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Genome shuffling of *Bacillus amyloliquefaciens* for improving antimicrobial lipopeptide production and an analysis of relative gene expression using FQ RT-PCR

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Abstract Genome shuffling is an efficient approach for the rapid improvement of the yield of secondary metabolites. This study was undertaken to enhance the yield of surfactin produced by *Bacillus amyloliquefaciens* ES-2-4 using genome shuffling and to examine changes in SrfA expression of the improved phenotype at the transcriptional level. Six strains with subtle improvements in lipopeptide yield were obtained from populations generated by ultraviolet irradiation, nitrosoguanidine, and ion beam mutagenesis. These strains were then subjected to recursive protoplast fusion. A strain library that was likely to yield positive colonies was created by fusing the lethal protoplasts obtained from both ultraviolet irradiation and heat treatments. After two rounds of genome shuffling, a high-yield recombinant F2-38 strain that exhibited 3.5- and 10.3-fold increases in surfactin production in shake flask and fermenter respectively, was obtained. Comparative analysis of synthetase gene expression was conducted between the initial and shuffled strains using FQ (fluorescent quantitation) RT-PCR. Delta CT (threshold cycle) relative quantitation analysis revealed that surfactin synthetase gene (*srfA*) expression at the transcriptional level in the F2-38 strain was 15.7-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.

Keywords Genome shuffling · *Bacillus amyloliquefaciens* · Antimicrobial lipopeptide · CT (threshold cycle) · Housekeeping gene · The gene of interest

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Introduction

Bacillus strains produce many kinds of bioactive lipopeptides synthesized nonribosomally by a large multifunctional enzyme complex [25]. Of these, the lipopeptide surfactin is well characterized at the genetic level. Surfactin is biosynthesized by three NRPSs, SrfA–C [20]; the thioesterase/acyltransferase enzyme SrfD stimulates the initiation of this process [22].

Surfactin is an extraordinarily powerful biosurfactant that is known to decrease the surface tension of water; it exerts a detergent-like action on biological membranes [3], and is distinguished by its exceptional emulsifying, foaming, antiviral and anti-mycoplasma activities [20]. Surfactin has a great number of potential applications in plant disease biocontrol [18] and biomedicine [14]. Moreover, lipopeptide can be widely used in the food [1], cosmetic [13] industries and for enhanced oil recovery [21] and for the bioremediation of oil-contaminated sites [17]. Both *Bacillus amyloliquefaciens* and *Bacillus subtilis* have been used as lipopeptide producers [24, 26]. There have been many attempts to increase lipopeptide production in these two organisms, but almost all of them have focused on fermentation optimization [12], isolation and purification

[7], or on the regulation of lipopeptide synthesis using genetic engineering methods [2, 23].

Although rational methods and global techniques have been successfully applied to strain improvement, the need to engineer more complex phenotypes requires a more combinatorial approach. The technology of genome shuffling has been suggested as a novel whole-genome engineering approach for the rapid improvement of complex cellular phenotypes. Two rounds of genome shuffling were shown to be sufficient to achieve results that had previously required 20 rounds of mutagenesis and screening [30]. The genome shuffling approach using recursive protoplast fusion with multi-parental strains offers the advantage of recombination throughout the whole genome without the need for genome sequence data or network information [10]. This method has been successfully used to improve acid tolerance [19], glucose tolerance [27], and degradation of pentachlorophenol in *Sphingobium chlorophenolicum* [5] to increase the production of tylosin in *Streptomyces fradiae* [30], hydroxycitric acid by *Streptomyces* sp. U121 [11], arachidonic acid in *Diasporangium* sp [31] and vitamin B12 in *Propionibacterium shermanii* [29].

Genome shuffling is an exciting and promising approach that can be used not only for producing improved strain but also as a source of information and data on complex metabolic and regulatory networks for an enormous variety of microorganisms. In the present study, genome shuffling was used to increase the yield of surfactin from *B. amyloliquefaciens*. The mechanisms that gave rise to the improved traits were explored by measuring the *srf* target mRNA.

