

Inactivation of the phosphoglucomutase gene *pgm* in *Paenibacillus polymyxa* leads to overproduction of fusaricidin

Ha-Rim Kim · Soo-Young Park · Seong-Bin Kim ·
Haeyoung Jeong · Soo-Keun Choi · Seung-Hwan Park

Received: 16 April 2014 / Accepted: 28 May 2014 / Published online: 18 June 2014
© Society for Industrial Microbiology and Biotechnology 2014

Abstract Fusaricidin, a lipodepsipeptide isolated from *Paenibacillus polymyxa*, has high antimicrobial activity against fungi and Gram-positive bacteria. Through mutagenesis, we obtained two mutant strains, NIU7 and N17U7, which produce 6.2- to 7.9-fold more fusaricidin than their parent strain. Causal mutations were identified by whole-genome sequencing, and the two strains each contained at least eleven point mutations, including four common mutations. A mutation in the PPE04441 gene (*pgm*), encoding an α -phosphoglucomutase, was found to be an important factor in fusaricidin overproduction by complementation experiments. Null mutation of *pgm* in the parental strain increased fusaricidin production by 5.2-fold. Increased growth and cell viability in stationary phase, reduced exopolysaccharide production, and increased *fusA* expression were observed in the *pgm* mutant strains, which might be related to fusaricidin overproduction. This is the first report revealing that PGM deficiency leads to an overproduction of fusaricidin.

Electronic supplementary material The online version of this article (doi:10.1007/s10295-014-1470-z) contains supplementary material, which is available to authorized users.

H.-R. Kim · S.-Y. Park · S.-B. Kim · S.-K. Choi · S.-H. Park (✉)
Super-Bacteria Research Center, Korea Research Institute
of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro,
Yuseong-gu, Daejeon 305-806, Republic of Korea
e-mail: shpark@kribb.re.kr

H.-R. Kim · H. Jeong · S.-K. Choi · S.-H. Park
Biosystems and Bioengineering Program, Korea University
of Science and Technology (UST), 217 Gajung-ro, Yuseong-gu,
Daejeon 305-350, Republic of Korea

H. Jeong
Korean Bioinformation Center, KRIBB, 125 Gwahak-ro,
Yuseong-gu, Daejeon 305-806, Republic of Korea

Keywords Fusaricidin · Phosphoglucomutase ·
pgm mutation · *Paenibacillus polymyxa*

Introduction

Cyclic lipodepsipeptides (CLDPs) containing one or more ester bonds, in addition to amide bonds, have been isolated from natural organisms such as fungi and bacteria, and are promising compounds for the development of new antibiotics [3, 34]. The biosynthesis of these peptides proceeds non-ribosomally through the catalytic function of non-ribosomal peptide synthetases (NRPSs) [33]. Daptomycin, ramoplanin, and fusaricidin (or LI-F) are well-known examples of CLDPs that have high antimicrobial activity. Daptomycin was the first lipopeptide agent to be released onto the market for the treatment of infections caused by multidrug-resistant Gram-positive organisms [1, 35, 37]. Ramoplanin is a promising agent for the treatment of *Clostridium difficile*-associated disease [10, 11, 37]. Fusaricidin has antimicrobial activity against fungi and Gram-positive bacteria, and shows potential as a new antibacterial drug [4, 17, 21].

Fusaricidins are a group of antibiotics isolated from *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*), and have a cyclic structure composed of six amino acid residues in addition to a 15-guanidino-3-hydroxyptadecanoic acid moiety. Various analogs of fusaricidins, called LI-F03a, LI-F03b, LI-F04a, LI-F04b, LI-F05a, LI-F05b, LI-F06a, LI-F06b, LI-F07a, LI-F07b, LI-F08a, and LI-F08b [9, 19–21], as well as fusaricidins A, B, C, and D [15–17], have been isolated and characterized. Fusaricidins have high antimicrobial activity against fungi such as *Aspergillus nidulans*, *Candida albicans*, *Fusarium oxysporum*, *Leptosphaeria maculans*, and *Penicillium expansum* [2, 19, 21],

and Gram-positive bacteria such as *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Micrococcus luteus* [17, 21]. The toxicity of the fusaricidins in mice, however, is low [21]. In 2012, Quinn et al. [29] reported that fusaricidin B is an important component of a lipopeptide complex with biofilm inhibition activity. Lee et al. [23] recently reported that fusaricidin can induce systemic resistance against bacterial and fungal infections at concentrations as low as 0.1 parts per million in tobacco and red pepper plants. These reports show that fusaricidins, and perhaps the entire LI-F family of natural products, have a unique structure, high antimicrobial activity, and low toxicity, and are an attractive source of candidates for the development of new antimicrobial agents to combat multidrug-resistant bacteria, and for the development of novel crop protection agents.

We previously reported the identification and functional analysis of the fusaricidin synthetase gene, *fusA*, from *P. polymyxa* E681 using whole-genome sequencing [7, 18]. Because fusaricidin production in wild-type strains is low, development of a fusaricidin-overproducing strain is necessary for industrial purposes. However, no such strain has been developed, although it was recently reported that metal ions such as Zn^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , and Cu^{2+} significantly affect the production of fusaricidin-type antifungal compounds [30, 31], and that the global transition state regulator, AbrB, plays a negative role in fusaricidin synthesis by directly binding onto the promoter region of the *fus* gene cluster [25]. In this study, we used both targeted and random mutagenesis to construct mutant strains that can overproduce fusaricidin, and analyzed the mutations of two high-producing strains using whole-genome sequencing. We found that a null mutation of the phosphoglucomutase (PGM) gene is an important factor in the overproduction of fusaricidin.

Materials and methods

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are described in Table 1. Unless otherwise described, the *P. polymyxa* strains described herein were grown in tryptic soy broth (TSB; Difco, Detroit, MI, USA) or on tryptic soy agar (TSA) at 30 °C. To analyze fusaricidin production, the *P. polymyxa* strains were grown in a TSBG medium (TSB, 8 g; glucose, 30 g; $(NH_4)_2SO_4$, 2 g; K_2HPO_4 , 0.1 g; Na_2HPO_4 , 0.1 g; $MgSO_4$, 0.01 g; and yeast extract, 1 g/L). *Escherichia coli* strains were grown in Luria–Bertani (LB; USB, Cleveland, OH, USA) broth or on LB agar at 37 °C. *M. luteus* was grown in TSB at 30 °C. When required, the media were supplemented with ampicillin (100 µg/mL), erythromycin (0.5 µg/mL), or spectinomycin (100 µg/mL).

