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Biosynthesis of Polymyxins B, E, and P Using Genetically Engineered Polymyxin Synthetases in the Surrogate Host *Bacillus subtilis*

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The development of diverse polymyxin derivatives is needed to solve the toxicity and resistance problems of polymyxins. However, no platform has generated polymyxin derivatives by genetically engineering a polymyxin synthetase, which is a nonribosomal peptide synthetase. In this study, we present a two-step approach for the construction of engineered polymyxin synthetases by substituting the adenylation (A) domains of polymyxin A synthetase, which is encoded by the *pmxABCDE* gene cluster of *Paenibacillus polymyxa* E681. First, the seventh L-threonine-specific A-domain region in *pmxA* was substituted with the L-leucine-specific A-domain region obtained from *P. polymyxa* ATCC21830 to make polymyxin E synthetase, and then the sixth D-leucine-specific A-domain region ($A_{6\text{-D-Leu}}$-domain) was substituted with the D-phenylalanine-specific A-domain region ($A_{6\text{-D-Phe}}$-domain) obtained from *P. polymyxa* F4 to make polymyxin B synthetase. This step was performed in *Escherichia coli* on a *pmxA*-containing fosmid, using the lambda Red recombination system and the *sacB* gene as a counter-selectable marker. Next, the modified *pmxA* gene was fused to *pmxBCDE* on the chromosome of *Bacillus subtilis* BSK4dA, and the resulting recombinant strains BSK4-PB and BSK4-PE were confirmed to produce polymyxins B and E, respectively. We also succeeded in constructing the *B. subtilis* BSK4-PP strain, which produces polymyxin P, by singly substituting the $A_{6\text{-D-Leu}}$-domain with the $A_{6\text{-D-Phe}}$-domain. This is the first report in which polymyxin derivatives were generated by genetically engineering polymyxin synthetases. The two recombinant *B. subtilis* strains will be useful for improving the commercial production of polymyxins B and E, and they will facilitate the generation of novel polymyxin derivatives.

Keywords: Polymyxin synthetase, A-domain engineering, polymyxin B, polymyxin E, heterologous expression, *Bacillus subtilis*

Introduction

Polymyxin is an old class of cyclic lipopeptide antibiotics that was discovered in 1947, which has excellent bactericidal activity against many gram-negative bacteria because of its ability to disrupt the cell membrane [5]. Among a number of different polymyxins that have been discovered, polymyxins B and E (also known as colistin) have been used clinically since the late 1950s, but were largely abandoned in the 1970s because of their toxicities, especially their nephrotoxicity [13, 16]. However, despite their toxicities, the emergence of extremely multidrug-resistant gram-negative bacteria has forced clinicians to re-instate polymyxins as the last-line therapy for gram-negative infections [12, 33]. Therefore, the development of less toxic polymyxin derivatives would be highly welcome. In the past few decades, total or semisynthesis, as well as modifications, of polymyxins were performed chemically or enzymatically to generate novel derivatives, and the resulting products were effectively used for structure-function studies that sought clues to reduce the toxicity of polymyxins [1, 9, 19, 25, 30, 31]. However, there are limitations to obtaining diverse derivatives using chemical or enzymatic approaches, and these limitations are related

to the structural complexity of polymyxins and the low efficiencies of the approaches. Therefore, the development of a platform that provides tools for the generation of polymyxin derivatives *via* the genetic engineering of polymyxin synthetase is greatly needed. Additionally, the molecular tools for the genetic manipulation of *Paenibacillus polymyxa* strains that produce polymyxins are very poor; thus, a surrogate host that is equipped with genetic tools and other characteristics, which are conducive to the introduction and expression of foreign genes while improving product safety, is also required. It is well known that *Bacillus subtilis* is a generally recognized as safe (GRAS) organism that can fulfill safety requirements, such as ensuring the absence of endotoxins. Well-developed genetic tools, such as transformation *via* natural competence and the ability to secrete peptides into culture media, also make *B. subtilis* a promising surrogate host for the expression of foreign nonribosomal peptide synthetase (NRPS) genes [3, 4, 34]. It can also potentially help improve polymyxin production and generate various derivatives through genome engineering.

Many pharmacologically important peptide antibiotics with modular structures, including polymyxins, are produced by NRPSs. Each module of an NRPS can be divided into different domains, such as the adenylation (A), thiolation (T; also referred to as the peptidyl carrier protein), condensation (C), epimerization (E), and termination (TE) domains. The A-domain plays a role in the selection and activation of amino acid monomers [17, 26]. Over the last two decades, many researchers have tried to obtain derivatives of peptide antibiotics by genetically modifying the modules or domains of NRPSs, especially through A-domain swapping. Stachelhaus *et al*. [27] and Schneider *et al*. [23] presented a general method for the targeted replacement of A-domains in the *srfA* operon, and they successfully modified the amino acid sequence of surfactin. Miao *et al*. [15] developed an NRPS *trans*-complementation system consisting of the substitution of NRPS subunits to generate novel peptide antibiotics. They generated various daptomycin derivatives (hybrid lipopeptides) by introducing expression plasmids carrying *dptD* or its substituents from other lipopeptide biosynthetic genes into a daptomycin-nonproducing *Streptomyces roseosporus dptD* deletion strain. However, polymyxin derivatives have not yet been constructed by genetically modifying a polymyxin synthetase.

Polymyxins are cyclic heptapeptides with a tripeptide side chain that is acylated by a fatty acid at its amino terminus, and they are produced by NRPSs consisting of 10 modules [10, 14, 28]. We previously identified the polymyxin A synthetase gene cluster (*pmxABCDE*) by whole genome sequencing of *P. polymyxa* E681, and succeeded in polymyxin A production by heterologous expression of the *pmx* gene cluster in a surrogate *B. subtilis* host [2, 8]. Recently, we identified two other *pmx* gene clusters encoding the polymyxin B and polymyxin E synthetases from *P. polymyxa* F4 and *P. polymyxa* ATCC21830, respectively, by genome sequencing [21]. The two *pmx* gene clusters were shown to have the same organization as that of the E681 strain (Fig. 1A). The *pmx* gene clusters responsible for the biosynthesis of polymyxins B and E have also been reported by Shaheen *et al*. [24] and Tambadou *et al*. [29], respectively.