Materials and methods

Microorganism and media

Bacillus amyloliquefaciens ES-2 was an endophytic bacterium isolated from the Chinese medicinal plant *Scutellaria baicalensis Georgi* [24]. *B. amyloliquefaciens* ES-2-4 was obtained with N^+ ion beam implantation (20 keV of energy and 2.60×10^{15} cm^{-2} in dose) [8]. The concentration of the lipopeptides in fermentation broth increased by 15.2% compared to ES-2. These microorganisms were conserved by Key Laboratory of Food Processing and Quality Control of Food Science and Technology College in Nanjing Agricultural University. *B. amyloliquefaciens* ES-2-4 was cultured in standard potato dextrose agar (PDA) media at 37°C. All microbial strains were maintained in BPY supplemented 20% (v/v) with glycerol and stored at −70°C. The regeneration medium (RM) was PDA with NaCl (0.6 mol/l). Seeds medium (BPY) (beef extract 5.0 g/l, peptone 10.0 g/l, yeast extract paste 5.0 g/l, glucose 10.0 g/l, NaCl 5.0 g/l) and modified Landy medium (glucose 42.0 g/l, L-sodium glutamate 4.0 g/l, $MgSO_4$ 0.5 g/l, KCl 0.5 g/l, KH_2PO_4 1.0 g/l, $FeSO_4$ 0.15 mg/l, $MnSO_4$ 5.0 mg/l, $CuSO_4$ 0.16 mg/l) were adjusted to pH 7.0. SMM, adjusted to pH 6.5, contained sucrose 171.14 g/l,$MgCl_2{\cdot}6H_2O$ 4.07 g/l, and maleic acid 2.32 g/l as a stabilizer. Lysozyme was purchased from Sigma and prepared with SMM, sterilized by filtration through a 0.22-μm membrane filter and stored at −20°C. PEG 6,000 (40%) was prepared with SMM. Surfactin standard sample was purchased from Sigma.

Mutagenesis

ES-2-4 cells were mutagenized with either nitrosoguanidine (0.5 mg/ml, 37°C, 30 min), ultraviolet irradiation (20 w, 30 cm, 60 s), or ion implantation [15] (implantation sources were produced by an ion-beam bioengineering instrument devised by Chinese Academy of Sciences, Institute of plasma physics) and then spread on PDA agar plates. The plates were incubated at 37°C for 24 h. The colonies were selected, diluted, and then spread on PDA plates and incubated in an incubator at 32°C for 36 h. The agar-well diffusion method [6] was used for its advantages of time savings, labor savings, and effectiveness. For lipopeptide production, *Escherichia coli* was used as an indicator [24]. The colonies with the largest killing halos were selected to carry out shake-flask analysis. The strains with the highest production were obtained and taken as the starter for genome shuffling.

Preparation of protoplasts

Strains were cultured at 37°C for 5 h in 50 ml BPY. Cells were harvested by centrifugation, washed, suspended and diluted in SMM buffer to give a suspension with an optical density of 2.0 at 600 nm. Lysozyme was then added to a final concentration of 0.2 mg/ml. After incubation at 37°C for 15 min, protoplasts of each strain were prepared. The appearance of spherical cells, as judged by light microscopy, was used as an indicator of protoplast formation. Protoplasts were collected by centrifugation at 2,500×*g* for 10 min and suspended in SMM.

Genome shuffling

An equal number of protoplasts from different populations were mixed and then divided equally into two fractions. One fraction was inactivated with UV for 60 min, and the other was heat treated at 100°C for 30 min. Both inactivated protoplast fractions were mixed in a cell ratio of 1:1, centrifuged, and resuspended in 0.2 ml of SMM. Nine volumes of 40% PEG 6000 in SMM were added to the

resuspended protoplast mixture and incubated for 12 min at 37°C. To terminate the effect of PEG, 5 ml SMM was added and the fused protoplasts were centrifuged, washed with SMM, resuspended in 2 ml of SMM, and serial dilutions of the suspension were spread on RM plates and incubated for 36 h at 37°C. The colonies that could regenerate on the RM plates were selected and a pooled library was built. The colonies with the biggest clearing halos were selected to carry out shake-flask analysis and the strains with the highest production were obtained and named F1. Two successive rounds of protoplast fusion were carried out; after each round, the production of lipopeptide increased. The yield of lipopeptide was analyzed using shake flasks. Samples from each of the fusion strains were saved for further analysis. Nonshuffled controls were prepared by the recursive formation and regeneration of protoplasts without any exposure to PEG.

Shake flask and bioreactor cultivation

Colonies from PDA (including 0.6 mol/l NaCl) plates were used to inoculate overnight cultures in 100 ml of BPY medium. The 200-ml fermentation medium in shake flasks (1 l) was inoculated with 10 ml of the overnight cultures at 150 rpm at 32°C. Each strain was cultured in three shake flasks. Precultures for bioreactor cultivations were grown in BPY medium at 37°C for 24 h. Two preinoculation steps were required before bioreactor cultivation. Bioreactor cultivation was performed in a 19-L bioreactor using 12 l of fermentation medium; inoculation was done at 32°C. During the course of pH-controlled batch fermentations, an agitation speed of 250 rpm was maintained. The fermentation pH (pH 7.0) was maintained with the automatic addition of 4.0 mol/l NaOH; silicone oil served as the anti-foam additive.