Null mutation of *trdA*

All primers used in this study are listed in Table 1. To knock out the putative tridecaptin synthetase gene (*trdA*) in *P. polymyxa* E681, an *E. coli* fosmid clone containing the putative *trdA* region in a pCC1FOS vector (called pTrdA) was isolated from a library which was constructed for genome sequencing in our previous study [18]. A 1.3-kb DNA fragment containing the entire spectinomycin resistance gene (*spc*) was amplified from plasmid pDG1730 [12] using primers trdspF and trdspR, giving 70-bp arms homologous to the target site at each end. Then, the amplified *spc*-containing DNA fragment was used to disrupt the putative *trdA* in the pTrdA by means of the lambda Red recombination system mediated by the pKD46 plasmid [8]. The resulting recombinant fosmid, called pDtrdA, was introduced into *P. polymyxa* E681 $\Delta pmxE$ which cannot produce polymyxin by knockout of the *pmxE* [6] by homologous recombination to generate a tridecaptin-defective mutant. *P. polymyxa* was transformed by electroporation, as previously described [7].

Mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)

NTG (Sigma-Aldrich Company, St Louis, MO, USA) was used for mutagenesis. Fresh *P. polymyxa* cells were grown in TSB at 30 °C with shaking to an OD_{600} of 0.7–1.0. To generate the mutant strains, a stock solution of NTG (2 mg/mL) was added to 1 mL of the *P. polymyxa* culture to a final concentration of 400 µg/mL. Following 10 min of incubation at room temperature without shaking, the cells were harvested through centrifugation (4 °C for 90 s), the mutagen was removed, and then the cells were washed with an equal volume of cold TSB and re-suspended in 1 mL of TSB. The cell suspension was diluted appropriately and then spread onto TSA plates. The plates were incubated at 30 °C for 1–2 days, and the colonies were assayed for fusaricidin production by bioassay against *M. luteus* as described below.

Mutagenesis using ultraviolet (UV) light

UV-induced mutants were obtained by exposing agar plates containing bacterial cells to UV light. A *P. polymyxa* culture freshly grown in TSB with an OD_{600} of 0.7–1.0 was serially diluted with a 0.1 M $MgSO_4$ solution, and three dilutions (10^{-4} , 10^{-5} , and 10^{-6}) were then used to make plates for UV irradiation by spread plating 0.1 mL of the diluted cell suspensions onto TSA plates. The plates were placed on an adjustable platform under a UV lamp (G30T8 UV lamp, 30 W; Sankyo Denki, Tokyo, Japan) fixed in a chamber. The UV irradiation was carried out by adjusting the distance between the UV lamp and culture plate from

Table 1 Bacterial strains, plasmids, and primers used in this study

| Strains, plasmids, and primers | Relevant characteristics/genotype/oligonucleotide sequences (5'-3') | References |
|--------------------------------|--|------------|
| Strains | | |
| <i>Paenibacillus polymyxa</i> | | |
| E681 | Wild-type strain containing intact polymyxin, fusaricidin, and tridecaptin synthetase genes. | [18] |
| E681 $\Delta pmxE$ | E681 <i>pmxE::cm</i> | [6] |
| E681 Δpt | E681 <i>pmxE::cm, trdA::spc</i> | This study |
| 4441p | E681 Δpt , PGM(Gln170Pro) | This study |
| 4441d | E681 Δpt , <i>pgm::erm</i> | This study |
| <i>Micrococcus luteus</i> | | |
| KCTC 2177 | Strain for monitoring of fusaricidin activity | ATCC10240 |
| Plasmids | | |
| pHPS9 | <i>E. coli/B. subtilis</i> shuttle vector | [13] |
| pHCMC05 | Pspac, <i>lacI</i> | [27] |
| pHPspac | IPTG-inducible vector | This study |
| pHR04441 | pHPspac-PPE04441 gene | This study |
| pHR00036 | pHPspac-PPE00036 gene | This study |
| pHR01127 | pHPspac-PPE01127 gene | This study |
| pHR01377 | pHPspac-PPE01377 gene | This study |
| pT4441p | Plasmid used for generating point mutation in <i>pgm</i> | This study |
| pT4441d | Plasmid used for generating deletion of <i>pgm</i> | This study |
| pET22b | Plasmid used for expression of His-tagged protein | Novagen |
| pET4441 | pET22b-PPE04441 | This study |
| Primers | | |
| emF | GAAGCAAACCTTAAGAGTGTG | This study |
| emR | TCCTTGGAAGCTGTCAGTAG | This study |
| spcF | GAATGGCGATTTTCGTTCTGTC | This study |
| spcR | TCATCATCGCTCATCCATGTC | This study |
| trdspF | CAGCCACAGCAACGGATTTGGTATACGGAGCTTCTGTATCCTAATCGGA ACACATCAACAATTATTGCAACTGGCGAATGGCGATTTTCGTTTC | This study |
| trdspR | GACAGGCTCCAGCACTGGCTCATGACCCGCACGCAATGCCAGGTAGCTGCC GAATACCTCCTGTGTCATATCATCATCGCTCATCCATGTC | This study |
| 00036F | CGGGATCCCTGATCTGGGAATAACG | This study |
| 00036R | TCCCCCGGGCCTCCGTGCTGGTTC | This study |
| 01127F | CGGGATCCGCAGGCTTTCTGATC | This study |
| 01127R | TCCCCCGGGTGGCAACATACAAGC | This study |
| 01377F | GGATCCTACGCGTCCGAAACGGCCAG | This study |
| 01377R | CCCGGGAACATAAAAGAAATAGTATG | This study |
| 4441F | CGGGATCCGCAGGCTTTCTGATC | This study |
| 4441R | TCCCCGGGCGTTGCCGGAATAAGCC | This study |
| 4441R' | CACACTCTTAAGTTTGCTTCCTTTGCCGTGAGAGGTCACC | This study |
| 4441FF | GAAAAGAGCAGTAGGCATTA | This study |
| 4441FR | CACACTCTTAAGTTTGCTTCCGGTCAGCATCAGGGTCTG | This study |
| 4441BF | CTACTGACAGCTTCCAAGGAGGCAGTACGCCGTGGAGGTC | This study |
| 4441BR | GATAATAGCCGTGCGATAA | This study |
| 4441X | CTCGAGAGCGTCTACGCGAGCCATAACAG | This study |
| 4441N | CATATGACGCAGTTGAGTAAAAAAGCATTAGAAG | This study |