In this study, we constructed recombinant *B. subtilis* strains that can produce commercially important polymyxin B and polymyxin E, respectively, by replacing the A-domain regions of the polymyxin A synthetase gene *via* a two-step approach consisting of targeted modification of the A-domain using a fosmid clone as a template in *Escherichia coli*, followed by integration and expression of the modified *pmx* genes in a surrogate *B. subtilis* host. In our previous study, the recombinant *B. subtilis* strain BSK4, which can produce polymyxin A without extracellular addition of L-2,4-diaminobutyric acid, was constructed by introducing the *ectB* gene of *P. polymyxa* [22], and the *B. subtilis* BSK4dA strain derived from strain BSK4 by deleting *pmxA* was used as a host in this study. This work demonstrates that the two-step approach used to engineer polymyxin A synthetase to construct polymyxin B and E synthetases works well and may facilitate the development of novel derivatives of polymyxins.

Materials and Methods

Bacterial Strains, Plasmids, Primers, and Culture Conditions

The bacterial strains, plasmids, and PCR primers used in this study are described in Tables 1 and 2. *E. coli* DH5α was used as a cloning host for recombinant plasmids and as a test strain for assaying the antibacterial activity of polymyxins. *E. coli* EcNRK, a derivative of the EcNR2 strain, was constructed as described below and used as a cloning host for fosmids. The EcNR2 constructed in George Church's laboratory (Harvard Medical School, MA, USA) was kindly provided by Professor Duhee Bang (Yonsei University, Seoul, Korea) [32]. *B. subtilis* strains were grown in LB broth or LB agar medium at 37°C for general purposes, and in Cal18 broth with shaking at 37°C to analyze polymyxin production [7]. Spectinomycin (100 μg/ml), chloramphenicol (5 μg/ml for *B. subtilis*, 20 μg/ml for *E. coli*), erythromycin (1 μg/ml for *B. subtilis*, 100 μg/ml for *E. coli*), kanamycin (40 μg/ml), and ampicillin (100 μg/ml) were used when required. For positive selection of transformants using the counter-selectable marker *sacB*, sucrose (5% final concentration) was added to the LB medium.

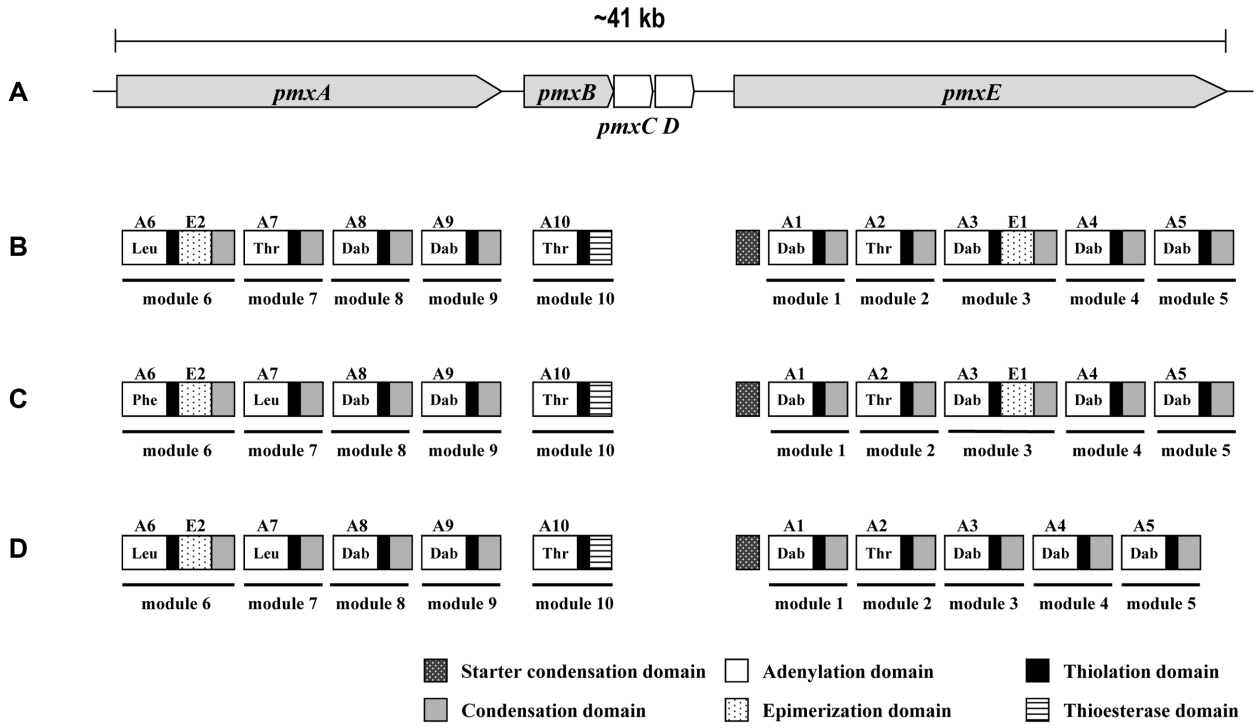

Fig. 1. The organization of the *pmx* gene clusters for the biosynthesis of polymyxins A, B, and E in *P. polymyxa* E681, F4, and ATCC21830, respectively (**A**), and the module and domain arrangements of the NRPSs for the biosynthesis of polymyxin A (**B**), polymyxin B (**C**), and polymyxin E (**D**).

