration of the increasing use in industrial and medical area, LAB are intensively studied on their genetics and metabolism [9, 10]. Therefore, efficient genome engineering tools are necessary for target gene(s) deletion or insertion for functional analysis or rerouting the metabolic flux [38]. In this study, a seamless negative selectable mutagenesis system PheS*/pG+host9 was developed. We also demonstrated its feasibility by constructing strains bearing the targeting seamless deletion of a 709 bp fragment in lactococcal galactose operon and *aldB* gene. Expectedly, the ratio of the double-crossover event was 100% after counterselection by p-Cl-Phe.

To our knowledge, this is the first report that the mutated *pheS* allele can be used as a counterselection marker for efficient and rapid genomic engineering in *Lc. lactis*. Previously, the development of a *pheS* based counterselection system in *Streptococcus mutans*, which is a close relative to *Lc. lactis*, has been reported [28]. However, *S. mutans* is a pathogenic bacterium distributed in the dental caries and could not be applied in the food field and used as a cell factory [39]. We expected that combining the counterselectable marker *pheS* with the traditional genetic tool pG+host9 [16] would overcome the bottleneck of laboriously screening of the double-crossover recombinants, and this system has greatly potential in genome engineering in LAB.

蛋白质序列分析表明PheS蛋白的alanine残基在LAB中高度保守（图1）。在这里我们证明了*pheS*作为counterselectable marker在*Lc. lactis*和*L. casei*中是可行的，这些结果与之前在*Streptococcus mutans*和*Enterococcus faecalis*中的结果一致 [28, 32]。因此，我们推测该正常-负性突变基因*pheS*可能在多种乳酸菌属中被广泛用于counterselectable marker。然而，细胞对p-Cl-Phe的敏感性取决于特定的菌株，如15 mM p-Cl-Phe对*Lc. lactis* NZ9000有效，20 mM p-Cl-Phe对*S. mutans*有效 [28]。因此，优化PheS*的表达是需要在使用*pheS*作为counterselectable marker时需考虑的。在这一研究中，PheS*蛋白在控制下*p-ldh*具有抑制*Lc. lactis* NZ9000生长的能力，暗示可以使用*p-ldh*-pheS* cassette作为counterselectable marker在*Lc. lactis*. 然而，重组体中*p-ldh*-pheS* insert的表达可能没有完全抑制。这种意外的结果意味着筛选双交叉重组体的比率不会是100%。我们推测这种现象是由于PheS*表达水平低 [28]，因为*p-ldh*-pheS*从染色体上插入的比率低于在野生型pleiss-P-pheS*。较低产量的PheS*不足以与野生型PheS竞争，从而形成与phenylalanyl-tRNA synthetase beta subunit (PheT)的复合物。
Fig. 5 Construction of the PheS*/pG+host9 counterselectable system in Lc. lactis NZ9000. a An efficient counterselectable system PheS*/pG+host9 used to create gene deletions in Lc. lactis NZ9000. The "pheS* cassette" indicates the pheS* gene under the control of five cascading Pldh. "up" and "down" indicate the upstream and downstream homology arms of the targeted region. "Erm" indicates the erythromycin resistant gene. "OriT" indicates the temperature sensitive origin of replication. b Twenty-four p-CI-Phe-resistant colonies were amplified by PCR to screen for the deletion of 709 bp fragment of the galactose operon. The expected PCR fragment from the mutant type (∆) is approximately 2.0 kb, while the band from the wild-type (WT) is about 2.7 kb.

Fig. 6 Generation of a seamless in-frame aldB deletion mutant. Twenty-one p-CI-Phe-resistant colonies were amplified by PCR to screen for the deletion of the aldB gene (711 bp). The expected PCR fragment from the mutant type (∆) is approximately 2.0 kb, while the band from the wild-type (WT) is about 2.7 kb.
In these cases, a strategy of cascading promoters [41] was employed to improve the expression level of protein PheS*. Surprisingly, when protein PheS* was driven simultaneously by five copies of the \( P_{ldh} \), the generating integrant \( Lc.\ lactis \) IG5 was substantially inhibited in the presence of 15 mM \( p-\text{Cl-Phe} \) and the ratio of screening double-crossover recombinants was 100%, suggesting recombination among the promoters was not occurred and the use of repeated \( P_{ldh} \) promoters could not confer genetic instability [41]. This strategy provides a new idea to address the issue of the low expression of the exogenous protein(s) in LAB.

