Identification of Polymyxin Synthetase Gene Cluster of *Paenibacillus polymyxa* and Heterologous Expression of the Gene in *Bacillus subtilis*

Soo-Keun Choi,¹,† Soo-Young Park,¹,† Rumi Kim,² Seong-Bin Kim,¹ Choong-Hwan Lee,³ Jihyun F. Kim,¹ and Seung-Hwan Park¹*

¹Industrial Biotechnology & Bioenergy Research Center, KRIIBB, 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea

²Institute of Hadong Green Tea, Hadong 667-805, Republic of Korea

³Division of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

†S.-K.C. and S.-Y.P. contributed equally to this study.

*Corresponding author: Tel: +82-42-860-4410

Fax: +82-42-860-4488

E-mail: shpark@kribb.re.kr

Running title: A polymyxin synthetase gene cluster
ABSTRACT

Polymyxin, a long-known peptide antibiotic, has recently been reintroduced in clinical practice because it is sometimes the only available antibiotic for the treatment of multidrug-resistant Gram-negative pathogenic bacteria. Lack of information on the biosynthetic genes of polymyxin, however, has limited the study of structure-function relationships and the development of improved polymyxins. During whole genome sequencing of *Paenibacillus polymyxa* E681, a plant growth-promoting rhizobacterium, we identified a gene cluster encoding polymyxin synthetase. Here, we report the complete sequence of the gene cluster and its function in polymyxin biosynthesis. The gene cluster spanning the 40.6-kilobase region consists of five open reading frames, designated *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE*. The *pmxC* and *pmxD* genes are similar to genes that encode transport proteins, while *pmxA*, *pmxB*, and *pmxE* encode polymyxin synthetases. The insertional disruption of *pmxE* led to a loss of the ability to produce polymyxin. Introduction of the *pmx* gene cluster into the *amyE* locus of the *Bacillus subtilis* chromosome resulted in the production of polymyxin in the presence of extracellularly added L-2,4-diaminobutyric acid. Taken together, our findings demonstrate that the *pmx* gene cluster is responsible for polymyxin biosynthesis.
INTRODUCTION

Since polymyxin was first isolated from *Bacillus polymyxa* in 1947 (1, 4, 47), at least 15 unique polymyxins have been reported (31, 49). Because of its excellent bactericidal activity against Gram-negative bacteria, polymyxin antibiotics (polymyxin B and polymyxin E) were used until early 1970 as therapies against many diseases caused by pathogenic microorganisms. However, because they carried serious side effects, including fever, skin eruption, and pain, and also induced severe nephrotoxicity and neurotoxicity (18, 37), it was rapidly replaced by other, better-tolerated antibiotics. In recent years, its application has been restricted to use as an ointment on local surface wounds.

Due to the increased and often unnecessary use of antibiotics, pathogenic microorganisms with resistance to antibiotics have become more widespread (2, 14, 30, 38). Under the limited therapeutic options available to treat multidrug-resistant Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, polymyxins are sometimes the only available active antibiotics, and have now become important therapeutic agents (13, 25, 28, 29, 55). Many recent reports have shown that patients infected with multidrug-resistant Gram-negative pathogens improved upon treatment with polymyxins (19, 27, 44, 48). In addition, polymyxins have been applied to prevent septic shock by removing
circulating endotoxin to polystyrene fibers in an immobilized form (8). Therefore, the clinical value of polymyxin, an antibiotic discovered six decades ago, is currently being reappraised. However, until now, we have had a very limited understanding of various characteristics of this agent, especially its biosynthetic genes.