Construction of the *E. coli* EcNRK Strain

To construct the *E. coli* EcNRK strain, the chloramphenicol resistance gene (*cat*) integrated at the *mutS* gene of strain EcNR2 was replaced with a kanamycin resistance (*km*) gene using the lambda Red recombination system. The *km* gene was obtained from pKD4 by PCR with the P1-muts and P2-muts primer set bearing 45 bp homologous arms that bind to the flanking regions of the *cat* gene of EcNR2, and it was introduced into the *mutS* locus of the EcNR2 strain by double-crossover recombination.

Construction of the Fosmid p8H3-*em*

To construct p8H3-*em* from fosmid p8H3, which contains the *pmxABC* genes and a truncated *pmxD* gene of *P. polymyxa* strain E681, the erythromycin resistance (*em*) gene was amplified from pDG1664 with the pmxAup-emF and pmxAup-emR primers bearing 50 bp arms that bind to the upstream region of the *pmxA* of p8H3. The PCR fragment containing the *em* gene (1.1 kb) was inserted upstream of *pmxA* by double-crossover recombination.

Construction of the *B. subtilis* BSK4dA Strain

To delete the *pmxA* region from the *pmx* gene cluster of the *B. subtilis* BSK4 strain, the *cat* gene was amplified from pDG1662 by PCR with primers cmF and cmR, and it was used to replace the *pmxA* region. The 5′- and 3′-flanking regions of *pmxA* (1.0 and 1.9 kb, respectively) were amplified from the chromosomal DNA of *P. polymyxa* E681 by PCR using primer sets pmxdFf2 and pmxdFr2, and pmxA3dF2 and pmxB5dR2, respectively. These two PCR fragments contain sequences that allow them to assemble with a PCR fragment containing the *cat* gene. In turn, these three PCR fragments were joined by fusion PCR and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) to construct pGT-dA, which was subsequently introduced into the *pmxA* locus of strain BSK4 by homologous recombination to construct strain BSK4dA (Fig. 2). Transformation of *B. subtilis* was conducted as described by Harwood and Cutting [6]

Construction of the *sp-sacB* Cassette

The spectinomycin resistance gene (*sp*) was amplified using primers spF and spR from pDG1730, and the *sacB* gene was amplified with primers sacF and sacR from *B. subtilis* genomic DNA. To assemble the two PCR fragments, primer sacF was designed to have a 21 bp overlap at the junction regions. The two PCR fragments were joined by fusion PCR with primers spF and sacR. The constructed *sp-sacB* cassette was amplified with primers A6spF and A6sacR such that it had 50 bp homologous arms that bind to each end of the gene encoding the sixth A-domain region, and the resulting PCR fragment was termed the A_6sp-sacB cassette. The *sp-sacB* cassette was also amplified with primers A7spF and A7sacR such that it had 50 bp homologous arms that bind to each end of the gene encoding the seventh A-domain region.

Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics/genotype	Reference
Strains		
Bacillus subtilis		
BSK4	Strain containing the entire polymyxin A synthetase gene cluster on the *amyE* locus, functional *sfp* gene, *srfC*::*etcB-tet*, *sp*	[22]
BSK4dA	BSK4 Δ*pmxA*	This study
BSK4-PE	Recombinant strain producing polymyxin E	This study
BSK4-PB	Recombinant strain producing polymyxin B	This study
BSK4-PP	Recombinant strain producing polymyxin P	This study
Escherichia coli		
DH5α	F′ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk^-, mk^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Enzynomics
EcNR2	MG1655 with λ-prophage::*bioA/bioB*, *mutS*$^-$, *amp*, *cm* Strain for DNA manipulation using λ-Red recombination system	[32]
EcNRK	Derivative of EcNR2, *cat* was replaced by the *km*	This study
Paenibacillus polymyxa		
E681	Wild type, produces polymyxin A	[8]
ATCC21830	Produces polymyxin E	[11]
F4	Wild type, produces polymyxin B	[21]
Plasmids/fosmids		
pGEM-T Easy	Plasmid for cloning PCR products, *amp*	Promega
pGT-dA	pGEM-T Easy containing *cat* cassette with *pmxA* flanking regions	This study
p8H3	Fosmid containing the polymyxin A synthetase gene cluster with a deletion of *pmxDE*	This study
p8H3-*em*	*em* was integrated upstream of *pmxA* in p8H3	This study
p8H3-SS_7	The $A_{7\text{-L-Thr}}$-domain gene was replaced by the *sp-sacB* cassette in p8H3-*em*	This study
p8H3-A_7Leu	*sp-sacB* was replaced by the $A_{7\text{-L-Leu}}$-domain gene in p8H3-SS_7	This study
p8H3-A_7Leu-SS_6	The $A_{6\text{-D-Leu}}$-domain gene was replaced by the *sp-sacB cassette* in p8H3-A_7Leu	This study
p8H3-A_6Phe-A_7Leu	*sp-sacB* was replaced by the $A_{6\text{-D-Phe}}$-domain gene in p8H3-A_7Leu-SS_6	This study
p8H3-A_6Phe	The $A_{6\text{-D-Leu}}$-domain gene was replaced by the $A_{6\text{-D-Phe}}$-domain gene in p8H3-*em*	This study

The resulting PCR fragment was termed the A_7sp-sacB cassette.

Screening for Polymyxin-Producing *B. subtilis* Transformants

E. coli DH5α cells that were grown overnight in 3 ml of LB at 37°C were mixed with 300 ml of LB agar medium, autoclaved, and cooled to <50°C to prepare the bioassay plate (EC plate). To test the antimicrobial activity of the *B. subtilis* BSK4dA transformants, the colonies were transferred onto an EC plate using toothpicks, and the plate was incubated at 37°C for 15 h. Transformants showing growth inhibition zones were selected for further analysis.