Several strategies have been employed to fulfill the genome engineering in LAB by homologous double-crossover using a solely conditional replication plasmid [38] or combining with other counterselectable system, such as \( upp \) [22] or \( oroP \) [24] based cassettes. Compared with those methods, the negative selectable system PheS*/pG\(^+\)host9 has several advantages. (1) It greatly simplifies the procedure for screening double-crossover recombinants. For example, taking only 2 days to screen double-crossover variants after the single-crossover integrants were subcultured at 28 °C. The ratio of the double-crossover recombinants was 100% after \( p-\text{Cl-Phe} \) counterselection. However, the ratio between the deletion and wild-type strains may not be the theoretical value (1:1), it can vary considerably depending on the function of gene(s) to be deleted. (2) To our knowledge, among all the reported counterselectable markers, only PheS* has the greatly potential to be widely utilized in wild-type LAB without pretreatment. In contrast to other counterselectable system, the variants required the counterselectable marker deficient strains, as in the case of \( upp \) [21–23] and \( oroP \) [24]. Recently, a new counterselective method for wild-type \( Lc.\ lactis \) genome engineering based on class IIa bacteriocin sensitivity was reported [42]. However, the li006Dlitation of this method to be widely used in LAB was the sensitivity to bacteriocins which would depend on the interaction between the listerial MpnC and the native PtnD [42]. (3) Strains without PheS* can naturally grow on GM9 medium with 15 mM \( p-\text{Cl-Phe} \). This means 15 mM \( p-\text{Cl-Phe} \) has no side-effect on the growth of the expected mutants.

Moreover, this mutagenesis system PheS*/pG\(^+\)host9 allowed gene deletion without any genomic scarring [15] in \( Lc.\ lactis \), as the case of the \( aldB \) gene. The generating genetically modified microorganisms (GMOs) [14] were seamless mutagenesis which means only leaving self-DNA in its native genome location [15]. Therefore, this system is useful in seamless gene deletions in industrial strains. However, this seamless mutagenesis system PheS*/pG\(^+\)host9 remains challenging in large DNA fragment deletions or insertions. In this study, the limited length of the targeted DNA fragment was mostly from the low efficient homologous recombination mediated by RecA [17]. In consideration of the ratio of the double-crossover recombinants was 100% after \( p-\text{Cl-Phe} \) counterselection, the ideal goal for deletion or insertion of large DNA fragment is the new genome engineering tools responsible for targeted fragments replacement by selection and the 5\( P_{ldh} \)-PheS* cassette responsible for selectable marker excision by counterselection [15].

Conclusions

A seamless mutagenesis system PheS*/pG\(^+\)host9 based on a counterselectable marker pheS* and a temperature sensitive plasmid pG\(^+\)host9 was developed in \( Lc.\ lactis \). Moreover, this system can be used for rapidly constructing a seamless mutagenesis (deleted or inserted) strain. We also extended the counterselectable marker pheS* to \( L.\ casei \). Although the feasibility of pheS* as a counterselectable marker used in other LAB remains to be demonstrated, we speculated that this counterselectable marker will accelerate the analysis of genes with unknown function and metabolic engineering research in LAB.

Abbreviations

LAB: lactic acid bacteria; pheS: gene of phenylalanyl-tRNA synthetase alpha subunit; pheS*: the mutant pheS gene; PheS*: phenylalanyl-tRNA synthetase alpha subunit with an A312G substitution; \( P_{ldh} \): promoter of the \( Ll\). lactis aldB gene. The genetic modification of the plasmid pG\(^+\)host9 was developed in \( NZ9000 \). We also extended the counterselectable marker pheS* to \( L.\ casei \). Although the feasibility of pheS* as a counterselectable marker used in other LAB remains to be demonstrated, we speculated that this counterselectable marker will accelerate the analysis of genes with unknown function and metabolic engineering research in LAB.