Extraction and detection of lipopeptide

Overnight cultures of *B. amyloliquefaciens* were inoculated (5%, v/v) into 200 ml of modified Landy medium, and then shaken 150 rpm for 36 h. At the end of cultivation, 200 ml of supernatant was treated with 6 mol/l HCl to adjust the pH to 2.0. The antibacterial peptides were then extracted with 5 ml of methanol for different times and adjusted to pH 7.0. The supernatants were analyzed by reversed-phase HPLC (C18 column, ODS 4.6 × 250 mm, AGILENT 1100 series). Lipopeptide was determined by high-performance liquid chromatography (HPLC) (AGILENT 1100 series) using a C18 column (ODS 4.6 × 250 mm) with a UV detector. The lipopeptide were eluted with acetonitrile-trifluoroacetic acid at a flow rate of 0.84 ml/min and monitored at 210 nm. The injection volume of the samples was 20 µl. For surfactin production, sheep blood was used as an indicator.

From the HPLC chromatograms, the peak areas of the lipopeptide were determined. The relation between concentration of surfactin (y) and the peak area (x) was expressed by the following criterion curve: $y = 0.1302\ x - 23.578$ ($R^2 = 0.9983$). By the above standard equation, the concentration of surfactin was calculated.

Analyses for antimicrobial lipopeptide, glucose, and dry cell weight

Total lipopeptide was determined by high-performance liquid chromatography (HPLC) with a UV detector. Glucose was estimated with DNS reagent by measuring the optical density at 540 nm with a Unic 7200 spectrophotometer (Unic, Shanghai, China). Dry cell weight was determined after centrifuging 10 ml of fermentation broth, washing once with distilled water, and drying to constant weight at 80°C. This was repeated three times for each sample.

Total RNA extraction

RNA was isolated using the EZgene^{TM} Bacterial RNA Kit (BIOMIGA R6616). RNA quality was determined before downstream application. Purity of the total RNA extracted was determined as the 260/280 nm ratio (an A260/A280 ratio of 1.8–2.0 corresponds to 90–100% pure nucleic acid) and the integrity was checked by denatured agarose gel electrophoresis with ethidium bromide staining. Several sharp bands for 28S and 18S ribosomal RNA and certain populations of mRNA should appear on the gels.

Reverse transcriptase reactions

A standard Super MLV RT-PCR kit (Biouniquer BU304) was applied to complete the reverse transcription reactions. The reactions contained the RNA template, 25 µmol/l random primer, 5 × super MLV buffer, 10 mmol/l of each of the dNTPs, 200 U/µl super MLV reverse transcriptase and 20 U/µl RNase inhibitor. The 20-µl reactions were incubated in a Bio-Rad Peltier Thermal Cycler in a 96-well plate for 10 min at 30°C, 50 min at 42°C, and 15 min at 70°C. All reverse transcriptase reactions were run in triplicate. cDNA was diluted 50-fold in nuclease-free water and stored at −20°C.

Real-time PCR

16S rDNA was selected as the housekeeping gene; the *srfA* gene is the gene of interest. The *B. amyloliquefaciens* nucleotide sequences for these two genes were obtained from the GenBank database at the National Center for

Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Primer pairs were designed from these sequences (80–200 bp product length, optimal Tm at 60°C, GC% between 40 and 60%) with the Primer Premier 5.0 software (Applied Biosystems); the 16S rDNA primers used were F341 (5′CCTACGGGAGGCAGCAG3′) and R518 (5′ATTACCGCGGCT GCTGG3′) and the *srfA* primers were F3726 (5′GAAGTCTTCAGCGGCGAACTG C3′) and R3879 (5′GGGTGGCTCCGTTTTTCTCG3′). After each run, melting curve analysis was performed to confirm the specificity of the amplification and the absence of primer dimers.