Antibacterial Activity Assay and Electrospray Ionization–Liquid Chromatography Mass Spectrometry (ESI-LC/MS) Analysis

Antibacterial activity against *E. coli* DH5α was assayed as follows. Recombinant *B. subtilis* strains were grown in Cal18 medium at 37°C with vigorous shaking (220 rpm) for 24 h [22]. Following centrifugation at 15,000 ×*g* for 15 min at 4°C, the supernatant was collected, and a 10 μl aliquot of each supernatant was dropped onto 6 mm paper disks, dried, and placed on an EC plate to observe the growth inhibition zone. The polymyxin in the culture supernatant was analyzed using ESI-LC/MS (Thermo Fisher Scientific, Waltham, MA, USA), as previously described by Park *et al.* [22].

Results and Discussion

As described above, we previously identified the polymyxin A synthetase gene cluster (*pmxABCDE*) of *P. polymyxa* E681. The polymyxin A synthetase consists of 10 modules that are encoded by three genes, *pmxA*, *pmxB*, and *pmxE*. Based on the structure of polymyxin, the order of the modules for amino acid assembly during polymyxin synthesis might be PmxE-PmxA-PmxB [2]. Recently, we

Table 2. Primers used in this study.

Primers	Oligonucleotide sequences (5′-3′)	References
P1-muts	ATA AAA ACC ATC ACA CCC CAT TTA ATA TCA GGG AAC CGG ACA TAAGTGTAGGCT GGAGCTGCTTC	This study
P2-muts	TTA ATA TTC CCG ATA GCA AAA GAC TAT CGG GAA TTG TTA TTA CACATGGGAATTAGC CATGGTCC	This study
cmF	TGTTTGACAGCTTATCATCG	This study
cmR	CCACGCCGAAACAAGCGCTC	This study
pmxdFf2	TCTAGCAGAGGCGCAGTGAAG	This study
pmxdFr2	CGATGATAAGCTGTCAAACAATGGCATGAACGTGACAGCC	This study
pmxA3dF2	GAGCGCTTGTTTCGGCGTGGGGATGGAGCGGATCAGCTTTAC	This study
pmxB5dR2	TCCAGATGGAGTGGCTCCACC	This study
pmxAup-emF	AGGCAGCATGGAATTCACCGCCAATCCAAGGGCTGTCACGTTCAT GCCATGAAGCAAACTTAA GAGTGTG	This study
pmxAup-emR	GACGATTAGATGTGACGATGGTGCTGCATGAGGCTCAAAGAATGC TCCGTTCCTTGGAAGCTG TCAGTAG	This study
sacF	GATGGATGAGCGATGATGACCCATCACATATACCTGCCG	This study
sacR	GATATGCCGCCCGGTAGTC	This study
spF	GAATGGCGATTTTCGTTCGTG	This study
spR	TCATCATCGCTCATCCATGTC	This study
sacredF	GTGTTCGATAAAGACAGCATC	This study
sacredR	GTCAATCGATCCTTCCAGCAG	This study
PA6F2	ACTCCGAGTTCATGACTCGG	This study
PE253	ATTACGATCGTCTCCTGCAC	This study
A7spF	CTCGTCCATCTGCTGGAGCAGGTGACGGATAACCCGGAAATTACA GTGAATGGCGATTTTCGT TCGTG	This study
A7sacR	AGCTGATTCAGGATAAACAGCCGCTTCTGAGCTGAGGAAAGCGGA TAATATGATATGCCGCCC GGTAGTC	This study
A6spF	CGTGGGGCTTCACGAGTTGGAGCTGGACATCGTGCGGGCGGACAT GCTGTGAATGGC GATTTTCGTTCGTG	This study
A6sacR	TCAATCTCGCCGATCTCGATCCGGAATCCACGGATTTTCACCTGATGG TCGATATGCCGCCCG GTAGT C	This study
A7F	AGCACGGCGACCTGACGATC	This study
A7R	GTCGAAAGGCCGGATGAAGG	This study
A6F	AAGGAGCTGCATCTGGACG	This study
A6R	GCGAAGATACTTGAATGG	This study

The underlined sequences indicate the targeted regions for lambda Red recombination.

identified two more *pmx* gene clusters encoding the polymyxin B and polymyxin E synthetases by genome sequencing of *P. polymyxa* F4 and *P. polymyxa* ATCC21830, respectively [21]. As shown in Figs. 1B–1D, the domain structures of the three polymyxin synthetases have a very similar modular organization. Only one difference was found between the 10 A-domains of the polymyxin A and polymyxin E synthetases. Specifically, the A-domain of the seventh position of the former is an L-threonine-specific domain ($A_{7\text{-L-Thr}}$-domain), whereas that of the latter is an L-leucine-specific domain ($A_{7\text{-L-Leu}}$-domain). There were two differences in the sixth and seventh A-domains of polymyxin B synthetase; that is, the D-phenylalanine-specific domain ($A_{6\text{-D-Phe}}$-domain) and $A_{7\text{-L-Leu}}$-domain, compared with the D-leucine-specific domain ($A_{6\text{-D-Leu}}$-domain) and $A_{7\text{-L-Thr}}$-domain of polymyxin A synthetase. These results suggest that polymyxin A synthetase can be genetically engineered to produce polymyxin B or polymyxin

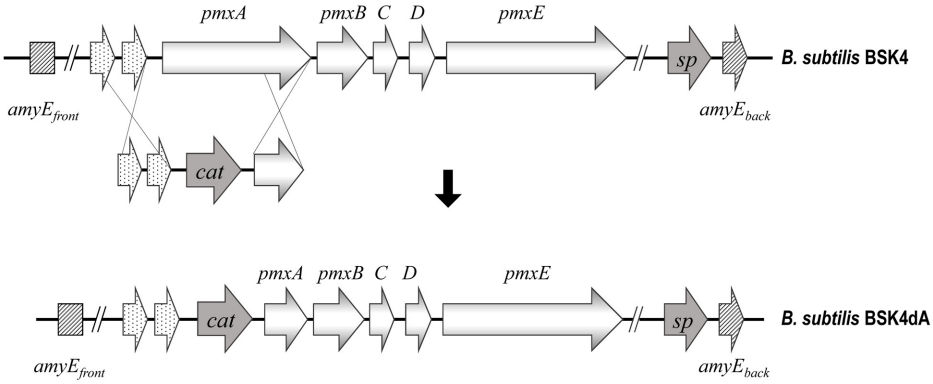

Fig. 2. Schematic diagram showing the strategy for the construction of *B. subtilis* BSK4dA.
The BSK4dA strain was constructed by replacing the *pmxA* region of the *pmx* gene cluster on the BSK4 chromosome with the *cat* gene by homologous recombination.