40 to 80 cm, with exposure times of 10–60 s. The plates were incubated at 30 °C for 48 h, and viable colonies were counted to determine the appropriate irradiation conditions.

Antimicrobial activity assay and electrospray ionization–liquid chromatography/mass spectrometry (ESI–LC/MS) analysis

Antimicrobial activity against *M. luteus* was assayed as follows. *M. luteus* KCTC 2177 cells grown overnight in 3 mL of TSB at 30 °C were mixed with 300 mL of TSA, autoclaved, and cooled to <50 °C to prepare the bioassay plate (ML plate). To test the antimicrobial activity of thousands of mutants derived from the *P. polymyxa* E681Δ*pmxE*Δ*trdA* strain (hereafter called E681Δ*pt*) which has a decreased antibacterial activity by double knockout of polymyxin and tridecaptin synthetase genes, the colonies were transferred onto the ML plate using toothpicks, and the plate was incubated at 30 °C for 24 h. Mutants showing larger growth inhibition zones than that of E681Δ*pt* were selected for further analysis. The selected mutant strains were grown in TSBG, the cells were harvested by centrifugation (4 °C for 10 min) and extracted using equal volumes of methanol, and 10 μL of each extract was then loaded onto 6-mm paper disks, dried, and placed on an ML plate for observation of the growth inhibition zone. The methanol-extracted fusaricidin was analyzed using ESI–LC/MS (Thermo Electron Co., San Jose, CA, USA), as previously described by Choi et al. [7]. The fusaricidin produced by *P. polymyxa* strains was quantitatively analyzed by electrospray ionization–liquid chromatography/mass spectrometry (ESI–LC/MS). ESI–LC/MS was operated under the XCALIBUR software system using a Finnigan Surveyor™ Modular HPLC System with XTerra MS C18 column (5 μm; 2.1 × 150 mm; Waters, Milford, MA, USA) and a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with a Finnigan electrospray source (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The mobile solvents were water (solvent A) and acetonitrile (solvent B), both containing 0.1 % formic acid. A gradient elution at a flow rate of 0.3 mL/min was performed as follows: 0–15 min, 5–80 % B (linear gradient), followed by 15–20 min of 100 % B (isocratic). Selected ion-monitoring mass spectra were obtained at *m/z* 883, 897, 911, and 925 [M + H]⁺ using three microscans and a maximum ion injection time of 200 ms. The amount of fusaricidin was calculated by measuring the peak area.

Genome sequencing

The genome sequences of *P. polymyxa* strains N1U7 and N17U7 were determined using a whole-genome shotgun strategy and an Illumina HiSeq 2000 instrument (Illumina,

San Diego, CA, USA). Pretreatment, reference mapping and variant detection analysis of Illumina paired-end reads (101 nt × 2) were performed using the CLC Genomics Workbench ver. 4.8. Quality-trimmed reads were aligned to the genome sequence of parental strain E681 (GenBank accession number CP000154) with length fraction cutoff of 0.8 and similarity cutoff of 0.9, which was more stringent than default mapping condition. Genomic variants as single nucleotide polymorphisms and deletion/insertion polymorphisms were then automatically reported from the CLC Genomics Workbench.

Complementation of mutations in *P. polymyxa*

To perform a complementation test on the four mutated genes in *P. polymyxa* N17U7, each gene was amplified using primer sets containing *Sma*I and *Bam*HI restriction enzyme sites. To generate an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible system, a pHPspac plasmid vector was constructed using pHPS9 [13] and pHCMC05 [27]. A 1.7-kb region including the Pspac promoter and *lacI* was obtained from pHCMC05 by cleaving with *Sma*I and *Eco*RI (Roche, Basel, Switzerland), and then inserted into pHPS9 treated with the same enzymes. The target genes, PPE00036, PPE01127, PPE01377, and PPE04441, were cloned into the *Bam*HI and *Sma*I sites of pHPspac. The resulting plasmids were introduced into N17U7, and the antimicrobial activity and fusaricidin production of the transformants were analyzed following culture in TSBG supplemented with IPTG for 30 h.

Construction of *P. polymyxa* mutant strains 4441p and 4441d

Point mutation and deletion of the PPE04441 were conducted using an overlap extension PCR technique. To construct a *P. polymyxa* mutant strain with only the PPE04441 point mutation and no other mutations, the PPE04441 gene and its downstream region (1.8 kb) were amplified from N17U7 genomic DNA using primer pairs of 4441F-4441R' and 4441BF-4441BR, respectively (4441R' and 4441BF contained 20 bp of nucleotides complementary to the 3' and 5' ends, respectively, of the erythromycin resistance gene, *erm*). The *erm* was amplified from the pDG1664 [12] plasmid using a primer pair of emF–emR, and used as a selectable marker. These three PCR products were purified and joined through an overlap extension PCR. The joined cassette was cloned into a pGEM-T Easy vector (Promega, Madison, USA) to construct pT4441p, and the cassette was then integrated into the *P. polymyxa* E681Δ*pt* chromosome by double cross-over recombination to obtain 4441p strain. To generate a PPE04441 deletion mutation, part of the PPE04441 gene (1 kb) and its upstream region (0.4 kb)

were amplified from *P. polymyxa* E681 using primers 4441FF and 4441FR. The *erm* and the downstream region of the PPE04441 gene were obtained in the same manner as described above. These three fragments were joined through an overlap extension PCR, cloned into a pGEM-T Easy vector to construct pT4441d, and then integrated into the *P. polymyxa* E681 Δpt chromosome using a double cross-over recombination to obtain 4441d strain.