Real-time PCR was performed using a standard real-time PCR Master Mix (SYBR Green) kit (Biouniquer BU-Q002) on a Rotor-Gene 3000 RT-PCR System. The 25-μl PCR reaction mixture contained 12.5 μl 2 × real-time PCR Mix, 1.25 μl template DNA, 1.25 μl 10 μmol/l forward primer and 1.25 μl 10 μmol/l reverse primer. All reactions were incubated in a 36-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s with a single fluorescence reading taken at the end of each cycle. Each reaction was run in triplicate. Following threshold-dependent cycling, melting was performed from 60 to 95°C at 0.2°C/s melt rates with a smooth curve setting averaging 1 point. Primer specificity was verified by melt curve analysis. The negative first derivative of the melt curve (fluorescence versus temperature) plotted against temperature should yield a single peak (Tm of product) if the primers are specific to the gene of interest.

The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes a fixed threshold. The CT values produced from real-time PCR instrumentation were imported into a Microsoft Excel spreadsheet. The data reported from a quantitative gene expression experiment were imported to a spreadsheet. The change in expression of the *srfA* target gene normalized to the 16S rDNA expression was monitored. Real-time PCR was performed on the corresponding cDNA synthesized from the shuffled and initial *B. amyloliquefaciens* strains. All samples including the minus controls were repeated three times.

CT values were determined by the Rotor-Gene 6 software using a fluorescence threshold manually set to 0.02051 for all runs and exported into Microsoft Excel for analysis. After confirming that the amplification efficiency of the reference gene was consistent with the gene of interest, comparative delta–delta CT analysis was performed. The data are presented as the fold change in gene expression normalized to the endogenous reference gene (16S rDNA) and relative to the initial control. The evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression relative to the initial control. The amount of target, normalized to the reference and relative to the initial, was calculated according to the following equation, where, $\Delta\Delta C_T = \left(CT_{,srfA} - CT_{,16S\,rDNA}\right)_{F2-38} - \left(CT_{,srfA} - CT_{,16S\,rDNA}\right)_{ES-2-4}$.

$F = 2^{-\Delta\Delta CT}$ [16]

Results

Strain mutagenesis and mutant screening

Genome shuffling almost imitates the features of natural evolution through the recursive genetic recombination. Thus, a diverse population of mutants with the desired phenotype (improved compared with the initial strain) is required as the starting point. NTG mutagenesis was used to generate the first population of heavy producing variants of ES-2-4. The cells of the initial strain were sensitive to 0.5 mg/ml NTG treatment for the clear inhibition zone and then the cells were scraped from different places on the lawn around the inhibition zone. About 38 colonies that showed the biggest transparent haloes were further tested for antimicrobial lipopeptide production in shake flasks. UV irradiation was used as the mutagenizing agent for the second population of mutants of ES-2-4. The cells spread on plates were exposed to UV irradiation at 20 W (30 cm) for 60 s during which a killing rate of approximately 90% was observed. The irradiated cells that characteristically produced the biggest zones of antimicrobial lipopeptide production were chosen; 29 colonies conformed to this standard. A third population of heavy producing variants was obtained by ion implantation mutagenesis using the energy at 10 keV and a dosage of 1.56×10^{15} N^+/cm^2 as the ion implantation parameters of ES-2-4. About 53 colonies were picked. All three mutant populations were assumed to possess the capability for the high production of antimicrobial lipopeptide. During the secondary screening in shake-flask evaluations, two NTG mutants, two UV mutants, and two ion implantation mutants were selected from the NTG, UV, and ion implantation populations. These six mutants showed small increases, from 40.5 to 45.8 mg/l (Fig. 1) in the production of surfactin. In addition, their high producing capacity was stable and was maintained after at least 20 transfers in shake-flasks. Consequently, these six mutants were used as the starting population for genome shuffling.

Genome shuffling to generate the antimicrobial lipopeptide strains

Genome shuffling is dependent on the recursive fusion of protoplasts to allow recombination. This recursive strategy permits the phenotype of interest to be obtained quickly.

The high frequency of protoplast formation and regeneration is the basis of the efficiency of genome shuffling. A suitable concentration of lysozyme was necessary to improve the frequency of protoplast formation as judged by osmotic fragility. The frequency of protoplast regeneration was low and so, in a successful attempt to improve this, we replaced sucrose in the RM with 0.6 mol/l sodium chloride. The protoplasts of the NTG, UV, and ion implantation mutants were subjected to a first round of pool-wise recursive protoplast fusion. The resulting populations were screened for individuals with improved antimicrobial lipopeptide production using the selective plates. After the first fusion, four colonies from the F1 generation with the biggest haloes on the PDA plates containing 0.6 mol/l sodium chloride were identified as producing more antimicrobial lipopeptide than the mutant parents in the shake flask. These four strains were then used in an additional round of shuffling. Four colonies from the second shuffled library with the biggest haloes on the plates containing 0.6 mol/l sodium chloride were identified as the F2 generation after the shake-flask test. The F2-38 mutants exhibited 124.1 mg/l (Fig. 1) surfactin yield, which is a 3.5-times improvement compared to the initial mutants. Figure 2 shows that compared to *B. amyloliquefaciens* ES-2-4, F2-38 produced more surfactin. These results clearly show the differences in antimicrobial lipopeptide production between the shuffled strain and the mutated strains. One of the best performers in the shuffled strain from F2 was F2-38, and so this strain was selected for scale-up fermentation. A control experiment was carried out using