To analyze structure-function relationships and to develop improved polymyxins with lowered toxicities, novel polymyxin derivatives must be generated. Recently total- or semi-synthesis or modifications of polymyxins were performed chemically or enzymatically, and the resulting products were effectively used for structure-function study (6, 20, 36, 45, 50, 52). There is a limitation to obtaining diverse derivatives using chemical or enzymatic approaches, however, and this limitation is related to the structural complexity of polymyxin. The basic structure of polymyxin is a cyclic heptapeptide with a tripeptide side-chain acylated by a fatty acid at the amino terminus (49). Normally, 6-methyloctanoic acid or 6-methylheptanoic acid is attached to the side-chain. This structure favors solubility of polymyxin in both water and organic solvent. Unlike other general ribosomally-translated peptides, polymyxin is produced by a non-ribosomal peptide synthetase (NRPS) (22, 31). NRPSs are multienzyme complexes that have modular structures (35, 46). A module is a distinct section of the multienzyme that is responsible for the incorporation of one or more specific amino acids into the final product. Each module can be
divided into different domains, each of which is responsible for a specific biochemical reaction. Three types of domains, the adenylation (A), thiolation (T; also referred to as the peptidyl carrier protein, PCP), and condensation (C) domains, are essential for nonribosomal peptide synthesis. The A-domain plays a role in the selection and activation of an amino acid monomer, the T-domain is responsible for transportation of substrates and elongation intermediates to the catalytic centers, and the C-domain catalyzes peptide bond formation. In addition to these core domains, there are the thioesterase domain (TE-domain), the epimerization domain (E-domain), and some other modification domains. Many NRPS gene clusters have been reported, but no polymyxin biosynthetic gene cluster has been reported to date.

During whole genome sequencing of *P. polymyxa* E681, a plant growth-promoting rhizobacterium, we found a gene cluster encoding polymyxin synthetase. In this study, the complete sequences of the polymyxin synthetase genes and the function of the gene cluster have been identified and analyzed by domain analysis, insertional mutagenesis, and heterologous expression of the genes, as well as by antibacterial assay and LC/MS analysis of the strains and their culture supernatants. The genome information and the heterologous expression of the polymyxin synthetase gene cluster will be useful for further studies on the regulation of *pmx* genes, their structure-function relationships, and the improvement of polymyxins.
MATERIALS AND METHODS

Strains and culture conditions

*P. polymyxa* E681 was isolated from the roots of winter barley in the Republic of Korea (41). *E. coli* DH5α and BW25113 carrying the Red recombinase of pKD46 (9) were used for cloning and λ Red recombination, respectively. *Bacillus subtilis* 168 was used as a host for heterologous expression of the *pmx* genes. *P. polymyxa* E681 was grown in TSB (Difco) for general purposes, BHI (Difco) containing 10% sucrose for transformation, and glucose-starch-CaCO$_3$ medium (GSC) (10) for analysis of polymyxin. *B. subtilis* strains were grown in LB medium for general purposes, and in GSC with or without 200 µg/ml of L-2,4-diaminobutyric acid (L-Dab, Sigma-Aldrich) for analysis of polymyxin.

LC/MS analysis

*P. polymyxa* E681 was grown in GSC medium under aerobic conditions at 30°C for 3 days, followed by centrifugation at 5000 × g for 10 min to obtain supernatant. LC/MS was performed with the supernatant using a high pressure liquid chromatography system provided by Thermo Electron Co. (USA) and an ion spectrometer. The sample was injected into a reverse-
phase column, YMC Pack Pro C18 (10 x 250 mm, 5 µm) or Terra MS C18 (2.1 x 50 mm, 3.5 µm), and was analyzed in a mixed solvent of acetonitrile and water containing 0.1% formic acid (0.2 ml/min). Analysis of metabolites from recombinant B. subtilis was conducted after solid phase extraction (SPE) using the general protocol. Bacillus cells were grown in 200 ml GSC medium with or without L-Dab (200 µg/ml) for 2 days. After the culture supernatant was extracted using the same volume of butanol, the butanol phase was evaporated and re-extracted with methanol. The final methanol extract was evaporated and dissolved in 2 ml water. After the concentrated sample was passed through a C18 column (SiliCycle Inc., Quebec, Canada), it was eluted using 3 ml of water-methanol gradient (10, 20, 40, 60, 80, and 100%). A 50-µl aliquot of each fraction was used to assay antimicrobial activity against E. coli, and the active fraction was subsequently used for LC/MS analysis.

**PCR-targeted mutagenesis**

The PCR primers used in this study are listed in Table 1. A deletion mutant of the pmxE gene was constructed using an E. coli fosmid clone. In brief, the fosmid DNA (PP12G04) harboring truncated pmxA and complete pmxB, pmxC, pmxD, and pmxE in a 38.1-kbp chromosomal DNA fragment cloned into pCC1fos (EPICENTRE Biotechnologies) was
introduced into *E. coli* BW25113 carrying the Red recombinase expression plasmid, pKD46 (9).