E by replacing the A_6- and A_7- or A_7-domain regions of the *pmxA* gene, respectively.

Here, we describe experiments in which two recombinant *B. subtilis* strains that produced polymyxin B or polymyxin E were constructed. There were two reasons for choosing this A-domain engineering approach instead of cloning the gene clusters entirely. First, we wished to develop a system for creating diverse polymyxin derivatives by A-domain engineering. Second, it is very difficult to introduce the entire polymyxin gene cluster, which spans about 41 kb, into the chromosome of *B. subtilis*. The engineering of polymyxin A synthetase to yield polymyxin B or polymyxin E synthetases was performed in two steps. In the first step, the substitution of the target adenylation domain in polymyxin A synthetase with the adenylation domain of the polymyxin B or polymyxin E synthetases was performed using a fosmid clone that contained the *pmxA* gene as template DNA in *E. coli*. In the second step, the modified *pmx* gene was introduced into a polymyxin-nonproducing *B. subtilis* Δ*pmxA* host strain.

Preparation of an *E. coli* Host Strain and Template DNA for Polymyxin A Synthetase Gene Recombineering

Although a variety of molecular tools for the genetic manipulation of *B. subtilis* are available, the low efficiency of recombination between short homologous nucleotide sequences causes some difficulties. Therefore, in the first step, recombination-mediated genetic engineering (recombineering) of the A-domain gene was performed by homologous recombination using the lambda Red recombination system in *E. coli*. The EcNRK strain was constructed by modifying the EcNR2 strain as a cloning host for fosmids carrying *pmx* genes with a *cm* marker, as described in Materials and Methods. Fosmid p8H3, which contains the *pmxABC* genes, as well a truncated *pmxD* gene, of the *P. polymyxa* E681 strain, was selected from our previous sequencing library, and it was modified into p8H3-*em* by introducing the *em* gene into the upstream region of *pmxA* so that it could serve as a selection marker for transformants in the next step, after introducing the modified *pmxA* gene into the *B. subtilis* host. p8H3-*em* was used as template DNA for the recombineering of the A-domain gene in *E. coli* EcNRK.

Preparation of a *B. subtilis* Host Strain for Polymyxin Synthetase Gene Recombineering and Expression

To efficiently construct and select the engineered polymyxin synthetase gene cluster *via* recombineering into the *B. subtilis* chromosome, the *B. subtilis* BSK4dA strain was constructed by modifying the *B. subtilis* BSK4 strain, which carries the entire *pmx* gene cluster (*pmxABCDE*). The 13.5 kb 5′ region of the *pmxA* gene (14.9 kb) of the BSK4 strain was removed, and the *cat* gene was integrated into the region as described in Materials and Methods (Fig. 2). The BSK4dA strain carrying *pmxBCDE* was used as a host for the introduction of the modified *pmxA* gene, as well as for the expression of the modified *pmx* gene cluster to produce polymyxins. When the modified *pmxA* gene is fused to the other *pmx* genes carried on the chromosome of strain BSK4dA, the entire gene cluster will be restored, and the strain will exhibit antibacterial activity against *E. coli*.

Preparation of Donor DNAs Encoding the $A_{6\text{-D-Phe}}$-Domain and $A_{7\text{-L-Leu}}$-Domain of Polymyxin Synthetase

DNA fragments encoding the $A_{6\text{-D-Phe}}$-domain (1.3 kb) and the $A_{7\text{-L-Leu}}$-domain (1.9 kb) were obtained by PCR

using the genomic DNAs of *P. polymyxa* F4 and *P. polymyxa* ATCC21830 as templates with the primer sets PA6F2-PE253 and sacredF-sacredR, respectively. The 5′- and 3′-end regions of the two PCR fragments were designed to be highly homologous to those of the sixth or seventh A-domain regions of the polymyxin A synthetase gene; that is, there were more than 95% identities in the nucleotide sequences of the 200–300 bp end regions and 100% identities in narrow regions of about 50 bp. Therefore, lambda Red-mediated homologous recombination between these regions was possible. Thus, the two DNA fragments were used to engineer the target A-domains of the polymyxin A synthetase gene.

Recombineering of the A_7-Domain Region of the Polymyxin A Synthetase Gene to Construct Polymyxin E Synthetase

To construct polymyxin E synthetase by engineering polymyxin A synthetase, the $A_{7\text{-L-Thr}}$-domain region of *P. polymyxa* E681 was substituted with the $A_{7\text{-L-Leu}}$-domain region of *P. polymyxa* ATCC21830, using the *pmxA* gene in fosmid p8H3-*em* as template DNA and *E. coli* EcNRK as a host for the recombineering reaction. This process was conducted in two steps, as shown in Fig. 3A. First, the $A_{7\text{-L-Thr}}$-domain region (1.9 kb) of the *pmxA* gene was deleted by inserting the A_7sp-sacB cassette (3.1 kb), which contains a spectinomycin resistance gene, the *sacB* gene, and 50 bp homologous arms to the $A_{7\text{-L-Thr}}$-domain region, using the lambda Red recombination system. A transformant carrying the recombinant fosmid containing the *sp-sacB* cassette was selected by its resistance to spectinomycin and sensitivity to 5% sucrose, and the recombination event was confirmed by PCR using primers A7F and A7R, which bind to each end of the A_7sp-sacB cassette. The resulting recombinant fosmid was designated as p8H3-SS_7. Second, the DNA fragment containing the $A_{7\text{-L-Leu}}$-domain region, which was obtained by PCR amplification of the chromosomal DNA of the *P. polymyxa* ATCC21830 strain, was integrated into p8H3-SS_7 by replacing the *sp-sacB* cassette *via* double-crossover recombination (Fig. 3A). The *E. coli* transformant carrying the recombinant fosmid, named p8H3-A_7Leu, was selected based on its sucrose-resistant and spectinomycin-susceptible phenotypes. The replacement of the A_7-domain region in the fosmid p8H3-A_7Leu was confirmed by PCR and nucleotide sequencing. The fosmid p8H3-A_7Leu was introduced into *B. subtilis* strain BSK4dA by homologous recombination to complete the polymyxin E synthetase gene cluster, and it was also used as a template to construct polymyxin B synthetase, as described below.