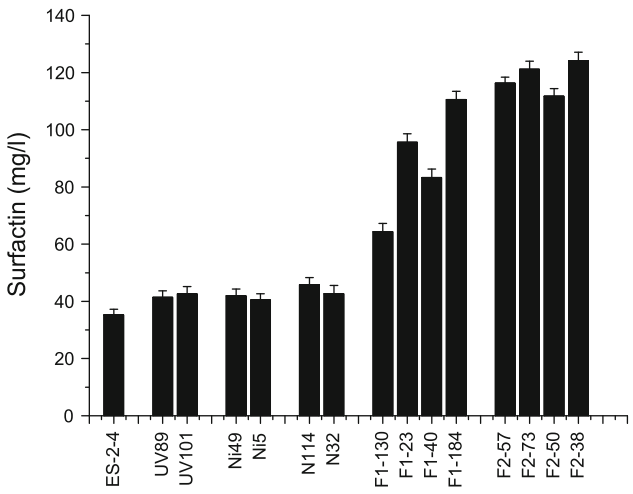

Fig. 1 Comparison of the wild-type and mutant strains of *Bacillus amyloliquefaciens* for surfactin production in BPY medium in shake flasks. ES-2-4, wild-type of *Bacillus amyloliquefaciens*; *UV*, UV mutant strains; *N*, NTG mutant stains; *Ni*, nitrogen ion implantation mutant stains, *F1*, strains from the first round of genome shuffling; *F2*, strains from the second round of genome shuffling

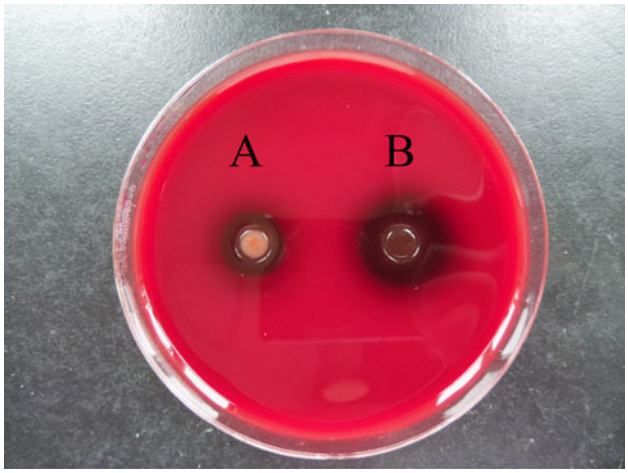

Fig. 2 Hemolytic activity of the supernatants obtained after growth of *Bacillus amyloliquefaciens* ES-2-4 (*A*) and F2-38 (*B*) in modified Landy medium. ES-2-4, wild-type of *Bacillus amyloliquefaciens*; F2-38, a strain produced after two rounds of genome shuffling

the selected populations of NTG, UV, and ion implantation mutants and F1 without exposure to PEG plated on the PDA plates containing 0.6 mol/l sodium chloride. In contrast to the results using the shuffled strains, no colonies were found on the corresponding plates under the same cultivation condition. The exposure of the protoplasts to PEG, which promotes fusion, would otherwise generate recombinants on the plate.

Characterization of surfactin production, glucose consumption and cell growth of ES-2-4 and F2-38 in a bioreactor

In an attempt to evaluate the effects of scale-up fermentation on the genome-shuffled strain, surfactin production, glucose consumption, and cell growth of F2-38 and ES-2-4 were compared in a 19-l bioreactor. Consistent with the shake-flask results, F2-38 yield of surfactin based on biomass (mg surfactin/g biomass) and yield of surfactin based on glucose (mg of surfactin/g glucose) were 58.1 and 10.0, respectively. The maximum surfactin concentration was 350.1 mg/l (Fig. 3), a 10.3-times increase compared to ES-2-4 at 42 g/l glucose concentration after 36 h. Moreover, F2-38 also exhibited better growth and more rapid glucose consumption than ES-2-4 under the same conditions. The dry cell weight of F2-38 was 6.0 g/l, whereas ES-2-4 was 5.1 g/l at the end of fermentation. When determined from the beginning to the end of the batch fermentation, the specific growth rate of F2-38 was 18.2% higher than that of ES-2-4. The cell growth rate was also consistent with the observed antimicrobial lipopeptide production. There was 6.7 g/l glucose left in the medium when cultivated with ES-2-4, while only 4.3 g/l glucose was left with F2-38 at the end of the fermentation. The rate of glucose consumption of the genome-shuffled