The chloramphenicol acetyl transferase (*cat*) gene of fosmid PP12G04 was replaced with a
tetracycline-resistance gene (*Tc*) using a λ Red recombination system to construct fosmid pPmx-
Tc. The *Tc* gene was amplified from pBC16 (5) with the Fosc-m-TCF and Fosc-m-TCR primers
bearing 70-bp side arms that bind to the flanking regions of the *cat* gene of pCC1fos. For
inactivation of the *pmxE* gene, a chloramphenicol resistance gene-kanamycin resistance gene
(*cat-kan*) cassette was introduced into the *pmxE* structural gene of pPmx-Tc using a λ Red
recombination system. The *cat-kan* cassette was constructed as follows. The *cat* gene was
amplified by PCR with primers CatF and CatR from pDG1661 (15), and was then introduced
into pGem7zf(+) (Invitrogen Inc.) with EcoRI and BamHI cleavage sites. The resulting plasmid
was digested with the NarI restriction enzyme, and was then ligated with the PCR product
containing the kanamycin resistance gene that was amplified from pKD4 (9) using the Kd4kanF
and Kd4kanR primer set. The constructed *cat-kan* cassette was amplified with primers PmxEckF
and PmxEckR, yielding 60-bp homologous arms of the target site to each of the ends. The
amplified *cat-kan* cassette was inserted into pPmx-Tc to construct the pDpmxE fosmid. To
remove the pKD46 plasmid completely, kanamycin-resistant transformants were transferred onto
fresh agar medium containing kanamycin, and were subsequently incubated at 37°C. The
disruption of *pmxE* with the *cat-kan* cassette was confirmed by PCR with primers *pmxEdelF* and *pmxEdelR*, which bind to the outer regions of the homologous arm. The pDpmxE fosmid was introduced into *P. polymyxa* E681 to generate a polymyxin-defective mutant. The mutant was also confirmed by PCR using the *pmxEdelF* and *pmxEdelR* primers. Transformation of *P. polymyxa* was performed according to a previously reported method (7).

Heterologous expression of the *pmx* gene cluster in *B. subtilis*

For the efficient transformation of the *Bacillus* host strain with large DNA fragments, the genes responsible for *BsuM* restriction and modification (RM) (16) were removed from *B. subtilis* 168 as follows. DNA fragments upstream of *ydiO* and downstream of *ydjA* were amplified by PCR with primers *ydiO-up-F* and *ydiO-up-R*, and *ydjA-down-F* and *ydjA-down-R*, respectively. The DNA fragments were inserted into the EcoRI and PstI sites of plasmid pBGSC6 (12) in tandem to construct plasmid pDBSUM. Transformation of *B. subtilis* was conducted using a previously reported method (17). After single-crossover integration of the pDBSUM plasmid into the chromosome of *B. subtilis* 168, cells were grown in LB medium without antibiotics, followed by screening for chloramphenicol-sensitive colonies. BSK1, a resultant recombinant strain with a disrupted RM system, was constructed without any marker gene.
Integration of the pmx gene cluster into the chromosome of BSK1 was conducted in two steps using fosmid clones, as shown in Fig. 3. Fosmid PP12B06 containing pmxABCD, a truncated pmxE, and a 5’-flanking region was digested with BamHI, and the DNA fragment containing pmx genes was ligated into the BamHI site of integration plasmid pDG1662 (15) to construct pDG-12B06. The pmx genes of pDG-12B06 were introduced into the amyE locus of strain BSK1 by homologous recombination to construct strain BSK2. To restore truncated pmxE, the PP12D08 fosmid containing an entire pmxE gene and a 3’-flanking region was used. A recombinant fosmid, PP12D08-sp'-amyEback, was constructed by integration of the sp’ (spectinomycin-resistant gene)-amyEback cassette amplified from plasmid pDG1730 (15) by PCR with primers 1730-12DF and 1730-12DR using a λ Red recombination system. Strain BSK3 containing the entire pmxABCD sequence was constructed by homologous recombination between PP12D08-sp'-amyEback and the chromosome of BSK2. Functional sfp was introduced into BSK1 and BSK3 by transferring the chromosomal DNA of B. subtilis CB114 (26) to construct BSK1S and BSK3S, respectively. Construction of BSK2, BSK3, and BSK3S was confirmed by PCRs with the primer sets of pmxAF/pmxAAR, pmxBF/pmxBR, pmxCF/pmxCR, pmxDF/pmxDR, and pmxEF/pmxEER. Introduction of the functional sfp was confirmed by observing reduced surface tension of the culture broth as described in a previous study (26).
Antibacterial activity assay