Preparation of crude extract from *P. polymyxa* cells

For the PGM activity assay, an overnight culture of the *P. polymyxa* strain was inoculated into 3 mL of TSB broth. After growing for 9 h, the cells were harvested by centrifugation at $15,000\times g$ for 10 min at 4 °C. The cell pellet was suspended in a buffer containing 50 mM Tris–HCl (pH 7.4) and 0.5 M NaCl, and then disrupted by sonication for 3 min (duty cycle 50 %, output control 4). Following centrifugation at $15,000\times g$ for 15 min at 4 °C, the supernatant was collected into new 1.5-mL microcentrifuge tubes and used for a PGM assay.

Purification of the PPE04441 gene product

The PPE04441 gene of E681 Δpt was amplified using primers 4441X and 4441N. The PCR product was cleaved with *Xho*I and *Nde*I and cloned into pET22b (Novagen, Inc., Madison, WI, USA) to construct pET4441. The plasmid was introduced into *E. coli* BL21(DE3) by CaCl_2 -mediated transformation, and the transformant was grown in LB broth supplemented with ampicillin and IPTG at 25 °C. The cells were disrupted by sonication for 1 min (duty cycle 50 %, output control 4) and the PPE04441 protein was purified using a His-Spin Protein Miniprep kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions.

Assay of PGM activity

PGM activity was assayed using a coupling enzyme reaction [38]. The standard reaction mixture for measuring the PGM activity contained 50 mM Tris–HCl (pH 7.4), 5 mM MgCl_2 , 10 μM glucose 1,6-diphosphate, 1 mM NADP^+ , 1 U of phosphoglucose dehydrogenase (coupling enzyme), and 1 mM glucose-1-phosphate. A 1-mL aliquot of a mixture containing 50 μL of crude extract or purified PPE04441 protein was used to measure the formation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm using an Ultrospec 7000 spectrophotometer (GE Healthcare Life Sciences, Uppsala, Sweden). The activity of the crude extract was expressed in arbitrary units ($(\Delta A_{340}/\text{OD}_{600}) \times 100$) \pm SEM, and the activity of the purified protein was expressed in units/mg protein \pm SEM.

One unit of enzyme activity is defined as the amount required for the formation of 1.0 μmol of NADPH per minute under the conditions described above.

Statistical analysis

Each assay was repeated three times and the data were analyzed using Analysis Of Variance (ANOVA). Comparison of data was made using Fisher's protected least significant difference (LSD) at $p = 0.05$. A p value <0.05 was considered statistically significant. All statistical tests were performed using JMP software version 5.0 (SAS Institute, Cary, NC, USA).

Results and discussion

Mutagenesis for fusaricidin-overproducing mutants

We used both targeted and random mutagenesis methods to develop *P. polymyxa* strains that could produce high levels of fusaricidin. We initially constructed a mutant *P. polymyxa* E681 strain that could not produce either polymyxin or a tridecaptin homolog, called E681 Δpt , to remove any possible interference caused by the co-production of unrelated antibiotics. Specifically, one of the putative tridecaptin synthetase-encoding genes, *trdA*, was knocked out by replacing it with *spc* in the *P. polymyxa* E681 $\Delta pmxE$. This strain was constructed in our previous study to block the biosynthesis of polymyxin by knockout of *pmxE*, and the resulting strain now defective in both *trdA* and *pmxE* was designated *P. polymyxa* E681 Δpt [6]. Tridecaptin, a lipopeptide antibiotic produced by *P. polymyxa*, was reported to have bactericidal activity against Gram-negative and -positive bacteria [32], and the putative tridecaptin synthetase genes, *trdA* and *trdB*, were discovered in the genome of the E681 strain (unpublished data). The resulting mutant, *P. polymyxa* E681 Δpt , showed very low antibacterial activity against *E. coli*, and slightly decreased activity against *M. luteus* KCTC 2177 compared to wild type, but maintained its antifungal activity (data not shown). The E681 Δpt strain was subjected to NTG treatment (six times) and UV irradiation (five times) to generate the fusaricidin-overproducing mutants. The overall mutagenesis procedure conducted in this study is shown in Fig. 1a. Fusaricidin-overproducing mutants were screened using a bioassay against *M. luteus* KCTC 2177 (ML), a Gram-positive bacterium that can grow on TSA and is sensitive to fusaricidin. After the first round of NTG mutagenesis, approximately 5,000 colonies grown on TSA plates were screened for antibacterial activity using an ML plate to isolate mutants showing increased antimicrobial activity. Then, a mutant strain showing the highest activity was selected and used for

Fig. 1 Scheme of the procedure used to construct fusaricidin-overproducing mutants (a) and a quantitative analysis of fusaricidin produced by N1U7 and N17U7 strains (b)

