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Enhancement of fengycin production in *Bacillus amyloliquefaciens* by genome shuffling and relative gene expression analysis using RT-PCR

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Abstract:Genome shuffling is an efficient approach for the rapid engineering of microbial strains with desirable industrial phenotypes. In this study, we used genome shuffling in an attempt to improve fengycin production of the wild-type strain *Bacillus amyloliquefaciens* ES-2-4 . After two rounds of genome shuffling, a high-yield recombinant F2-72 (FMB72) strain that exhibited 8.30- fold increases in fengycin production was obtained. Comparative analysis of synthetase gene (*fenA*) expression was conducted between the initial and shuffled strains using FQ (fluorescent quantitation) RT-PCR. Delta CT (threshold cycle) relative quantitation analysis revealed that fengycin synthetase gene (*fenA*) expression at the transcriptional level in the FMB72 strain was 12.77-fold greater than in the ES-2-4 wild type. The shuffled strain has a potential application in food and pharmaceutical industries. At the same time, the analysis of improved phenotypes will provide more valuable data for inverse metabolic engineering.

Key words: Genome shuffling , *Bacillus amyloliquefaciens*, Fengycin, CT (threshold cycle) , RT-PCR

Introduction

Bacillus strains can produce many types of bioactive peptides that are synthesized non-ribosomally by a large multifunctional enzyme complex. Of these, the lipopeptide fengycin has specific anti filamentous fungal activity (Vanittanakom et al. 1986). Fengycin is biosynthesized by five NRPSs, Fen1-Fen5, respectively is coded by the gene *fen A-E* (Steller et al. 1999). Fengycin consists of a β -hydroxy fatty acid connected to the N-terminus of a decapeptide including four D-amino acid residues and the rare amino acid L-ornithine. The C-terminal residue of the peptide moiety is linked to the tyrosine residue at position 3, forming the branching point of the acylpeptide and the eight-membered cyclic lactone (Stein et al. 2005; Wang et al. 2010). Fengycin has potential applications in plant disease biocontrol (Ongena et al. 2008), biomedicine, food (Bie et al. 2005) and cosmetics (Kanlayavattanakul et al. 2010) industries. Therefore, it is particularly significant to improve fengycin production in industrial *Bacillus* strains.

Genome shuffling is an efficient approach for the rapid improvement of microbial phenotypes (Zhang et al. 2002). Genome shuffling involves generation of mutant strains that have an improved phenotype, followed by multiple rounds of protoplast fusion to allow recombination between genomes (Zhang et al. 2013; Zhang et al. 2014; Lv et al. 2013). A strain with a high yield of a desired product can rapidly be obtained by genome shuffling without knowledge of the metabolic regulatory mechanism. Two rounds of genome shuffling were shown to be sufficient to achieve results that had previously required 20 rounds of mutagenesis and screening (Zhang et al. 2002). In the present study, genome shuffling was used to increase the yield of fengycin from *B. amyloliquefaciens*. The mechanisms that gave rise to the improved traits were explored by detecting the *fenA* target mRNA.

1 **Materials and methods**

2 **Strains and culture conditions**

3 *B. amyloliquefaciens* ES-2 is an endophyte isolated from the Chinese medicinal plant
4 *Scutellaria baicalensis* Georgi (Sun et al. 2006). *B. amyloliquefaciens* ES-2-4 was got by
5 20 keV N⁺ ion beam implantation (Fang et al. 2006). The yield of the peptides increased
6 by 15.2% compared to ES-2. These strains are conserved by the Key Laboratory of Food
7 Processing and Quality Control of the Food Science and Technology College at Nanjing
8 Agricultural University, Nanjing, China. *B. amyloliquefaciens* ES-2-4 was cultured in
9 potato dextrose agar (PDA) media at 37°C. All microorganisms were maintained in BPY
10 supplemented with 20% (v/v) glycerol and stored at -70°C. Seed medium (BPY) (peptone
11 10.0 g/L, beef extract 5.0 g/L , yeast extract paste 5.0 g/L, glucose 10.0 g/L, NaCl 5.0
12 g/L) and fermentation medium (modified Landy) (L-sodium glutamate 4.0 g/L, glucose
13 42.0 g/L , CuSO₄ 0.16 mg/L ,MgSO₄ 0.5 g/L, KCl 0.5 g/L , KH₂PO₄ 1.0 g/L, FeSO₄ 0.15
14 mg/L , MnSO₄ 5.0 mg/L) were adjusted to pH 7.0. SMM, adjusted to pH 6.5, contained
15 MgCl₂·6H₂O 4.07 g/L, sucrose 171.14 g/L , and maleicacid 2.32 g/L as a stabilizer.
16 Lysozyme was dissolved in SMM, sterilized by filtration through a 0.22 µm membrane
17 filter and stored at -20°C. PEG 6000 (40%) was prepared with SMM.