The antibacterial activity was analyzed using freshly prepared *E. coli* plates. *E. coli* cells grown overnight in 3 ml of LB medium at 37°C were mixed with 300 ml of LB agar, autoclaved, and cooled below 50°C to prepare the plates. When necessary, L-Dab was added at a final concentration of 200 µg/ml. To analyze the antibacterial activity of culture supernatants of *P. polymyxa* strains and their extracts, 50 µl of each sample was loaded on a paper disk and transferred to the *E. coli* plates. Recombinant *B. subtilis* cells grown overnight in 3 ml of LB medium at 37°C were inoculated directly onto the *E. coli* plates by dropping 5 µl of the culture onto plates. Each plate was then incubated at 37°C for 24 hours to observe the growth inhibition effect.

Nucleotide sequence accession number

The GenBank accession number for the polymyxin synthetase gene cluster is EU371992.
RESULTS

Domain analysis of the polymyxin synthetase

During the whole genome sequencing of *P. polymyxa* E681 that was recently completed in our laboratory (Kim *et al.*, unpublished results), an NRPS gene cluster was identified as a potential polymyxin synthetase gene cluster. The gene cluster consisted of five open reading frames, *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE* (Fig. 1A). The results of a BLAST search suggested that *pmxC* and *pmxD* may encode membrane transporters, while *pmxA*, *pmxB*, and *pmxE* encode polymyxin synthetase.

The domains of the polymyxin synthetase were analyzed based on the method of Ansari *et al.* (3). The PmA containing 4953 amino acids comprises four modules and a C domain (Fig. 1A). The substrate specificities of the four PmA A-domains were predicted to activate the amino acid substrates, Leu, Thr, Dab, and Dab, respectively (Table 2). The PmxB, an 1102 amino acid polypeptide, comprises one module containing A-T-TE domains. The predicted amino acid specificity of the A-domain of PmxB was Thr (Table 2). Due to the presence of the TE-domain, PmxB may contribute to the termination of polymyxin synthesis. The PmxE, a 6312 amino acid polypeptide, has five modules and a C domain. The substrate specificities of the five PmxE A-
domains were predicted to activate the amino acid substrates Dab, Thr, Dab, Dab, and Dab, respectively (Table 2). Based on the polymyxin structure, the order of modules for amino acid assembly during polymyxin synthesis should be PmxE-PmxA-PmxB, and the last C domains of PmxE and PmxA should become one module with the A-T-E domains of PmxA and the A-T-TE domains of PmxB, respectively. The third module of PmxE contains an E-domain, which suggests that the third amino acid, Dab, may be a D-form in polymyxin produced by the E681 strain. Taken together, these findings suggest that the polymyxin synthetase of *P. polymyxa* E681 may synthesize polymyxin A (Fig. 1B), the structure of which was reported by Wilkinson and Lowe in 1966 (54).

**Analysis of polymyxin in *P. polymyxa* E681**

The composition of the supernatant of *P. polymyxa* E681 grown in GSC medium was analyzed using an LC/MS system (Fig. 2A, 2C). The (M+H)+, (M+2H)2+, and (M+3H)3+ ion peaks were observed at 1157, 579, and 386, respectively (Fig. 2C). The molecular weight of the polymyxin was the same as those of polymyxins A and M (31, 54). The only difference between polymyxin A and M is the D/L-configuration of the third amino acid, Dab; a D-Dab is present in polymyxin A, and a L-Dab is found in polymyxin M. From the results of domain analysis, we
concluded that the polymyxin produced by *P. polymyxa* E681 is a polymyxin A.