Authors’ contributions

YPX, TTG and JK conceived and designed the experiments, YPX carried out the experimental work, YPX, TTG, YLM and JK wrote and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

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References
1. Cavanagh D, Fitzgerald GF, McAuliffe O. From field to fermentation: the origins of Lactococcus lactis and its domestication to the dairy environment. Food Microbiol. 2015;47:45–61.

2. Leroy F, De Vuyts L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci Technol. 2004;15:67–78.

3. Kleerebezem M, Hugenholz J. Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol. 2003;14:134–7.

4. Liu J, Dantoff SH, Wurtz A, Jensen PR, Solem C. A novel cell factory for efficient production of ethanol from dairy waste. Biotechnol Biofuels. 2016;9:33.

5. Guimaraes VO, Innocentini S, Levefre F, Azevedo V, Wal JM, Langella P, Chatel JM. Use of native lactococci as vehicles for delivery of DNA into mammalian epithelial cells. Appl Environ Microbiol. 2006;72:7091–7.

6. Kruger C, Hu Y, Pan Q, Marcotte H, Hultberg A, Delbar D, van Dalen PJ, Pouwels PH, Leer RJ, Kelly CG, et al. In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. Nat Biotechnol. 2002;20:702–6.

7. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Renaud E. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science. 2000;289:1352–5.

8. Wyszynska A, Koberecka P, Bardowski J, Jagusztyn-Krynicka E, Kaczmarski S. Lactic acid bacteria—20 years exploring their potential as live vectors for mucosal vaccination. Appl Microbiol Biotechnol. 2015;99:2967–77.

9. Rossi M, Amaretti A, Raimondi S. Folate production by probiotic bacteria. Nutrients. 2011;3:118–34.

10. Thakur K, Tomar SK, De S. Lactic acid bacteria as a cell factory for riboflavin production. Microb Biotechnol. 2016;9:441–51.

11. van Pijkeren JP, Britton RA. High efficiency recombining in lactic acid bacteria. Nucleic Acids Res. 2012;40:e67.

12. Oh JH, van Pijkeren JP. CRISPR-Cas9-assisted recombining in Lactobacillus reuteri. Nucleic Acids Res. 2014;42:e131.

13. Yang P, Wang J, Qin Q. Prophage recombining-mediating genome engineering in Lactobacillus plantarum. Microbiol Cell Fact. 2015;14:154.

14. van Pijkeren JP, Britton RA. Precision genome engineering in lactic acid bacteria. Microbiol Cell Fact. 2014;13(Suppl 1):S10.

15. Wang H, Bian X, Liu L, Ding X, Muller R, Zhang Y, Fu J, Stewart AF. Improved seamless mutagenesis by recombining using dCas8 for counterselection. Nucleic Acids Res. 2014;42:e37.

16. Maguin E, Duvat P, Hege T, Ehrlich D, Gruss A. New thermosensitive plasmid for gram-positive bacteria. J Bacteriol. 1992;174:5633–8.

17. Blowes I, Gruss A, Ehrlich SD, Maguin E. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol. 1993;175:3628–35.

18. Guo T, Kong J, Zhang L, Zhang C, Hu S. Fine tuning of the lactate and diacetyl production through promoter engineering in Lactococcus lactis. PLoS ONE. 2012;7:e36296.

19. Lu W, Kong J, Kong W. Construction and application of a food-grade expression system for Lactococcus lactis. Mol Biotechnol. 2013;54:170–1.

20. Wang T, Lu W, Su S, Kong J. Protective role of glutathione against oxidative stress in Streptococcus thermophilus. Int Dairy J. 2015;45:41–7.

21. Goh YJ, Ascarate-Peril MA, O’Flaherty S, Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR. Development and application of a upp-based counterselective gene replacement system for the study of the S-layer protein SLPX of Lactobacillus acidophilus NCFM. Appl Environ Microbiol. 2005;71:3093–105.

22. Martinussen J, Hammer K. Cloning and characterization of upp, a gene encoding uracil phosphoribosyltransferase from Lactococcus lactis. J Bacteriol. 1994;176:6457–63.