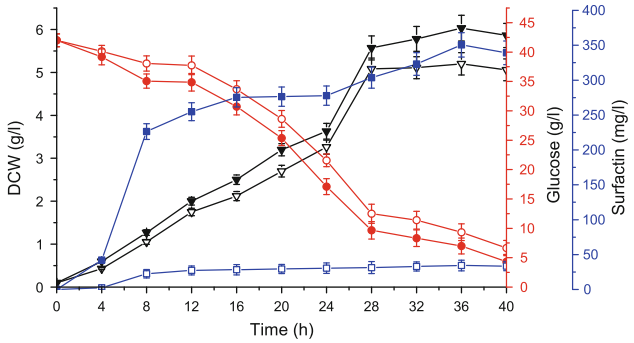

Fig. 3 Comparison of cell growth (*triangle*), glucose consumption (*circle*), and surfactin production (*square*) by *Bacillus amyloliquefaciens* ES-2-4 (*open*) and *Bacillus amyloliquefaciens* F2-38 (*closed*) in a 19-l bioreactor. Samples of the experiments with initial glucose concentrations of 42 g/l were analyzed every 4 h. *DCW* dry cell weight

strain was higher than that of ES-2-4 when 42 g/l initial glucose was used.

Comparative mRNA analysis between the shuffled and initial strains

The initial strain and shuffled strain were assayed using real-time PCR and the melt curves for the reference gene (16S rDNA) and for the gene of interest (*srfA*) were obtained at 60–95°C. Melt-curve analysis showed only one reference gene melting peak and one melting peak for the gene of interest. The two melting peaks were consistent with the Tm of the amplicons generated by the gene-specific primer pairs. After each run, melting-curve analysis was performed to confirm the specificity of the amplification and the absence of primer dimers.

The normalized expression was calculated by averaging three CT values for the reference gene (16S rDNA) and for the gene of interest (*srfA*). As shown in Table 1, the expression of *srfA* in the shuffled strain was 15.7-fold times its expression in the initial strain.

Discussion

Genome shuffling can be integrated with rational methods to promote the evolution of complex phenotypes, such as, in this case, increasing the yield of secondary metabolites. In this study, we used this approach to improve the production of lipopeptide in *B. amyloliquefaciens*. Compared to classical strain improvement strategies and rational genetic methods, genome shuffling offers more advantages.

The efficiency of genome shuffling for phenotypic improvement is significantly higher than that of classical strain improvement methods. First, it was very important to obtain a diverse protoplast of mutants that already shows some improvement in the trait of interest, as compared to the same trait in the initial strain. Then UV, NTG, and ion implantation were used to amplify the genetic diversity of the population in later generations. UV radiation can lead to the DNA molecules to form thymine dimers, NTG-induced mutations mainly GC–AT conversion. More attention is now being paid to implanting low-energy ions into industrial microorganisms to produce mutants with improved properties [28]. Accumulating evidence has shown that three factors including energy absorption, mass deposition, and charged ion exchange may play an essential role in low-energy ion bio-effects. Previous studies showed that the mutation effects of low-energy ion bombardment led to high product yield and wide mutation spectrum [4]. This may be the main advantage of our approach, which made it much more efficient for strain improvement. Before fusion, the protoplasts were divided into two equal groups and then inactivated either in a boiling water bath or using ultraviolet, which can cause lethal damage to physiological structures without actually killing them. Only the fusants that possessed the functions of the parents could regenerate on the regeneration medium. Thus, genome shuffling can be used to enhance lipopeptide production in *B. amyloliquefaciens*. A high-yield recombinant F2-38 strain was obtained after two rounds of genome shuffling. In a 19-l bioreactor the yield of surfactin in F2-38 reached 350.1 mg/l, 10.3-fold higher than surfactin production in the initial strain.

