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Improvement of Fengycin Production by *Bacillus amyloliquefaciens* via Promoter Replacement at the Fengycin Operon with the P_{59} and P_{repU} Promoters

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In this present study, a method was established to enhance the production of fengycin by *Bacillus amyloliquefaciens* ES-2 via replacement of the native promoter of the *pps* operon with one of two constitutive promoters, P_{59} and P_{repU}. This resulted in the production of two recombinant bacteria, *B. amyloliquefaciens* ES-2-1 and ES-2-2, that contained the P_{59} and P_{repU} promoters, respectively. Bioassays against *Aspergillus oryzae* and *Rhizopus stolonifer* confirmed increases in fengycin production in the recombinant strains. Furthermore, enhanced fengycin production was validated by high performance liquid chromatography (HPLC), and HPLC peaks for recombinant *B. amyloliquefaciens* ES-2-1 and ES-2-2 showed similar patterns of lipopeptides to the wild type strain. Fengycin production in *B. amyloliquefaciens* ES-2-1 and ES-2-2 was increased compared to the wild type strain by 65% and 20%, respectively. These results demonstrate the possibility to increase fengycin production by replacement of the native promoter of the *pps* operon.

Key words: *Bacillus amyloliquefaciens*; fengycin production; promoter replacement.

The application of biological fungicides is an attractive alternative to the extensive use of chemical fungicides. Some *Bacillus amyloliquefaciens* strains produce a wide variety of antifungal antibiotics, which are lipopeptides of the iturin and fengycin families[1]. Among them, fengycin has strong antifungal activity and is inhibitory for the growth of a wide range of plant pathogens[2-7].

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Fengycin (synonymous with plipastatin[8] combines several exceptional structural properties, including cyclization and the presence of branching and unusual constituents. Fengycin acts specifically against filamentous fungi [4] and is biosynthesized by fengycin synthetase, which is composed of five non-ribosomal peptides (Fen1 to Fen5) that are encoded by *ppsA* to *ppsE*, respectively[9-12]. At present, large-scale production of fengycin is difficult because fengycin is produced by spore-forming bacterium and the yield is very low. If fengycin can be produced industrially it could be used as a new biological fungicide. Thus, the enhancement of fengycin production is critical for its future application in the field.

Previous research on fengycin has concerned its isolation and purification, structural

identification, production by fermentation, and the cloning and sequencing of its gene[13-15]; however, present research is focused to enhance fengycin production.

B. amyloliquefaciens strain FZB42 is a naturally occurring isolate that colonizes plant roots. It is distinguished from the model organism *Bacillus subtilis* 168 by its abilities to stimulate plant growth and suppress plant pathogens. A complete genome sequence is available for *B. amyloliquefaciens* strain FZB42.

Previously, an endophytic bacterium, *B. amyloliquefaciens* ES-2, was isolated from the Chinese medicinal plant *Scutellaria baicalensis* Georgi [16]. This bacterium produces three families of secondary metabolites with broad spectrum antibacterial and antifungal activities. Culture filtrate of *B. amyloliquefaciens* ES-2 is antagonistic against certain phytopathogens and food-borne pathogenic and spoilage bacteria and fungi owing to the presence of antimicrobial compounds. The antimicrobial lipopeptides produced by *B. amyloliquefaciens* ES-2 include fengycins, surfactins and iturins, of which the fengycins are the principal constituents[16].

The aim of this present study was to enhance the productivity of fengycin by genetically modifying the *pps* operon that is responsible in fengycin synthesis by replacing the promoter with either the P_{59} or P_{repU} promoters .

MATERIALS AND METHODS

Bacterial strains, plasmids and culture media

The microorganisms and plasmids used in this study are listed in Table 1. *B. amyloliquefaciens* ES-2 strains were grown at 33°C in Landy medium[17] and at 37°C Luria-Bertani (LB) medium. *Escherichia coli* DH5α was cultured at 37°C in LB medium supplemented, when required, with chloromycin at 5 µg/ml (Sigma, USA) and erythromycin at 1 µg/ml (Sigma).

DNA extraction and polymerase chain reaction (PCR) conditions

Total genomic DNA was prepared and purified from *B. amyloliquefaciens* ES-2 using the UNlQ-10 Column Genome Extraction Kit, while plasmid DNA was prepared from *E. coli* using the UNlQ-10 Column Plasmid Mini-Prep Kit. PCR amplification products were purified using the UNlQ-10 Column PCR Production Purification Kit (Shanghai Sangon Biological Engineering Technology & Services).