18 **Mutagenesis**

19 The induction of mutation was carried out by treatment of *B. amyloliquefaciens* ES-2-4
20 cells with 0.3 mg /mL nitrosoguanidine (NTG) for 30 min or antibiotics (0.5 U /mL
21 penicillin, 0.15 U /mL streptomycin or 3 U /mL amikacin sulfate) for 15 min and then
22 spread on the resistance media plate. The plates were incubated at 30°C for 2 days. The

1 colonies were selected to carry out shake flask analysis. The strains with higher fengycin
2 concentration were obtained and taken as the starters for genome shuffling.

3 **Preparation of protoplast**

4 The cells of each mutant strain were statically cultured at 37°C for 5 h in 50 mL BPY
5 seed medium. Cells were harvested by centrifugation, washed, and resuspended in SMM.
6 Protoplasts were prepared by addition of lysozyme to final concentration of 0.2 mg /mL
7 (37°C for 15 min) and collected by centrifugation at 2500 rpm for 10 min and then
8 suspended in SMM (Zhao et al. 2012) .

9 **Genome shuffling**

10 Equal number of protoplasts from different populations was mixed and divided equally
11 into two parts. One part was treated with UV for 60 min, and the other was heated at
12 100°C for 30 min. Both inactivated protoplasts (10^6 /mL) were mixed in a cell ratio of 1:1,
13 centrifugated and resuspended in 0.2 mL of SMM. Nine volumes of 40% PEG 6000 in
14 SMM were added to the resuspended protoplast mixture and incubated for 12 min at
15 37°C. The fused protoplast was centrifugated, washed, resuspended in 2 mL SMM, and
16 0.1 mL of sample were regenerated on RM at 37°C for 36 h. The colonies were selected
17 to carry out shake flask analysis and the strains with higher fengycin concentration were
18 obtained and named F1. Subsequent rounds of genome shuffling were carried out by
19 repeating the protoplast fusion described above (Zhao et al. 2012).

20 **Shake flask and bioreactor cultivation**

21 Colonies from PDA (including 0.6 mol/L NaCl) plates were used to inoculate

1 overnight cultures in 100 mL BPY medium. The 180 mL fermentation medium in shake
2 flasks (1 L) was inoculated with 8 mL of the overnight cultures at 33°C, 180 rpm. Each
3 strain was cultured in three shake flasks.

4 Precultures for bioreactor cultivations were grown in BPY medium at 37°C for 24 h.
5 Two preinoculation steps were required before bioreactor cultivations. Bioreactor
6 cultivation were performed in a 20-L bioreactor using 12 L of fermentation medium and
7 cultured at 33°C. During the batch fermentation course, pH was controlled at 7.0 with
8 automatic addition of 3.0 mol/L NaOH and an agitation speed of 250 rpm was maintained.
9 Silicone oil was served as anti-foam additive.

10 **Extraction and detection of fengycin**

11 Overnight cultures of *Bacillus amyloliquefaciens* were inoculated (5%, v/v) into
12 200 mL modified Landy medium, and then shaken at 33°C and 180 rpm for 36 h. At the
13 end of cultivation, 200 mL supernatant was treated with 6 mol/L HCl to adjust the pH to
14 2.0 and then the antibacterial peptides was extracted with 5 mL methanol for different
15 times and adjusted the pH to 7.0. The supernatants were analyzed by reversed-phase
16 HPLC (C18 column, ODS 4.6×250 mm, AGILENT 1100 series). Total lipopeptides
17 were determined by high-performance liquid chromatography (HPLC) (AGILENT 1100
18 series) using a C18 column (ODS 4.6×250 mm) with UV detectors. The eluent used was
19 Methyl Cyanides with 1% trifluoroacetic acid at a flow rate of 0.6 mL/min. The injection
20 volume of the sample was 20 µL. For fengycin production, *Rhizopus stolonifer* was used
21 as an indicator.

22 **Analysis for fengycin, glucose and dry cell weight**

1 Fengycin was determined by high-performance liquid chromatography (HPLC).
2 Glucose was estimated with DNS by measuring the OD (optical density) at 600 nm with
3 Unic 721 spectrophotometer (Shanghai). Dry cell weight was determined after
4 centrifuging 10 mL fermentation broth, washed twice with distilled water, and dried to
5 constant weight at 85°C. The same sample was repeated three times.