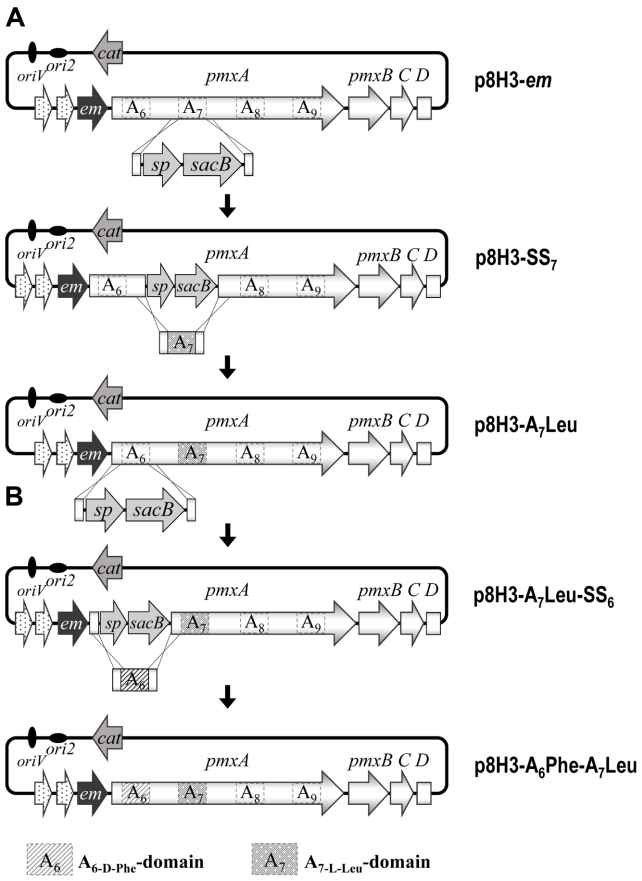

Fig. 3. Schematic diagram showing the strategy for the construction of polymyxin B and E synthetases by modifying the specificity of the A_6- and A_7- or A_7-domains of polymyxin A synthetase, respectively.

(**A**) Recombineering of the A_7-domain coding region of *pmxA* was performed in two steps; the $A_{7\text{-L-Thr}}$-domain region was replaced by the *sp-sacB* cassette, and then the *sp-sacB* cassette was replaced with a DNA fragment encoding the $A_{7\text{-L-Leu}}$-domain, using the lambda Red recombination system. (**B**) Recombineering of the $A_{6\text{-D-Leu}}$-domain coding region of *pmxA* was performed similarly to that described in (**A**) by introducing a DNA fragment encoding the $A_{6\text{-D-Phe}}$-domain.

Recombineering of the A_6-Domain Region to Construct Polymyxin B Synthetase

The construction of the polymyxin B synthetase gene was performed by substituting the $A_{6\text{-D-Leu}}$-domain region of the *pmxA* gene of *P. polymyxa* E681 in fosmid p8H3-A_7Leu with the $A_{6\text{-D-Phe}}$-domain region of *P. polymyxa* F4. This process was also conducted in two steps as described above (Fig. 3B). First, the $A_{6\text{-D-Leu}}$-domain region (about 1.3 kb) of the *pmxA* gene of the fosmid p8H3-A_7Leu was deleted by inserting the A_6sp-sacB cassette (about 3.1 kb) and confirmed

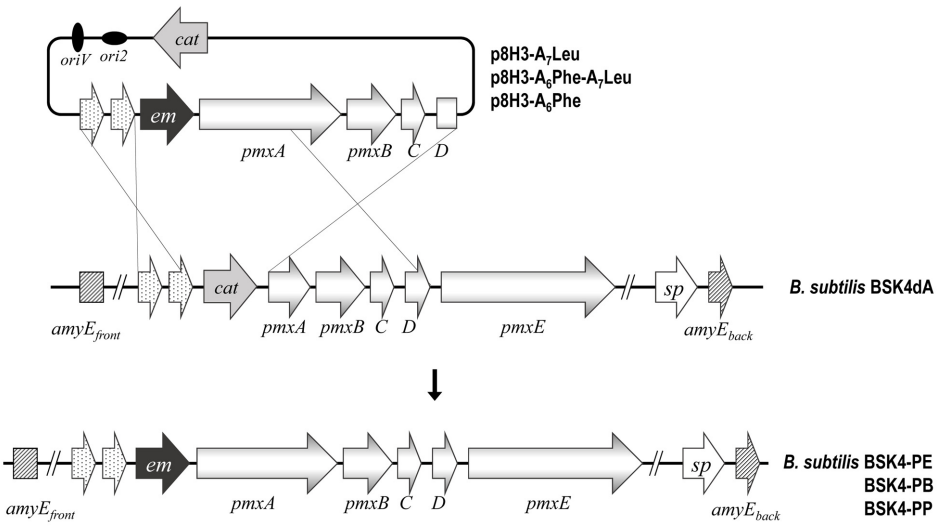

Fig. 4. Schematic diagram showing the strategy for the construction of the *B. subtilis* strains BSK4-PB, BSK4-PE, and BSK4-PP.
The three recombinant *Bacillus* strains were constructed by introducing the modified *pmxA* genes of the fosmid clones p8H3-A_7Leu, p8H3-A_6Phe-A_7Leu, and p8H3-A_6Phe, respectively, into the chromosome of BSK4dA.