The P_{59} promoter was amplified from the PHB201 plasmid using the forward primer 5'-ctttattgttttgccatt-3' and reverse primer 5'-ggataagaaagtgaaataacaaactgtcaaataaagta-3'. The P_{repU} promoter was amplified from the pMK3 plasmid using the forward primer 5'-acgaagtcgagatcagggaa-3' and reverse primer 5' -ggataagaaagtgaaataaacaaagtaatcaacattagcg-3'. The P_{59}- and P_{repU}-integrated plasmids (Fig. 1) were designed to facilitate promoter exchange by a single homologous recombination step in *B. amyloliquefaciens* ES-2. These plasmids were engineered using homologous fragments of the 450 base pair (bp) fengycin biosynthesis enzyme gene *fen*1 that was PCR-amplified using primers designed based on the previously published sequence of the *B. amyloliquefaciens pps* operon (PubMed nucleotide accession no. AJ576102). The homologous fragments to be linked to P_{59} were amplified using the forward primer 5'-tactttatttgacagtttgttatttcactttcttatcc-3' and reverse primer 5'-ccaaacttcttcgtctg-3', while the homologous fragments to be linked to P_{repU} were amplified using the forward primer 5'-cgctaatgttgattactttgtttatttcactttcttatcc-3' and reverse primer 5'-ccaaacttcttcgtctg-3'.

Then the promoter fragments and the homologous fragments were linked using the SOE-PCR method of Horton *et al*.,[18]. The PCR conditions for the promoter fragments and the homologous fragments consisted of an initial denaturation step at 95°C for 2 min, followed by 30 cycles of 50 s at 95°C, 50 s at 53°C, and 50 s at 72°C. The final extension step was for 2 min at 72°C.

Transformations

DNA transformations were performed in *E. coli* DH5α via the $CaCl_2$ heat method. Competent *B. subtilis* cells were prepared as described previously[19], and these cells were transformed using a BioRad Gene Pulser Xcell operating at 2 kV for 5 ms.

Fengycin fermentation and extraction

Landy liquid medium was inoculated with *B. amyloliquefaciens* ES-2, ES-2-1 or ES-2-2 using a seed culture (5% v/v). After incubating for 48 h at 32°C and 180 rpm/min, the culture was centrifuged at 11,000 ×g for 15 min. The supernatant

was adjusted to pH 2 with HCl, and allowed to precipitate overnight. Then the supernatant was centrifuged and the precipitate was collected, neutralized with NaOH, extracted three times with MeOH, and this yielded an extract containing the antimicrobial compounds including fengycin.

Analysis of fengycin production

First, fengycin production was assayed to investigate whether it inhibited the growth of *Aspergillus oryzae* and *Rhizopus stolonifer*. The fungi were incubated for 48 h before the spores were collected and diluted to 1.0×10^5 spores/ml. Then, 100 µl of spore solution was used to inoculate an agar plate. In total 200 µl of each extract from *B. amyloliquefaciens* ES-2, ES-2-1 and ES-2-2 cultures (containing the fengycin) was put into Oxford Cups. Plates were incubated for 48 h at 28°C and then growth inhibition zones were measured.

Second, a method for detecting the production of fengycin using high performance liquid chromatography (HPLC) was established. Fengycin production was detected at 210 nm (UV) within 80 min on a COSMOSIL 5C18-AR- (4.6×250 mm, Agilent) chromatographic column using a mobile phase of acetonitrile (0.1% trifluoroacetic acid [TFA]) and water (0.1% TFA) at a flow rate of 0.84 ml/min by gradient elution. Three characteristic peaks of fengycin were eluted at 24, 26.5 and 30 min. Fengycin production was calculated from the heights and areas of the peaks.