6 RT-PCR analysis

7 Total RNA was extracted from *B. amyloliquefaciens* cultures using TRIzol Reagent
8 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then
9 treated with RNase-free DNase. First-strand cDNA was synthesized from total RNA
10 using an RT-PCR kit (Fermentas, Vilnius, Lithuania). Real-time PCR was performed
11 using a real-time PCR SYBR Green Master Mix kit (Toyobo Biologics, Osaka, Japan) on
12 a Rotor-Gene 3000 RT-PCR System (Corbett Research, Australia). Each 25-μL PCR
13 reaction contained 10 μL of real-time PCR mix, 1 μL of template DNA (100 ng), and 2 μL
14 of 10 μmol/L primers. The PCR conditions were as follows: 95°C for 5 min, followed by
15 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Each reaction was
16 performed in triplicate. Following threshold-dependent cycling, melting was performed
17 from 45-95°C at a 0.1°C/s melt rate with a smooth curve setting averaging one point.
18 Primer specificity was verified by melt curve analysis. Band intensities were normalized
19 to the 16S rDNA transcript band for $2^{-\Delta\Delta CT}$ relative quantification (Livak et al. 2001).
20 16S rDNA was selected as the housekeeping gene, the *fenA* gene is the gene of interest.
21 The *B. amyloliquefaciens* nucleotide sequences for these two genes were obtained from
22 the GenBank database at the National Center for Biotechnology Information (NCBI,
23 <http://www.ncbi.nlm.nih.gov/>). Primer pairs were designed from these sequences (80-200

1 bp product length, optimal T_m at 60°C, GC% between 40 and 60%) with the Primer
2 Premier 5.0 software (Applied Biosystems), the 16S rDNA primers used were F
3 (5-CCTACGGGAGGCAGCAG-3) and R (5-ATTACCGC GGCTGCTGG-3) and the
4 *fenA* primers were F (5- CCATCGGCACTTATTACGGAAAC -3) and
5 R(5-TGCGGACAAAGG ACAGGAACT -3).

6 **Results and discussion**

7 **Strain mutagenesis and mutant screening**

8 Genome shuffling accelerates directed evolution by facilitating recombination within a
9 diverse mutant population. An improved starting point for breeding was required.
10 Genome shuffling amplifies genetic diversity by homologous recombination using
11 protoplast fusion within the selected mutant population (Hida et al. 2007).
12 Consequently, in order to increase the genetic diversity of mutant population, NTG was
13 used to generate the first population of heavy producing mutants of ES-2-4. The cells of
14 the initial strain were sensitive to 0.3 mg /mL NTG treatment for the clear inhibition zone
15 and then the cells were scraped from different places on the lawn around the inhibition
16 zone. About 115 colonies that showed the biggest transparent haloes were further tested
17 for fengycin production in shake flasks. Antibiotics (0.5 U/mL penicillin, 0.15 U/mL
18 streptomycin, 3 U/mL amikacin sulfate) were used as the mutagenizing agent for the
19 second population of mutants of ES-2-4. The cells spread on plates were exposed in
20 antibiotics solutions during which a killing rate of approximately 90% was observed. The
21 cells that characteristically produced the biggest zones of fengycin production were
22 chosen; 111 colonies conformed to this standard. Two mutant populations were assumed
23 to possess the capability for the high production of fengycin. During the secondary

1 screening in shake flask evaluations, 3 NTG mutants, 3 antibiotics mutants were selected
2 from the NTG and antibiotics populations. These six mutants showed small increases,
3 from 7.5% to 62.0% (Fig.1) in the production of fengycin . In addition, their high
4 producing capacity was stable and was maintained after at least 20 transfers in
5 shake-flasks. Consequently, these six mutants were used as the starting population for
6 genome shuffling.

7 **Genome shuffling**

8 Genome shuffling is dependent on the recursive fusion of protoplasts to allow
9 recombination. This recursive strategy permits the phenotype of interest to be obtained
10 quickly. The high frequency of protoplast formation and regeneration is the basis of the
11 efficiency of genome shuffling. A suitable concentration of lysozyme was necessary to
12 improve the frequency of protoplast formation as judged by osmotic fragility. The
13 frequency of protoplast regeneration was low and so, in a successful attempt to improve
14 this, we replaced sucrose in the RM with 0.6 mol/L sodium chloride. The protoplasts of
15 the NTG, and antibiotics mutants were subjected to a first round of pool-wise recursive
16 protoplast fusion. The resulting populations were screened for individuals with improved
17 fengycin production using the selective plates. After the first fusion, 147 colonies were
18 assayed for fengycin production in shake flask. We found two isolates showing higher
19 concentration of fengycin, named as *B. amyloliquefaciens* F1-140 (65.0 mg/L) and *B.*
20 *amyloliquefaciens* F1-142 (65.2 mg/L), respectively (Fig. 1). These two strains were then
21 used in an additional round of shuffling. The resulting second round population of 134
22 isolates was assayed for fengycin production in shake flask. Three colonies (F2) were

1 found to show higher concentration of fengycin. The mutant showing the highest
2 fengycin production level was selected and named as *B. amyloliquefaciens* F2-72 (Fig. 1).
3 The F2-72 (FMB72) mutants exhibited 81.4 mg/L (Fig. 1) fengycin yield which is 2.03
4 times improvement compared to the initial strain.