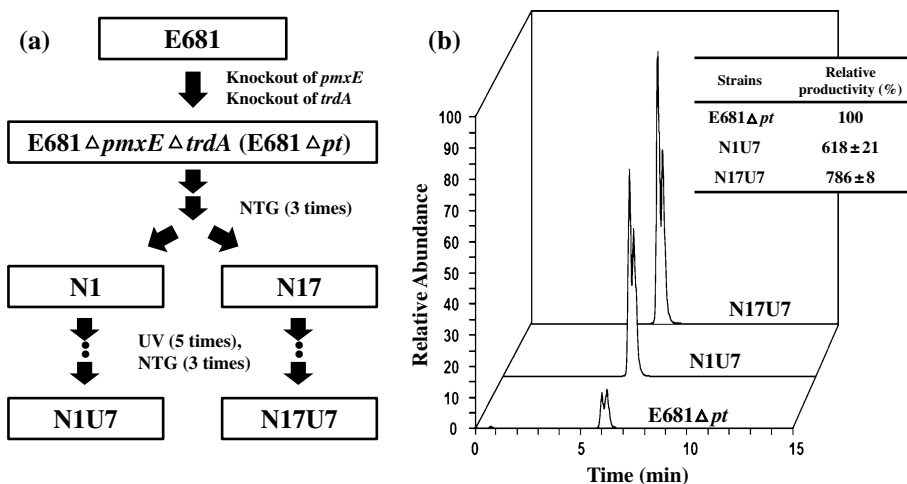


Table 2 Genes with mutations in N1U7 and N17U7 strains

| Strain | Gene | Highest BLAST hit | Point mutation |
|--------------|----------|--|----------------|
| N1U7 & N17U7 | PPE00036 | <i>N</i> -Acetylglucosamine-1-phosphate uridylyltransferase | Val235Ile |
| | PPE01127 | Glycosyltransferase WecG/TagA superfamily | Ser73Phe |
| | PPE01377 | Histidine kinase | Gly329Val |
| | PPE04441 | Phosphoglucomutase | Gln170Pro |
| N1U7 | PPE00823 | Hypothetical protein | Ile68Asn |
| | PPE02827 | Hypothetical protein | Thr106Ile |
| | PPE04186 | ABC-type multidrug transport system (ATPase/permease components) | Thr488Met |
| N17U7 | PPE00194 | Central glycolytic genes regulator | Ala60Val |
| | PPE00238 | Response regulator containing CheY-like receiver domain and AraC-type DNA-binding domain | Gly335Asp |
| | PPE01083 | Hypothetical protein | Gly73Asp |
| | PPE01945 | Dihydrodipicolinate synthase (DHDPS) | Pro240Ser |
| | PPE02356 | Hypothetical protein | Ala146Val |
| | PPE02686 | Glutamate 5-kinase | Gly235Asp |
| | PPE03671 | UDP- <i>N</i> -acetylmuramate-L-alanine ligase | Gly263Glu |

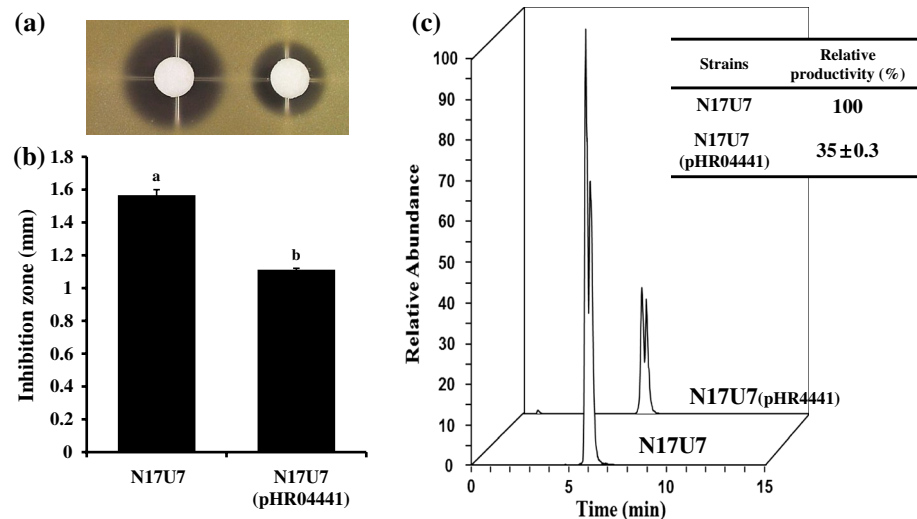
further mutagenesis. The second round of NTG mutagenesis was conducted in the same way, and after the third round mutagenesis, two mutant strains, N1 and N17, were selected for further development. In the next step, the two mutant strains were mutagenized using UV irradiation and NTG treatment. Finally, two mutant strains, N1U7 and N17U7, were selected as the highest fusaricidin producers in each line. An analysis of the LC/MS data showed that N1U7 and N17U7 strains produced 6.2- and 7.9-fold more fusaricidin, respectively, than the E681 Δpt strain (Fig. 1b).

Genome-wide analysis of two mutant strains

The two fusaricidin-overproducing mutants, N1U7 and N17U7, were analyzed to identify mutations using whole-genome sequencing (Illumina HiSeq 2000; Illumina, San Diego, CA, USA). By comparing the genomic sequences of the mutants with that of the parental strain, E681, we found

that the N1U7 strain had eleven point mutations, including seven missense mutations and three synonymous substitutions in coding regions and one mutation in a non-coding region, and that the N17U7 strain had 22 point mutations, including eleven missense mutations and seven synonymous substitutions in coding regions and four mutations in non-coding regions. The missense mutations in coding regions of the two strains are shown in Table 2. Through a comparative analysis of the mutations, five genes with mutations in both the N1U7 and N17U7 strains were found and these mutations might be involved in enhancing fusaricidin production. Among the five mutations, the four missense mutations in PPE00036, PPE01127, PPE01377, and PPE04441 were confirmed by PCR and sequencing and selected for further analysis to identify the effective mutations. One mutation in a 651-bp non-coding region was excluded because the point mutation seemed to be a silent mutation in our preliminary analysis of the mutation region.

Fig. 2 Antibacterial activities of the N17U7 and N17U7(pHR04441) strains against *Micrococcus luteus* (a and b), and quantitative analysis of fusaricidin produced by the strains (c). Cells harvested from 30-h cultures of the two strains were extracted with equal volumes of methanol, and 10 μ L of the extract was dropped onto a 6-mm paper disk, dried, and placed on an *M. luteus* plate. The means with different letters are significantly different ($p < 0.05$). The error bars indicate standard deviations ($n = 3$)



Among the four common missense mutations, one point mutation, which causes an amino acid change from glycine to valine at position 329 of the PPE01377, was in a sporulation histidine kinase [28]. The second point mutation causes a valine to isoleucine substitution at position 235 of the PPE00036, which has high similarity to the *N*-acetylglucosamine-1-phosphate uridylyltransferases of *Bacillus subtilis* 168 (61.2 % amino acid identity; GenBank accession number CAB11826.1) and *E. coli* K12 (41.4 % amino acid identity; GenBank accession number AAC76753.1). The other two mutations were found in PPE01127 (Ser73Phe) and PPE04441 (Gln170Pro), which showed high similarities to the glycosyltransferase WecG/TagA superfamily and PGM, respectively.