RESULTS

Construction of the homologous recombination plasmid

The homologous recombination plasmid was constructed using the SOE-PCR method. Fig. 2a and Fig. 2b show the homologous plasmids carrying an 826 bp fragment containing the P_{59} promoter and an 850 bp fragment containing the P_{repU} promoter, which indicated that the P_{59} and P_{repU} promoter DNA fragments were correctly linked to the homologous fragments. Then, the two linked fragments were recovered from agarose gel and linked to the pMD19-T vector, forming pMD19-T1 and pMD19-T2 for the P_{59} and P_{repU} promoters, respectively. A neomycin resistance gene (neo^r) was inserted into the EcoRI site of pMD19-T1 and pMD19-T2 as a screening marker,

Table 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
Escherichia coli DH5a	Host strain for gene clone	Laboratory stock
Bacillus amyloliquefaciens ES-2	Wild-type. Produces surfactin, fengycin, iturin A. The *pps* operon undercontrol of native promoter	Laboratory stock
Bacillus amyloliquefaciens ES-2-1	ES-2 derivative overproducing fengycin, Neo^R The *pps* operon under control of P_{59} promoter	This study
Bacillus amyloliquefaciens ES-2-2	ES-2 derivative produced a new fengycinhomologen Neo^R, The *pps* operon under control of P_{repU} promoter	This study
pMK3	Prepu carrier	BGSC
pHB201	P_{59} carrier	BGSC
pMD19-T	Cloning vector, Am^R	Takara

Neo^R, neomycin resistance gene. Am^R, ampicillin resistance gene.

Table 2. Fengycin HPLC production analysis

Strains	Fengycin production (mg/L)
ES-2	3455.05±113.84
ES-2-1	5704.77±258.89
ES-2-2	4052.47±168.76

Data are expressed as the mean ± SD of three independent cultures.

forming pMD19-T3 and pMD19-T4 as described in Fig. 2. Then the pMD19-T3 and pMD19-T4 plasmids were transformed into *B. amyloliquefaciens* ES-2 by homologous recombination.

Identifying the transformants

After *B. amyloliquefaciens* ES-2 was transformed with pMD19-T3 and pMD19-T4, seven neo^r transformants containing the P_{59} promoter and seven neo^r transformants with the P_{repU} promoter were identified using PCR (Fig. 3). For each of the seven P_{59}-derived transformants an 826 bp fragment was amplified, but for the seven P_{repU}-derived transformants an 850 bp fragment was amplified in only three strains. The 826 bp and 850 bp DNA fragments could not be amplified from the wild type strain, indicating that the native promoter had been replaced by the P_{59} and P_{repU} promoters.

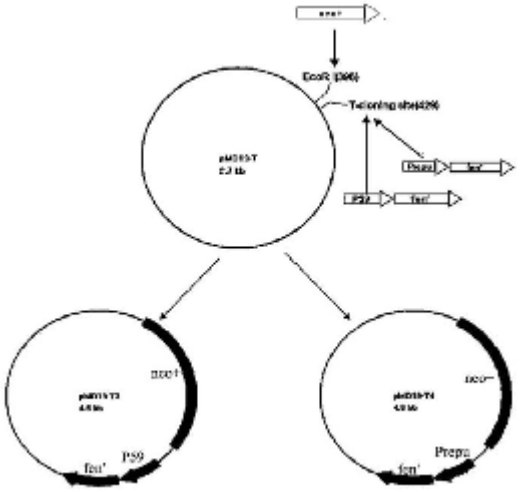

Fig. 1. Construction of the P_{59}-integreted and P_{repU}-integreted plasmids. The P_{59}-fen' fragment and P_{repU}-fen' fragment were generated by SOE-PCR. The resulting amplicons were inserted into the TA-cloning site of plasmid pMD19-T. The *neo*$^+$ gene was inserted into the *EcoR*I site of plasmid pMD19-T. The resulting pMD 19-T3 (P_{59}-integreted) and the pMD 19-T4 (Prepu-integrated) plasmids were generated

Assay of fengycin production

Fengycin can inhibit the growth of *A. oryzae* and *R. stolonifer*. Thus, fengycin production by *B. amyloliquefaciens* ES-2-1 (carrying the P_{59} promoter), *B. amyloliquefaciens* ES-2-2 (carrying the P_{repU} promoter) and the wild type strain was assessed by measuring the antifungal properties of these three strains (Figs. 4a and 4b). The inhibition zone diameters against *A. oryzae* and *R. stolonifer* of substances from *B. amyloliquefaciens* ES-2-1 and ES-2-2 were larger than those formed by the wild type strain. Substances from *B. amyloliquefaciens* ES-2-1 gave the largest zones of fungal growth inhibition. The results indicate that the fengycin productivity of *B. amyloliquefaciens* ES-2-1 and ES-2-2 were enhanced by replacement of the native promoter at the fengycin synthesis operon with stronger promoters.