5 **Characterization of fengycin production, glucose consumption and cell growth of** 6 **ES-2-4 and FMB72 in bioreactor**

7 The fengycin production, glucose consumption and cell growth of FMB72 were
8 compared with ES-2-4 in the 20 L bioreactor. In an attempt to evaluate the effects of
9 scale-up fermentation on the genome shuffled strain for fengycin production with cell
10 growth, glucose consumption. Consistent with shake flask results, FMB72 obtained
11 fengycin production of 450.51 mg/L, which was 8.30 times increase compared to the
12 ES-2-4 at 42 g/L glucose concentration after 36 h. It also exhibited better growth and
13 more rapid glucose consumption under the same condition (Fig. 2). The cell growth
14 characterization was consistent with fengycin production. The dry cell weight of FMB72
15 reached 6.13 g/L, whereas ES-2-4 was 5.24 g/L. The specific growth rate of FMB72 was
16 16.98% higher than ES-2-4, when it was determined from the beginning to the end of the
17 batch fermentation. The rate of glucose consumption of genome-shuffled strain was
18 higher than that of ES-2-4. There was 6.64 g/L residual glucose left in the medium when
19 cultivated with ES-2-4, while only 4.23g/L glucose left with FMB72 at the end of the
20 fermentation. The results showed the FMB72 produced more antimicrobial lipopeptides
21 compared with ES-2-4. As is shown in Fig.2 that the final fengycin concentration of
22 FMB72 was 8.30 times greater than ES-2-4.

23 **Comparative mRNA analysis between the shuffled and initial strains**

1 The initial strain and shuffled strain were assayed using real-time PCR respectively.
2 The data are presented as the fold change in gene expression normalized to an
3 endogenous reference gene and relative to the initial control. The evaluation of $2^{-\Delta\Delta CT}$
4 indicates the fold change in gene expression relative to the initial control. The amount of
5 target, normalized to an endogenous reference and relative to the initial, is calculated
6 according to the following equation;

7
$$F = 2^{-\Delta\Delta CT} \text{ (Livak et al. 2001)}$$

8 Where $\Delta\Delta C_T = (CT_{fenA} - CT_{16S \text{ rDNA}})_{FMB72} - (CT_{fenA} - CT_{16S \text{ rDNA}})_{ES-2-4}$. The
9 normalized expression was calculated by averaging three CT values for the reference
10 gene (16S rDNA) and for the gene of interest (*fenA*). As shown in Fig. 4, the expression
11 of *fenA* in the shuffled strain was 12.77 times its expression in the initial strain.

12 In conclusion, the production of fengycin in *B. amyloliquefaciens* can be improved by
13 genome shuffling and the expression of *fenA* in the shuffled strain was higher than its
14 expression in the initial strain. The analysis of improved phenotypes will provide more
15 valuable data for inverse metabolic engineering.

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1 **Fig. 1** Comparison of the wild-type and mutant strains of *Bacillus amyloliquefaciens* for
2 fengycin production in BPY medium in shakeflasks. ES-2-4, wild-type of *Bacillus*
3 *amyloliquefaciens*; N, NTG mutant stains; K, antibiotics mutant stains, F1, strains from
4 the first round of genome shuffling; F2, strains from the second round of genome
5 shuffling.

6 **Fig.2** Comparison of cell growth (triangle), glucose consumption (circle) and fengycin
7 production (square) by *Bacillus amyloliquefaciens* ES-2-4 (open) and *Bacillus*
8 *amyloliquefaciens* FMB72 (closed) in a 20-L bioreactor. Samples of the experiments with
9 initial glucose concentrations of 42 g/L were analyzed every 4 h. DCW, dry cell weight.

10 **Fig.3** Suppressive effect of the supernatants on *Rhizopus stolonifer* obtained after growth
11 of (A) *Bacillus amyloliquefaciens* ES-2-4 and (B) FMB72 in modified Landy medium.
12 ES-2-4, wild-type of *Bacillus amyloliquefaciens*; FMB72, a strain produced after two
13 rounds of genome shuffling.

14 **Fig.4** qRT-PCR analysis of mRNA expression of *fenA* gene. Asterisks indicate a
15 statistically significant difference ($P < 0.05$) between the parental strain ES-2-4 and
16 recombination strain FMB72.

17

Figure 1

Figure 2

Figure 3

Figure 4

Draft