Effect of complementation of the four point mutations on fusaricidin production

To investigate the effects of the four selected mutations on fusaricidin production in *P. polymyxa* E681, complementation plasmids were generated for the PPE00036, PPE01127, PPE01377, and PPE04441 mutated genes using an IPTG-inducible vector pHPspac as listed in Table 1. The plasmids were introduced into N17U7 strain to prepare four complementation strains, and the strains were used for growth inhibition assays against *M. luteus*. Three transformants of N17U7, containing the complementation plasmids of pHR00036, pHR01127, and pHR01377, showed the same level of antimicrobial activity as that of N17U7 (data not shown). However, the antimicrobial activity of N17U7(pHR04441) was significantly lower than N17U7 (Fig. 2a, b). Quantitative analysis of fusaricidin produced by N17U7 and N17U7(pHR04441) using ESI-LC/MS (Fig. 2c) showed that the latter strain had 65 % lower production than the former. These results suggest that the point

mutation in the PPE04441 gene, which encodes a PGM homolog, has a significant effect on fusaricidin production in *P. polymyxa* E681.

Effect of null mutation of the PPE04441 gene on the fusaricidin production of *P. polymyxa* E681

P. polymyxa N17U7, which produces a high level of fusaricidin, has 22 point mutations, including four that are also seen in the N1U7 strain. With the complementation of the PPE04441 gene in the N17U7 strain, we observed a significant decrease in antimicrobial activity, and therefore hypothesized that PPE04441 mutation plays an important role in fusaricidin overproduction. To support this finding, we generated two different *P. polymyxa* E681 Δ pt strains with PPE04441 gene defects: a mutant containing a point mutation at position 509 of the PPE04441 gene, mimicking the N17U7 PPE04441 gene, and a mutant with deletion of PPE04441, to completely disrupt its function. The resulting mutant strains were designated *P. polymyxa* 4441p and *P. polymyxa* 4441d, respectively. The antibacterial activity and fusaricidin production of the two strains are shown in Fig. 3. An approximately fivefold increase in fusaricidin production was observed in both *P. polymyxa* 4441p and *P. polymyxa* 4441d by ESI-LC/MS quantitative analysis (Fig. 3c).

Functional analysis of the PPE04441 gene

The PPE04441 gene of *P. polymyxa* E681 was predicted to encode a 572 amino acid protein with high similarity to several known α -PGMs from other bacteria (*B. subtilis* pgcA, 53 %, GenBank accession number AJ784890.1; *Streptococcus thermophilus* pgmA, 46 %, GenBank accession number AJ243290.2; *S. gordonii* pgm, 46 %, GenBank

Fig. 3 Antibacterial activities of E681 Δpt , 4441p, and 4441d strains against *Micrococcus luteus* (a and b), and quantitative analysis of fusaricidin produced by the strains (c). Cells harvested from 30-h cultures of the three strains were extracted with equal volumes of methanol, and 10 μ L of the extract was dropped onto a 6-mm paper disk, dried, and placed on an *M. luteus* plate. The means with different letters are significantly different ($p < 0.05$). The error bars indicate standard deviations ($n = 3$)

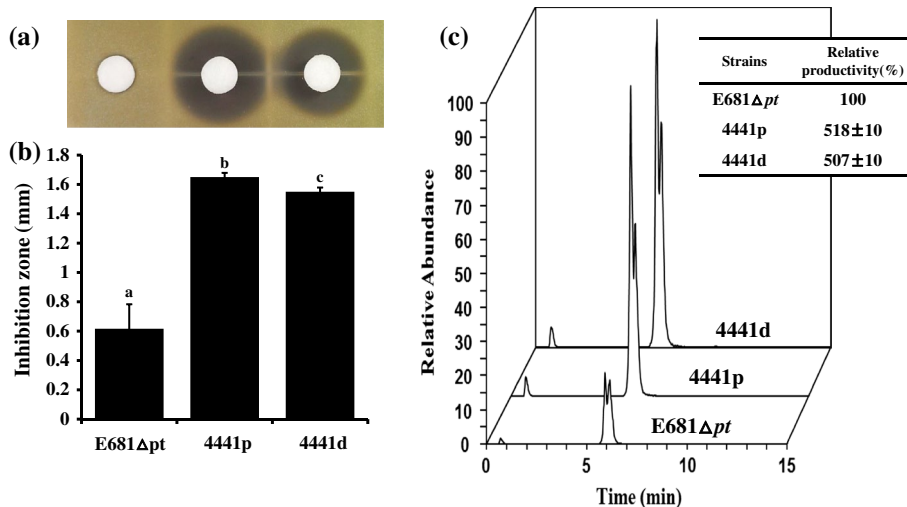


Table 3 Analysis of phosphoglucumutase activity

| Strains | PGM activity | |
|-------------------------------------|----------------------------|-------------------------------|
| | Crude extract ^a | Purified protein ^b |
| <i>P. polymyxa</i> E681 Δpt | 30.0 \pm 1.6 | 13.4 \pm 0.2 |
| <i>P. polymyxa</i> 4441p | 0.7 \pm 0.3 | ND ^c |
| <i>P. polymyxa</i> 4441d | 0.4 \pm 0.3 | ND |

^a PGM activity is expressed in arbitrary units ($(\Delta A_{340}/OD_{600}) \times 100$) \pm SEM

^b Units/mg protein

^c Not determined

accession number DQ234767.1) [5, 22, 24]. To confirm the function of the PPE04441 gene, PPE04441 protein purified from *E. coli* (pET4441) was analyzed for its PGM activity by measuring the NADPH generated during the coupling enzyme reaction [38]. As shown in Table 3, the PPE04441 protein of E681 Δpt had specific PGM activity of 13.4 units/mg. *P. polymyxa* E681 Δpt and the two mutant strains, 4441p and 4441d, were also analyzed for their PGM activity. A crude extract of E681 Δpt , which has an intact PPE04441 gene, had PGM activity of 30.0 AU, whereas the PGM activity of the two mutant strains was nearly abolished. These results show that PPE04441 is the only gene encoding PGM, which was supported by our finding that there is no other ORF showing significant identity to known PGMs in the genome of *P. polymyxa* E681.