Insertional disruption of the polymyxin synthetase gene cluster

To confirm that the *pmx* gene cluster is involved in polymyxin biosynthesis, we constructed and characterized a *pmxE* mutant strain. The antibacterial activity of the *pmxE* mutant of *P. polymyxa* E681 was completely abolished in a bioassay against *E. coli* (Fig. 2D). LC/MS data supported our earlier results by showing that the peak corresponding to polymyxin could not be detected in the *pmxE* mutant (Fig. 2B). Taken together, these results demonstrated that the *pmx* gene cluster is essential for polymyxin biosynthesis.

Heterologous expression of the *pmx* gene cluster in *B. subtilis*

As described in Materials and Methods, a recombinant *B. subtilis* strain BSK1 having a disrupted RM system was constructed, and showed at least 100 times higher transformation efficiency than the parent 168 strain with large DNA fragments 30-70 kb in length (data not shown). For heterologous expression, the entire *pmx* gene cluster was integrated into the *amyE* locus of *B. subtilis* BSK1 using fosmid clones containing *pmx* genes. The integration was carried out in two steps because no fosmid clone containing the entire *pmx* gene cluster was present in
our fosmid library. The scheme of the integration is shown in Figure 3. In the first step, a DNA
fragment (36.8 kb) containing the \( \text{pmx} \text{ABCD} \), truncated \( \text{pmxE} \), and an 8.2 kb of upstream region
was introduced into the \( \text{amyE} \) locus of \( B. \text{subtilis} \) BSK1 by homologous recombination using a
recombinant plasmid, pDG-12B06, containing the \( \text{pmx} \) genes and a flanking region. The
resulting strain, BSK2, was then transformed with a recombinant fosmid, PP12D08-sp\(^{r}\)-am\( \text{E}_{\text{back}} \),
which contained an intact \( \text{pmxE} \) and its 22.7 kb downstream region. Through this second step of
homologous recombination, strain BSK3 containing the entire \( \text{pmxABCDE} \) and its flanking
regions was constructed. Strain BSK3, however, did not show antibacterial activity against \( E. \text{coli} \) (Fig. 4). For the synthesis of nonribosomal peptide antibiotics, functional Sfp, a
phosphopantetheinyl transferase, is required (24). Because Sfp in \( B. \text{subtilis} \) 168 is nonfunctional
due to a mutation of the \( \text{sfp} \) gene (51), a functional \( \text{sfp} \) gene from \( B. \text{subtilis} \) CB114 (26) was
introduced into the BSK3 to construct strain BSK3S. However, the introduction of intact \( \text{sfp} \) still
did not induce antibacterial activity (Fig. 4). We found that the synthetic mechanism of an amino
acid, Dab, which is a major amino acid in polymyxin, was absent in \( B. \text{subtilis} \) 168. When Dab
was added extracellularly in growth medium, the antimicrobial activity of strain BSK3S against
\( E. \text{coli} \) was successfully detected (Fig. 4). LC/MS analysis of the supernatant of BSK3S grown in
GSC medium containing Dab showed that the polymyxin peak of BSK3S had the same mass
profile as that of *P. polymyxa* E681, thus demonstrating that *B. subtilis* BSK3S produced polymyxin (Fig. 5).

**DISCUSSION**

The excellent antibacterial activities of polymyxins against multidrug-resistant pathogenic Gram-negative bacteria have led to its reemergence among the antibiotics currently used in clinical practice in order to cope with such bacteria. However, widespread use of these antibiotics has been limited by their severe side effects, which include nephrotoxicity and neurotoxicity (18, 37). The development of polymyxin analogues with reduced toxicity has been limited because of the structural complexity of polymyxin and the lack of information on relevant biosynthetic genes. This report represents the complete sequence of the polymyxin synthetase gene cluster. Information on the sequence of the gene cluster may facilitate the development of a polymyxin analogue with reduced toxicity, as well as novel polymyxin-based antibiotics.

An interesting feature of the polymyxin gene cluster is the presence of *pmxC* and *pmxD*...
genes encoding transporter-like proteins within the gene cluster. The deduced gene products, PmxC (608 aa) and PmxD (577 aa), are 32.4% identical. PmxC and PmxD share 40.5% and 43.5% identities, respectively, with TycD and TycE, members of the ABC transporter family, of *Brevibacillus brevis* (34). Analysis of PmxC and PmxD in the TC database (42) showed the presence of five and seven transmembrane helices, respectively. The locations of the two tandem transporters within the polymyxin gene cluster suggest a role in conferring resistance against polymyxin via secretion by the producer cell. Work is in progress to clarify the potential roles of the ABC transporters, PmxC and PmxD, in the secretion of polymyxin.