by PCR using primers A6F and A6R. The resulting recombinant fosmid was designated as p8H3-A_7Leu-SS_6. Second, the DNA fragment containing the $A_{6\text{-D-Phe}}$-domain region, which was obtained from the *P. polymyxa* F4 strain by PCR, was integrated into p8H3-A_7Leu-SS_6 by replacing the *sp-sacB* cassette to construct p8H3-A_6Phe-A_7Leu (Fig. 3B). The replacement of the A_6-domain region in the fosmid was confirmed by PCR and nucleotide sequencing. The resulting fosmid, p8H3-A_6Phe-A_7Leu, was introduced into the *B. subtilis* BSK4dA strain to complete the polymyxin B synthetase gene cluster, as described below.

Completion of Polymyxin Synthetase Gene Clusters and Biosynthesis of Polymyxin B and E in *B. subtilis*

Fosmids p8H3-A_7Leu and p8H3-A_6Phe-A_7Leu containing *pmxA* genes with altered amino acid sequences in the seventh or sixth and seventh A-domains of the polymyxin A synthetase gene were introduced into *B. subtilis* BSK4dA harboring intact *pmxBCDE* genes, but lacking the *pmxA* gene (Fig. 4). As we previously reported, polymyxin synthetase is encoded by three *pmx* genes: *pmxA*, *pmxB*, and *pmxE* [2]. The deleted *pmxA* region of the BSK4dA strain will be restored by homologous recombination with the modified *pmxA* regions of the fosmids p8H3-A_7Leu or p8H3-A_6Phe-A_7Leu. After separately transforming the BSK4dA strain with the two fosmids, more than 100 transformants (Em^r Cm^s) were selected and assayed for their antibacterial activity against *E. coli*, using an EC plate, and we found that about 50% of the Em^r Cm^s transformants showed antibacterial activity in both cases. These results showed that the upstream region of the *pmx* genes on the BSK4dA chromosome, including the *cat* gene, were successfully replaced with the modified *pmxA* gene and *em* gene, thereby restoring the entire polymyxin synthetase gene cluster in both cases. Some of the *B. subtilis* transformants that were Em^r Cm^s, but which did not exhibit antibacterial activity, were analyzed by PCR and nucleotide sequencing. It was found that the recombination events occurred in non-target regions, and that some regions of *pmx* genes are missing in these transformants. This is probably because there is a somewhat high degree of homologies between domains consisting of multi-modules of polymyxin synthetase. The recombinant *B. subtilis* strains obtained by transformation with p8H3-A_7Leu and p8H3-A_6Phe-A_7Leu were named BSK4-PE and BSK4-PB, respectively. We confirmed the correct integration of the modified A-domains into the target sites by PCR and nucleotide sequencing, and the expression of the engineered polymyxin synthetase gene clusters of the two recombinant *B. subtilis* transformants was confirmed by LC/MS analysis.

The two recombinant strains, BSK4-PB and BSK4-PE, as well as BSK4, were grown in Cal18 medium, and culture supernatants were collected and assayed for antibacterial activity against *E. coli* DH5α by the disk diffusion method using an EC plate (Fig. 5A). As was expected, all three strains, BSK4, BSK4-PE, and BSK4-PB, showed antibacterial activity against *E. coli*. The culture supernatant samples were analyzed using an ESI-LC/MS system to detect