PGM catalyzes the interconversion of α -glucose 1-phosphate and glucose 6-phosphate, and plays an important role in synthesizing uridine diphosphate glucose, which is a precursor for the synthesis of cell envelope components, capsules, and exopolysaccharides [1, 14, 22, 24]. Many reports have shown that PGM is required for the biosynthesis of biofilm, capsular polysaccharide, and exopolysaccharides in *Bacillus*, *Lactococcus*, *Streptococcus*, and

other bacterial species [14, 24, 26, 36]. In this study, we observed that cultures of the two mutant strains, 4441p and 4441d, had significantly lower viscosity than that of E681 Δpt when the strains were grown in TSBG medium (Fig. S1), which suggests that EPS production was reduced by null mutation of *pgm*. Reduced culture viscosity caused by decreased EPS production is itself a desirable feature for a bacterial culture, aside from any effect on antibiotic production, because it enables higher oxygen transfer rates and low-cost agitation in industrial fermentations.

In other species, it has been reported either that *pgm* is an essential gene, or that *pgm* mutation reduces growth, reduces viability in the stationary phase, and increases autolytic activity [5, 24, 26]. However, *pgm* null mutations in *P. polymyxa* E681 produced no such traits. There was no reduced growth in 4441p and 4441d strains when compared with the parent strain, E681 Δpt , in the logarithmic growth phase, and more interestingly, in stationary phase, the two mutant strains showed higher levels of growth and cell viability and lower autolysis than the parent strain. The OD₆₀₀ values of *pgm* mutants increased continuously in TSBG medium for 24 h, but the OD₆₀₀ of the parent strain decreased after 16 h (Fig. S2). We observed that fusaricidin production in the mutant strains was initiated in the early stationary growth phase and lasts for 12 h (data not shown); therefore, the higher levels of growth and cell viability of the mutant strains in stationary phase probably played a role in increasing productivity of fusaricidin.

We also found that the expression of the fusaricidin synthetase gene *fusA* was significantly higher in the *pgm* mutant strains. When the transcript level of *fusA* was analyzed by quantitative RT-PCR of a 10-h culture, the mutants showed about fourfold higher levels than the parent strain (Fig. S3). Although further studies are required to elucidate the mechanism involved in the regulation of gene

expression, the increased transcript level of *fusA* might be related to fusaricidin overproduction.

Conclusions

Random mutagenesis conducted in combination with targeted mutagenesis to disrupt two unnecessary NRPS genes, *pmxE* and *trdA*, was successful to obtain fusaricidin-overproducing *P. polymyxa* strains, N1U7 and N17U7. Through functional analysis of four mutations shared by the two mutant strains, a mutation in *pgm* which plays an important role in fusaricidin overproduction could be easily detected. A null mutation in *pgm* led to 5.2-fold higher fusaricidin production than the parent strain and the mutation seems to be a major factor contributing to fusaricidin overproduction in the mutant strains. The increased growth and cell viability in stationary phase, reduced viscosity of the culture, and increased transcript level of *fusA* in the *pgm* mutant strains might be related to fusaricidin overproduction. This is the first study to address the relationship between α -PGM and fusaricidin production, which should be useful for developing strains for industrial purposes.

Acknowledgments This study was supported by the New Industry R&D program funded by the Ministry of Trade, Industry, and Energy, and the KRIBB Research Initiative Program, Ministry of Science, ICT and Future Planning, Republic of Korea.