One of the greatest concerns in polymyxin biosynthesis is the mechanism of incorporation of the fatty acid moiety to the peptide. The N-terminal C-domains (named starter C-domains) in first subunits of NRPSs clearly distinguishable from the other downstream C-domains were proposed to have a role in coupling a fatty acid to an amino acid (32, 33). Recent phylogenetic studies of C-domains showed that many other NRPSs have these starter C-domains (39, 40). The PmxE also contains a starter C-domain, which suggests that the C-domain may mediate a fatty acyl tailing of polymyxin. In contrast, Komura and Kurahashi suggested that a separate acyltransferase is necessary for the fatty acyl tailing of polymyxin (21, 23). In this study, the pmx gene cluster was introduced into the *amyE* locus of the *B. subtilis* chromosome, with 8.2
kb of upstream flanking region and 22.7 kb of downstream flanking region. The upstream and downstream flanking regions contain 7 and 24 putative open reading frames, respectively (data not shown). Among them, we could not find any gene that was potentially involved in the incorporation of a fatty acyl group into the polymyxin. If the suggestion of Komura and Kurahashi is correct, *P. polymyxa* E681 and *B. subtilis* may contain acyltransferases with the same specificity, because polymyxins produced by the two species showed the same mass profiles (Fig. 5).

Synthesis of polymyxin in *B. subtilis* 168 harboring entire *pmx* genes was induced only in Dab-containing medium (Fig. 4), which suggests that there is no synthetic mechanism of Dab in the strain. Synthesis of Dab is mediated by 2,4-diaminobutyrate aminotransferase encoded by *ectB* (43). There is no homolog of *ectB* in *B. subtilis* 168. The *ectB* is composed of an operon structure with *ectA* and *ectC* encoding 2,4-diaminobutyrate acetyltransferase and ectoine synthase, respectively, in *Halobacillus halophilus* (43). The *ectABC* genes responsible for ectoine biosynthesis have usually been found in halophilic bacteria. The order of these genes was found to be highly conserved, even in a Gram-negative bacterium, *Halomonas elongate* (43). In *P. polymyxa* E681, the amino acid sequence of the *ectB* homolog shares 51% identity with that of *H. halophilus*. Interestingly, the *ectB* gene of *P. polymyxa* E681 is not part of an operon. There is
no homolog of *ectA* and *ectC* in the genome, which suggests that *P. polymyxa* E681 does not produce ectoine. Therefore, in *P. polymyxa* E681, Dab synthesized by the EctB may not be used as an intermediate for the synthesis of ectoine, resulting in an increase of its concentration in the cell. This condition may be favorable to the cell in terms of the synthesis of polymyxin.

Many bacterial isolates producing natural products such as peptide antibiotics are usually difficult to handle because of our lack of knowledge of their physiological and genetic traits, and the low transformation efficiencies of these isolates. Therefore, studies on the production of natural products and development of novel analogs through biosynthetic engineering often encounter difficulties from their initiation. Many reports have dealt with the heterologous expressions of natural product pathways from the original microbial organisms to well-developed surrogate hosts (11, 51, 53). Although we succeeded in constructing a *pmxE* knock-out mutant in this study, the low level of transformation efficiency of *P. polymyxa* E681 remains a bottleneck in genetic studies. Therefore, heterologous expression of the polymyxin biosynthetic gene cluster in *B. subtilis* may accelerate structure-function study and engineering of *pmx* genes for the generation of novel analogs.
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FIGURE LEGENDS

Fig. 1. The pmx gene cluster. A, Genetic structure of pmx genes and domain organization of the Pmx enzymes. B, Primary structure of the polymyxin A. MOA indicates 6-methyloctanoic acid.

Fig. 2. Analysis of polymyxin synthesis in P. polymyxa E681. A and B, LC analysis of culture supernatants of E681 and the pmxE mutant, respectively, using a YMC Pack Pro C18 column. C, MS data for polymyxin A produced by P. polymyxa E681. The arrow indicates the peak for polymyxin A. D, Antibacterial activities of the culture supernatants of wild-type E681 and the pmxE mutant strains against E. coli DH5α.