Furthermore, fengycin production by *B. amyloliquefaciens* ES-2-1, ES-2-2 and ES-2 was quantified by HPLC assay after culturing for 48 h under identical growth conditions. The peak patterns for *B. amyloliquefaciens* ES-2, ES-2-1 and

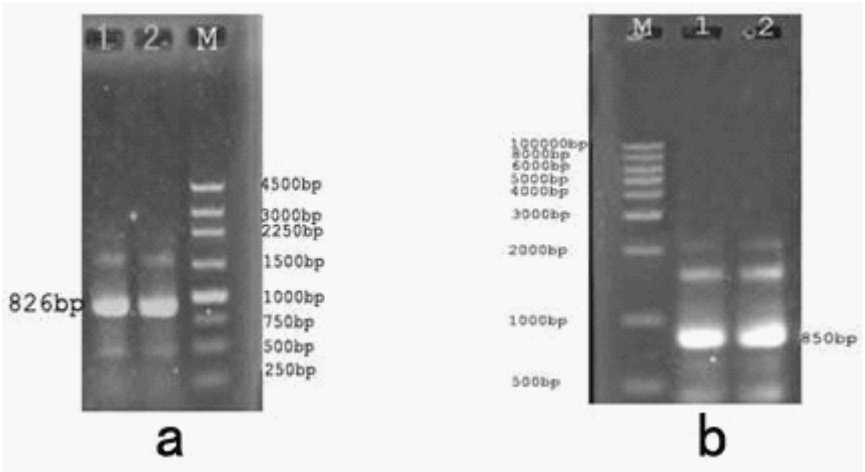

Fig. 2.The P_{59} and P_{repU} promoters with their respective homologous fragments. a) Lane M, DNA markers; lane 1, P_{59} linked to homologous fragment fen'. b) Lane M, DNA markers; lane 1 and 2, promoter P_{repU} and homologous fragment

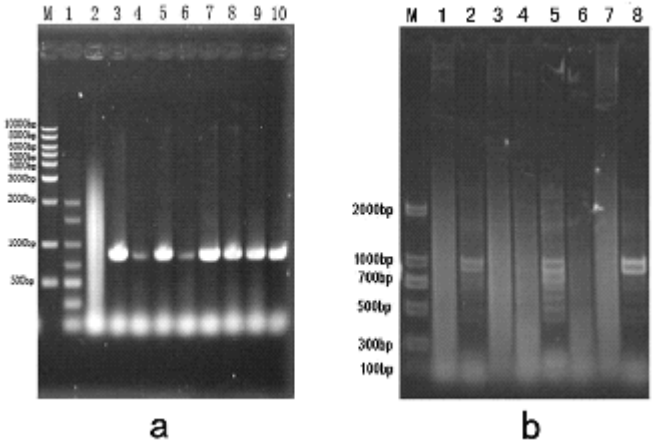

Fig. 3 Identification of P_{59} and P_{repU} transformants. a) Lane M1 and M2, DNA markers; lane 2, ES-2 control; lane 3, positive control; lane 4-10, 7 neomycin-resistant P_{59} transformants. b) Lane M, DNA markers; lane 1, ES-2 control; lane 2-8, 7 neomycin-resistant P_{repU} transformants

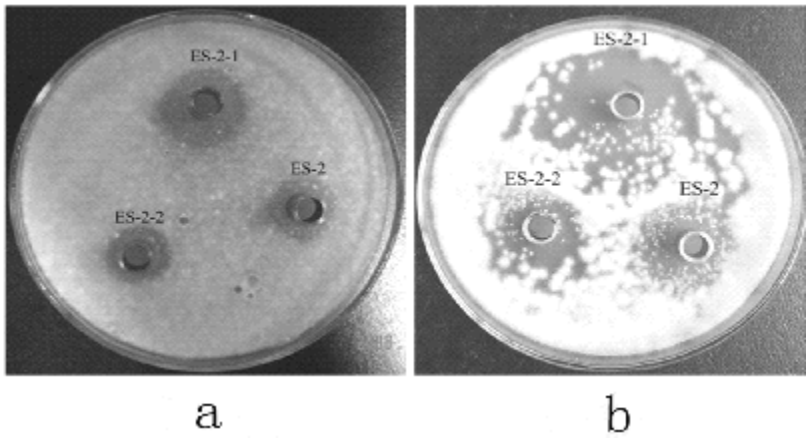

Fig. 4(a) *A. oryzae* and b) *R. stolonifer* growth inhibition following the addition of ES-2, ES-2-1 and ES-2-2 cell extracts