The technology of genome shuffling has been presented as a novel whole-genome engineering approach for the rapid improvement of cellular phenotypes without the necessity for genome sequence data or network information. It is well known that the profile of an ideal cell depends on the expression of a large number of genes that are often poorly understood, mostly unknown, and broadly distributed throughout the genome. Recombinant *B. subtilis* fmbR-1 was obtained by replacing the native surfactin

Table 1 Delta–delta CT relative quantitation analysis of the shuffled and initial strains of *B. amyloliquefaciens*

Replicate name	GOI CT[a]	Norm. CT[b]	Delta CT	Delta–delta CT	Relative conc.
ES-2-4[c]	21.5 ± 1.16	15.88 ± 0.49	5.62 ± 0.67	0	1.00
F2-38[d]	17.70 ± 1.12	16.05 ± 0.65	1.65 ± 0.47	−3.97 ± 0.2	15.72 ± 0.34

[a] GOI CT, CT (threshold cycle) of the gene of interest (*srfA*). [b] Norm. CT, CT of the reference gene (16S rDNA). [c] *B. amyloliquefaciens* ES-2-4 (initial strain). [d] The recombinant F2-38 strain obtained after two rounds of genome shuffling. For each measurement, $n = 3$

promoter with the inducible Pspac promoter and this increased surfactin production by about fivefold [23] compared to the wild-type. In signal factor mutants, the production of lipopeptide was increased to about threefold [2] more than that of the wild-type. In the present study, genome shuffling increased the production of surfactin by 10.3-fold over its production in the initial strain. Therefore, we can conclude that to improve the strain, genome shuffling is better than direct genetic manipulation through the control of a specific gene. Compared to other molecular breeding techniques genome shuffling is convenient and easy to use and, because the technique is based on protoplast fusion, the strains produced by genome shuffling are not considered to be "genetically modified". Importantly, therefore, the public distaste reserved for genetically modified organisms (GMOs) can be avoided if genome shuffling is used.

Genome shuffling can also be used to obtain information on desired phenotypes. The recombination of genetic traits among multiple parent strains can be completed in the process of genome shuffling. Evolved phenotypes from shuffling experiments are very likely to have altered network regulation, rebalanced fluxes, altered transport mechanisms, and/or increased substrate availability [10]. Until now, the only related research that explores the mechanisms of improved phenotypes is that of Zhang Ying et al. [29] who reported improved vitamin B12 production in *Propionibacterium shermanii* by genome shuffling and who carried out a comparative proteome analysis. Currently, RT-PCR is routinely used to amplify cDNA products reverse transcribed from mRNA and to study low abundance gene expression [9]. In this study, $2^{-\Delta\Delta CT}$ relative quantitation analysis was used, which revealed that the mRNA of the shuffled strain was 15.7-fold that of the initial strain. These mRNA transcriptional level changes explained the enhanced flux to lipopeptide in the shuffled strain compared to in the initial strain. Comparative analyses of the initial and shuffled mRNA showed that over-production of lipopeptide was accompanied by significant changes of a key synthetase involved in the surfactin biosynthesis pathway. Genome shuffling resulted in an increase in the transcriptional level of the synthetase SrfA, an important enzyme in the biosynthesis of surfactin in *B. amyloliquefaciens*. These results may provide information that can be used for the metabolic engineering of *B. amyloliquefaciens* for overproduction of surfactin and this research is proceeding in our groups. In addition to the development of this platform, the analysis of improved phenotypes will provide more valuable data for inverse metabolic engineering.

In summary, we have demonstrated that genome shuffling can be used for rapid strain improvement in *B. amyloliquefaciens*. We showed that the production of lipopeptide in *B. amyloliquefaciens* can be improved by genome shuffling and, for the first time, rationally explored the mechanisms of the improved phenotype at the transcriptional level. By combining the use of ion implantation to create genetic diversity, the advanced technology for inducing protoplast fusion and high-through-put screening method to select desired strains, genome shuffling is poised to play a very important role in strain improvement in the future [10].