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

References

- Arbeit RD, Maki D, Tally FP, Campanaro E, Eisenstein BI (2004) The safety and efficacy of daptomycin for the treatment of complicated skin and skin-structure infections. *Clin Infect Dis* 38:1673–1681
- Beatty PH, Jensen SE (2002) *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agent of blackleg disease of canola. *Can J Microbiol* 48:159–169
- Bionda N, Cudic P (2011) Cyclic lipodepsipeptides in novel antimicrobial drug discovery. *Croat Chem Acta* 84:315–329
- Bionda N, Pitteloud JP, Cudic P (2013) Solid-phase synthesis of fusaricidin/li-f class of cyclic lipopeptides: guanidinylation of resin-bound peptidyl amines. *Biopolymers* 100:160–166
- Bizzini A, Majcherczyk P, Beggah-Moller S, Soldo B, Entenza JM, Gaillard M, Moreillon P, Lazarevic V (2007) Effects of alpha-phosphoglucomutase deficiency on cell wall properties and fitness in *Streptococcus gordonii*. *Microbiology* 153:490–498
- Choi S-K, Park S-Y, Kim R, Kim S-B, Lee C-H, Kim JF, Park S-H (2009) Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *J Bacteriol* 191:3350–3358
- Choi S-K, Park S-Y, Kim R, Lee C-H, Kim JF, Park S-H (2008) Identification and functional analysis of the fusaricidin biosynthetic gene of *Paenibacillus polymyxa* E681. *Biochem Biophys Res Commun* 365:89–95
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645
- Deng Y, Lu Z, Bi H, Lu F, Zhang C, Bie X (2011) Isolation and characterization of peptide antibiotics LI-F04 and polymyxin B6 produced by *Paenibacillus polymyxa* strain JSa-9. *Peptides* 32:1917–1923
- Farver DK, Hedge DD, Lee SC (2005) Ramoplanin: a lipoglycopeptide antibiotic. *Ann Pharmacother* 39:863–868
- Fulco P, Wenzel RP (2006) Ramoplanin: a topical lipoglycopeptide antibacterial agent. *Expert Rev Anti Infect Ther* 4:939–945
- Guerout-Fleury AM, Frandsen N, Stragier P (1996) Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* 180:57–61
- Haima P, van Sinderen D, Bron S, Venema G (1990) An improved beta-galactosidase alpha-complementation system for molecular cloning in *Bacillus subtilis*. *Gene* 93:41–47
- Hardy GG, Caimano MJ, Yother J (2000) Capsule biosynthesis and basic metabolism in *Streptococcus pneumoniae* are linked through the cellular phosphoglucomutase. *J Bacteriol* 182:1854–1863
- Kajimura Y, Kaneda M (1996) Fusaricidin A, a new depsipeptide antibiotic produced by *Bacillus polymyxa* KT-8. Taxonomy, fermentation, isolation, structure elucidation and biological activity. *J Antibiot* 49:129–135
- Kajimura Y, Kaneda M (1997) Fusaricidins B, C and D, new depsipeptide antibiotics produced by *Bacillus polymyxa* KT-8: isolation, structure elucidation and biological activity. *J Antibiot* 50:220–228
- Kaneda M, Kajimura Y (2002) New antifungal antibiotics, bacillopeptins and fusaricidins. *Yakugaku Zasshi* 122:651–671
- Kim JF, Jeong H, Park S-Y, Kim S-B, Park YK, Choi S-K, Ryu C-M, Hur C-G, Ghim S-Y, Oh TK, Kim JJ, Park CS, Park S-H (2010) Genome sequence of the polymyxin-producing plant-probiotic rhizobacterium *Paenibacillus polymyxa* E681. *J Bacteriol* 192:6103–6104
- Kuroda J, Fukai T, Konishi M, Uno J, Kurusu K, Nomura T (2000) LI-F antibiotics, a family of antifungal cyclic depsipeptides produced by *Bacillus polymyxa* L-1129. *Heterocycles* 53:1533–1549
- Kuroda J, Fukai T, Nomura T (2001) Collision-induced dissociation of ring-opened cyclic depsipeptides with a guanidino group by electrospray ionization/ion trap mass spectrometry. *J Mass Spectrom JMS* 36:30–37
- Kurusu K, Ohba K, Arai T, Fukushima K (1987) New peptide antibiotics LI-F03, F04, F05, F07, and F08, produced by *Bacillus polymyxa*. I. Isolation and characterization. *J Antibiot* 40:1506–1514
- Lazarevic V, Soldo B, Medico N, Pooley H, Bron S, Karamata D (2005) *Bacillus subtilis* alpha-phosphoglucomutase is required for normal cell morphology and biofilm formation. *Appl Environ Microbiol* 71:39–45
- Lee SH, Cho YE, Park S-H, Balaraju K, Park JW, Lee SW, Park K (2013) An antibiotic fusaricidin: a cyclic depsipeptide from *Paenibacillus polymyxa* E681 induces systemic resistance against *Phytophthora* blight of red-pepper. *Phytoparasitica* 41:49–58
- Levander F, Radstrom P (2001) Requirement for phosphoglucomutase in exopolysaccharide biosynthesis in glucose- and lactose-utilizing *Streptococcus thermophilus*. *Appl Environ Microbiol* 67:2734–2738
- Li S, Zhang R, Wang Y, Zhang N, Shao J, Qiu M, Shen B, Yin X, Shen Q (2013) Promoter analysis and transcription regulation of fus gene cluster responsible for fusaricidin synthesis of

- Paenibacillus polymyxa* SQR-21. Appl Microbiol Biotechnol 97:9479–9489
26. Neves AR, Pool WA, Castro R, Mingote A, Santos F, Kok J, Kuipers OP, Santos H (2006) The alpha-phosphoglucomutase of *Lactococcus lactis* is unrelated to the alpha-D-phosphohexomutase superfamily and is encoded by the essential gene *pgmH*. J Biol Chem 281:36864–36873
 27. Nguyen HD, Nguyen QA, Ferreira RC, Ferreira LC, Tran LT, Schumann W (2005) Construction of plasmid-based expression vectors for *Bacillus subtilis* exhibiting full structural stability. Plasmid 54:241–248
 28. Park S-Y, Park S-H, Choi S-K (2012) Characterization of sporulation histidine kinases of *Paenibacillus polymyxa*. Res Microbiol 163:272–278
 29. Quinn GA, Maloy AP, McClean S, Carney B, Slater JW (2012) Lipopeptide biosurfactants from *Paenibacillus polymyxa* inhibit single and mixed species biofilms. Biofouling 28:1151–1166
 30. Raza W, Wu H, Shen Q (2010) Use of response surface methodology to evaluate the effect of metal ions (Ca^{2+} , Ni^{2+} , Mn^{2+} , Cu^{2+}) on production of antifungal compounds by *Paenibacillus polymyxa*. Bioresour Technol 101:1904–1912
 31. Raza W, Yang X, Wu H, Huang Q, Xu Y, Shen Q (2010) Evaluation of metal ions (Zn^{2+} , Fe^{3+} and Mg^{2+}) effect on the production of fusaricidin-type antifungal compounds by *Paenibacillus polymyxa* SQR-21. Bioresour Technol 101:9264–9271
 32. Shoji J, Hino H, Sakazaki R, Kato T, Wakisaka Y, Mayama M, Matsuura S, Miwa H (1978) Isolation of tridecaptins A, B and C (studies on antibiotics from the genus *Bacillus*. XXIII). J Antibiot (Tokyo) 31:646–651
 33. Sieber SA, Marahiel MA (2003) Learning from nature's drug factories: nonribosomal synthesis of macrocyclic peptides. J Bacteriol 185:7036–7043
 34. Stawikowski M, Cudic P (2007) Depsipeptide synthesis. Methods Mol Biol 386:321–339
 35. Steenbergen JN, Alder J, Thorne GM, Tally FP (2005) Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J Antimicrob Chemother 55:283–288
 36. Torino MI, Mozzi F, Font de Valdez G (2005) Exopolysaccharide biosynthesis by *Lactobacillus helveticus* ATCC 15807. Appl Microbiol Biotechnol 68:259–265
 37. Woodford N (2003) Novel agents for the treatment of resistant Gram-positive infections. Expert Opin Investig Drugs 12:117–137
 38. Ye RW, Zielinski NA, Chakrabarty AM (1994) Purification and characterization of phosphomannomutase/phosphoglucomutase from *Pseudomonas aeruginosa* involved in biosynthesis of both alginate and lipopolysaccharide. J Bacteriol 176:4851–4857