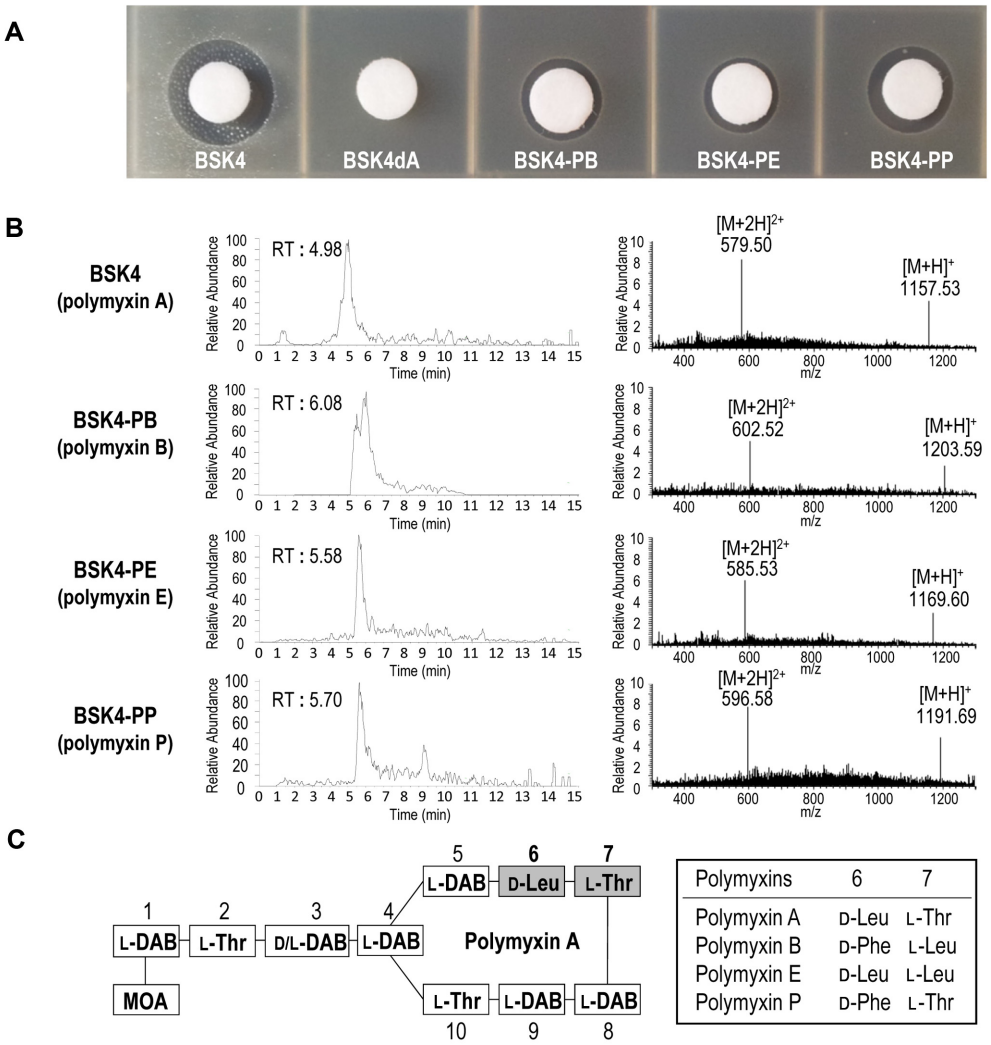

Fig. 5. Analysis of polymyxins synthesized by the recombinant *B. subtilis* strains BSK4-PB, BSK4-PE, and BSK4-PP.
(**A**) Antibacterial activities of the cell-free culture supernatants of BSK4 and the three recombinant strains against *E. coli* DH5α. (**B**) LC/MS data for polymyxins A, B, E, and P produced by *B. subtilis* strains BSK4, BSK4-PB, BSK4-PE, and BSK4-PP, respectively. (**C**) The primary structures of the polymyxins A, B, E and P.

polymyxins, and the BSK4-PB and BSK4-PE strains were found to produce polymyxin B and polymyxin E, respectively, as was expected (Fig. 5B). The $(M+H)^+$ ion peaks of BSK4-PB and BSK4-PE were detected at *m/z* 1,203.59 and *m/z* 1,169.60, respectively, which correspond to the known mass values of polymyxin B_1 (*m/z* 1,203.6) and polymyxin E_1 (*m/z* 1,169.77) [20, 29]. The mass values of the polymyxins produced by BSK4-PB and BSK4-PE were 46.06 and 12.07 greater than that of BSK4 (*m/z* 1,157.53), respectively. These differences coincide well with the differences in the molecular weights of the amino acids at the sixth and seventh or seventh positions of the modified polymyxins; the mass difference between threonine (119.12) and leucine (131.17) is 12.05, and the mass difference between threonine (119.12) and phenylalanine (165.19) is 46.07. We also succeeded in constructing the *B. subtilis* BSK4-PP strain, which can produce polymyxin P by engineering of polymyxin A synthetase. As shown in Fig. 5C there is only one difference between the primary structure of polymyxin A and that of polymyxin P; that is, the amino acid of the sixth position of the former is D-leucine, whereas that of the latter is D-phenylalanine. The construction was done by substituting the $A_{6\text{-D-Leu}}$-domain region of the polymyxin A synthetase gene from *P. polymyxa* E681 with the $A_{6\text{-D-Phe}}$-domain region from *P. polymyxa* F4 using the same strategy as described above (Fig. 4). The

culture supernatant of the BSK4-PP strain also showed antibacterial activity against *E. coli*, and it was confirmed to produce polymyxin P by ESI-LC/MS (Figs. 5A and 5B). The *m/z* (1,191.69) of the polymyxin produced by the BSK4-PP strain coincided well with the known molecular mass of polymyxin P (*m/z* 1,191.9) produced by *Paenibacillus polymyxa* M-1 [18].

To determine the polymyxin productivity of the three recombinant *B. subtilis* strains producing polymyxins B, E and P, respectively, the antibacterial activities in their culture supernatants were compared with serially diluted standard solutions of polymyxin B. The range of productivities of the strains were estimated to be 200 mg/l or lower levels (data not shown). These levels were obtained without optimizing the expression of the *pmx* gene clusters or the fermentation conditions; therefore, yield improvements should be possible by further genetic engineering of the early-stage transformants or fermentation development. To improve the environmental safety of the recombinant *B. subtilis* strains that produce polymyxins, the removal of antibiotic resistance marker genes from the strains is under process.

In our two-step approach, the target A-domain region of *P. polymyxa* E681 was substituted with a donor A-domain region from *P. polymyxa* ATCC21830 and/or *P. polymyxa* F4 on a fosmid containing the *pmxA* gene, and then the entire *pmx* gene cluster was restored *via* recombination of the modified *pmxA* gene into the chromosome of the *B. subtilis* BSK4dA strain containing *pmxBCDE*. The above-mentioned results show that this approach works well, and that we have succeeded in constructing the three recombinant *B. subtilis* strains producing polymyxins B, E and P. The former two strains produce the commercially important polymyxins B and E that will be useful for improving polymyxin production because of the advantages of using *B. subtilis* as a host. *B. subtilis* 168, the parent strain of the BSK4 strain used in this study, is a non-pathogenic strain with GRAS status, and therefore, downstream processing can be simplified. The biosynthesis of surfactin in strain BSK4 was completely blocked by knockout of the *srfC* gene during the construction of the strain in our previous study [22]. If necessary, we can also block the biosynthesis of other compounds that possibly hinder purification of polymyxins, by disruption of the relevant genes without difficulty. This will be an advantage for producing polymyxin or its derivatives in pure form. The three recombinant *B. subtilis* strains will also facilitate the generation of novel polymyxin derivatives as a platform for further engineering polymyxin synthetases. The two-step approach might be used to generate modifications in any A-domains of polymyxin synthetases by introducing various A-domain genes, with different amino acid specificities, obtained from other polymyxin synthetase genes or possibly other NRPS genes.

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