23. Martinussen J, Hammer K. Powerful methods to establish chromosomal markers in Lactococcus lactis: an analysis of pyrimidine salvage pathway mutants obtained by positive selections. Microbiology. 1995;141(Pt 8):1883–90.

24. Solem C, Defoor E, Jensen PR, Martinussen J. Plasmid pCS1966, a new selection/counterselection tool for lactic acid bacterium strain construction based on the orop gene, encoding an orotate transporter from Lactococcus lactis. Appl Environ Microbiol. 2008;74:4772–5.

25. Carr JF, Danziger ME, Huang AL, Dahlberg AE, Gregory ST. Engineering the genome of Thermus thermophilus using a counterselectable marker. J Bacteriol. 2015;197:1135–44.

26. Kino Y, Nakayama-Imaohji H, Fujita M, Tada A, Yoneda S, Murakami K, Hashimoto M, Hayashi T, Okazaki K, Kuwahara T. Counterselection employing mutated phet for markerless genetic deletion in Bacteroides species. Anaerobe. 2016;42:81–8.

27. Kast P, Hennecque H. Amino acid substrate specificity of Escherichia coli phenylalanine-tRNA synthetase altered by distinct mutations. J Mol Biol. 1991;222:99–124.

28. Xie Z, Okinaga T, Qi F, Zhang Z, Merritt J. Cloning-independent and counterselectable markerless mutagenesis system in Streptococcus mutans. Appl Environ Microbiol. 2011;77:8025–33.

29. Chu LL, Pandey RP, Jung N, Jung HJ, Kim EH, Sohng JK. Hydroxylation of diverse flavonoids by CYF450 BM3 variants: biosynthesis of eriodictyol from naringenin in whole cells and its biological activities. Microb Cell Fact. 2016;15:135.

30. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. Clustal X version 2.0. Bioinformatics. 2007;23:2947–8.

31. Robert X, Gouet P. Deciphering key features in protein structures with the I-TASSER server. Nucleic Acids Res. 2014;42:W520–4.

32. Kristich CJ, Chandler JR, Dunny GM. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugal delivery system and their use in genetic analysis of Enterococcus faecalis. Plasmid. 2007;57:131–44.

33. Kuipers OR, de Ruyter PGGA, Kleerebezem M, de Vos WM. Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol. 1998;64:15–21.

34. Le Loir Y, Gruss A, Ehrlich SD, Langella P. A nine-residue synthetic propetide enhances secretion efficiency of heterologous proteins in Lactococcus lactis. J Bacteriol. 1998;180:1895–903.

35. Holo H, Nes IF. High-frequency transformation, by electroporation, of Lactococcus lactis subsp. cremoris. Microb Cell Fact. 2007;6:12.

36. Hazebrouck S, Pothelune L, Azevedo V, Corthier G, Wal JM, Langella P. Improved production and secretion of bovine beta-lactoglobulin by Lactobacillus casei. Microb Cell Fact. 2007;6:12.

37. O’Sullivan DJ, Klaenhammer TR. High and low-copy-number plasmid shuttles: counterselectable markers for cloning in Lactococcus lactis. Microbiol Cell Fact. 2007;6:12.

38. Fang F, O’T oole PW. Genetic tools for investigating the biology of commensal lactic acid bacteria. Front Biosci (Landmark Ed). 2009;14:3111–27.

39. Legenova K, Budjakova H. The role of Streptococcus mutans in the oral biofilm. Epidemiol Mikrob I Infekc. 2015;64:179–87.

40. Merenshtain I, Finarov I, Klipcan L, Kessler N, Rozenberg H, Safro MG. Idiocrasy and identity in the prokaryotic Phc-system: crystal structure of E. coli phenylalanine-tRNA synthetase complexed with phenylalanine and AMP. Protein Sci. 2011;20:160–7.
41. Li M, Wang J, Geng Y, Li Y, Wang Q, Liang Q, Qi Q. A strategy of gene overexpression based on tandem repetitive promoters in Escherichia coli. Microb Cell Fact. 2012;11:19.

42. Wan X, Usvalampi AM, Saris PE, Takala TM. A counterselection method for Lactococcus lactis genome editing based on class Ila bacteriocin sensitivity. Appl Microbiol Biotechnol. 2016;100:9661–9.