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References

1. Bie XM, Lu ZX, Lu FX, Zeng XX (2005) Screening the main factors affecting extraction of the antimicrobial substance from *Bacillus* sp. fmbJ using Plackett–Burman method. World J Microbiol Biotechnol 21:925–928
2. Cao GQ, Zhang XH, Zhong L, Lu ZX (2010) A modified electrotransformation method for *Bacillus subtilis* and its application in the production of antimicrobial lipopeptides. Biotechnol Lett 33:1047–1051
3. Carrillo C, Teruel JA, Aranda FJ, Ortiz A (2003) Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim Biophys Acta 1611:91–97
4. Chen Y, Lin ZX, Zou ZY, Zhang F, Liu D, Liu XH, Tang JZ, Zhu WM, Huang B (1998) High yield antibiotic producing mutants of *Streptomyces erythreus* induced by low energy ion implantation. Nucl Instrum Methods Phys Res B 140:341–348
5. Dai MH, Copley SD (2004) Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC39723. Appl Environ Microbiol 70:2391–2397
6. Deng Y, Lu ZX, Lu FX, Wang Y, Bie XM (2011) Study on an antimicrobial protein produced by *Paenibacillus polymyxa* JSa-9 isolated from soil. World J Microbiol Biotechnol 27:1803–1807
7. Dimitrov K, Gancel F, Montastruc L, Nikov I (2008) Liquid membrane extraction of bioactive amphiphilic substances: recovery of surfactin. Biochem Eng J 42:248–253
8. Fang CJ, Lu ZX, Sun LJ, Lu FX, Bie XM (2006) Study on mutation breeding and fermentation of antimicrobial lipopeptides yielding bacterium with 20 keV N^+ ion beam implantation. J Radiat Res Radiat Process 24:333–336
9. Gibson UEM, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6:994–1001
10. Gong JX, Zheng HJ, Wu ZJ, Chen T, Zhao XM (2009) Genome shuffling: progress and applications for phenotype improvement. Biotechnol Adv 45:996–1005
11. Hida H, Yamada T, Yamada Y (2007) Genome shuffling of *Streptomyces* sp. U121 for improved production of hydroxycitric acid. Appl Microbiol Biotechnol 73:1387–1393
12. Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, Pauw ED, Thonart P (1999) Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett–Burman design. Appl Biochem Biotechnol 77:223–233
13. Kanlayavattanakul M, Lourith N (2010) Lipopeptides in cosmetics. Int J Cosmet Sci 32:1–8
14. Kowall M, Vater J, Kluge B, Stein T, Franke P, Ziessow D (1998) Separation and characterization of surfactin isoforms produced by *Bacillus subtilis* OKB 105. J Colloid Interface Sci 204:1–8

15. Li SC, Wu M, Yao JM, Pan RR, Yu ZL (2005) Mutation-screening in xylanase-producing strains by ion implantation. Plasma Sci Technol 7:2697–2700
16. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408
17. Mulligan CN, Yong RN, Gibbs BF (2001) Heavy metal removal from sediments by biosurfactants. J Hazard Mater 85:111–125
18. Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125
19. Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, del Cardayré S (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat Biotechnol 20:707–712
20. Peypoux F, Bonmatin JM, Wallach J (1999) Recent trends in the biochemistry of surfactin. Appl Microbiol Biotechnol 51:553–563
21. Schaller KD, Fox SL, Bruhn DF, Noah KS, Bala GA (2004) Characterization of surfactin from *Bacillus subtilis* for application as an agent for enhanced oil recovery. Appl Biochem Biotechnol 115:827–836
22. Steller S, Sokoll A, Wilde C, Bernhard F, Franke P, Vater J (2004) Initiation of surfactin biosynthesis and the role of the SrfD-thioesterase protein. Biochemistry 43:11331–11343
23. Sun HG, Bie XM, Lu FX, Lu YP, WuYun DL, Lu ZX (2009) Enhancement of surfactin production of *Bacillus subtilis* fmbR by replacement of the native promoter with the Pspac promoter. Can J Microbiol 55:1003–1009
24. Sun LJ, Lu ZX, Bie XM, Lu FX, Yang SY (2006) Isolation and characterization of a co-producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2, from Scutellaria baicalensis Georgi. World J Microbiol Biotechnol 22:1259–1266
25. Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56:845–857
26. Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A (2001) Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology 91:181–187
27. Yu L, Pei XL, Lei T, Wang YH, Feng Y (2008) Genome shuffling enhanced l-lactic acid production by improving glucose tolerance of *Lactobacillus rhamnosus*. J Biotechnol 134:154–159
28. Yu ZL (2000) Ion beam in application in genetic modification. IEEE Trans Plasma Sci 28:128–132
29. Zhang Y, Liu JZ, Huang JS, Mao ZW (2010) Genome shuffling of *Propionibacterium shermanii* for improving vitamin B12 production and comparative proteome analysis. J Biotechnol 148:139–143
30. Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WPC, del Cardayré S (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415:644–646
31. Zhao M, Dai CC, Guan XY, Tao J (2009) Genome shuffling amplifies the carbon source spectrum and improves arachidonic acid production in *Diasporangium* sp. Enzyme Microb Technol 45:419–425