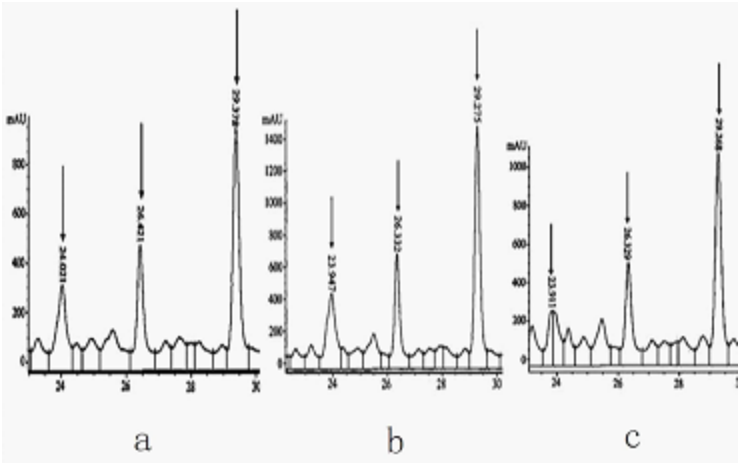

Fig. 5. Analysis of ES cell lysates by HPLC. a) ES-2, b) ES-2-1, c) ES-2-2. Retain times of fengycin peaks were between 24-35 min (X-axis, retain time; Y-axis, 230 nm UV absorption)

ES-2-2 were similar, except that the area of the peaks corresponding to fengycin differed between the recombinants and the wild type strain (Figs.5a, 5b and 5c). This indicated that promoter replacement did not change the production pattern of fengycin in the recombinants but enhanced its productivity. Fengycin production by the recombinant strains *B. amyloliquefaciens* ES-2-1 and ES-2-2 was 65% and 20% greater than the wild type strain (Table 2). This shows that P_{59} or P_{repU} promoters were able to regulate fengycin synthesis and promote the expression of fengycin synthesis genes, with a greater effect attributable to the P_{59} promoter compared with the P_{repU} promoter.

DISCUSSION

It has been assumed that rhizosphere competence and biocontrol functions of bacilli are partly due to nonribosomally produced cyclic lipopeptides that act against phytopathogenic viruses, bacteria, fungi and nematodes [9]. Fengycins have antifungal activities against some filamentous fungi *in vitro* [4]. But to date, there are no reports on the antifungal application of fengycins in practice, which is because fengycin production by *Bacillus* spp. is rather low. Thus, it is necessary to increase fengycin production by *Bacillus* spp. so that its application as a biocontrol agent can be evaluated. Most studies on fengycin have focused on its biochemical characterization, the gene cluster and enzymes responsible for its synthesis and its antifungal effects, but relatively few studies have reported methods for increasing fengycin production such as fermentation optimization.

Since the fengycin synthase gene cluster is too large (38 kb) to clone and express in *E. coli*, a promoter replacement strategy was developed to modify the expression of the fengycin synthase gene cluster to improve fengycin production. In the present study, the native fengycin synthesis *pps* promoter was replaced by P_{59} or P_{repU} promoters. The results showed that P_{59} and P_{repU} promoters were able to regulate fengycin synthesis and to promote the expression of fengycin synthesis genes.

In this present study, we have developed a method to increase fengycin production by 65% compared with the wild-type strain by replacing the native promoter with a stronger promoter. Specifically, fengycin production by *B. amyloliquefaciens* ES-2-1 strain was 5.7 g/l, which is more than the greatest production reported previously (5.2 g/L)[5] that was obtained by optimizing fermentation conditions.

The P_{repU} promoter has been used previously to improve the production of iturin by 10-fold and mycosubtilin by 15-fold[20,21]. In our earlier study, the native promoter of the surfactin operon in *B. subtilis* (*fmbR*) was replaced by the inducible promoter *Pspac* and it was possible to enhance the expression of many genes in *B. subtilis*. For example, surfactin production was increased by 10.85-fold[22]. The P_{59} promoter from *Lactococcal* has previously been shown to promoting strong expression of gene in *B. subtilis*. In the present study, fengycin production was increased by 65% when the native promoter of the fengycin operon was replaced with P_{59}. However, the P_{59} and P_{repU} promoters displayed different effects in *B. amyloliquefaciens* ES-2. P_{repU} did not increase fengycin synthesis as strongly as has been observed previously for iturin and mycosubtilin production, indicating that the effects of promoters on regulating gene expression differs according to the gene cluster involved.

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