Fig. 3. Scheme for the transfer of pmx genes into B. subtilis. The pmx gene cluster was integrated into the amyE locus of B. subtilis BSK1 containing a deleted BsuM restriction and a modification system from B. subtilis 168. The detailed protocol is described in Materials and Methods.

Fig. 4. Antibacterial activities of recombinant B. subtilis strains against E. coli under conditions with or without L-Dab. B. subtilis BSK1 derived from B. subtilis 168 contains a deleted BsuM restriction and modification system. Strain BSK1S was constructed by introducing a functional sfp from B. subtilis CB114 into BSK1. BSK3 contains complete pmx genes.
(pmxABCDE) in the amyE locus of BSK1. BSK3S was constructed by introducing a functional
sfp into the BSK3.

Fig. 5. Biosynthesis of polymyxin in B. subtilis. A, LC analysis of culture supernatants of P.
polymyxa E681 and B. subtilis BSK3S grown in GSC with or without L-Dab using a Terra MS
C18 column. Arrows indicate the peaks to be analyzed by mass spectrometry. B, MS data for
polymyxins produced by P. polymyxa E681 and B. subtilis BSK3S.
### TABLE 1. Primers used in this study.

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<th>Primers</th>
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<td>5’-TATCGAGATTTTCAGGAGCTAAGGAATGGAGAAATCGACGTCAGCTTATGGCGGAGATATCGATGTCAGCTTATGCT-3’</td>
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<td>pmxA</td>
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<tr>
<td>pmxAR</td>
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<tr>
<td>pmxB</td>
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<td>pmxC</td>
<td>ACTTACCAGTTTTATATGGAATACC-3’</td>
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<tr>
<td>pmxD</td>
<td>GAAAGCTGCGTCAAGGCAGGAGGAAATGGAGAAATCGACGTCAGCTTATGGCGGAGATATCGATGTCAGCTTATGCT-3’</td>
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<td>pmxE</td>
<td>GGGGCGTTTTATATGGAATACC-3’</td>
</tr>
<tr>
<td>pmxF</td>
<td>TCCACAACACTGCGTCAAGGCAGGAGGAAATGGAGAAATCGACGTCAGCTTATGGCGGAGATATCGATGTCAGCTTATGCT-3’</td>
</tr>
</tbody>
</table>

*The underlined sequences indicate the targeted regions for Red recombinase, and italicized forms indicate the synthetic restriction sites.*
TABLE 2. Specificity-conferring amino acids of adenylation domains in the polymyxin synthetase.

<table>
<thead>
<tr>
<th>A domain</th>
<th>Active site residues</th>
<th>Amino acid specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>235 236 239 278 299 301 322 330 331 517</td>
<td></td>
</tr>
<tr>
<td>PmxA A1</td>
<td>D A W I V G A I V K</td>
<td>Leu</td>
</tr>
<tr>
<td>PmxA A2</td>
<td>D F W N I G M V H K</td>
<td>Thr</td>
</tr>
<tr>
<td>PmxA A3</td>
<td>D V G E I S A I D K</td>
<td>Dab</td>
</tr>
<tr>
<td>PmxA A4</td>
<td>D V G E I S A I D K</td>
<td>Dab</td>
</tr>
<tr>
<td>PmxB A1</td>
<td>D F W N I G M V H K</td>
<td>Thr</td>
</tr>
<tr>
<td>PmxE A1</td>
<td>D V G E I S S I D K</td>
<td>Dab</td>
</tr>
<tr>
<td>PmxE A2</td>
<td>D F W N I G M V H K</td>
<td>Thr</td>
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<tr>
<td>PmxE A3</td>
<td>D V G E I S S I D K</td>
<td>Dab</td>
</tr>
<tr>
<td>PmxE A4</td>
<td>D V G E I S A I D K</td>
<td>Dab</td>
</tr>
<tr>
<td>PmxE A5</td>
<td>D V G E I S A I D K</td>
<td>Dab</td>
</tr>
</tbody>
</table>
Fig. 1

(A) 14.9 kb 3.3 kb 1.7 kb 18.9 kb


1.8 kb

PmxE

Dab  Thr  Dab  Dab  Dab  Dab

module 1 module 2 module 3 module 4 module 5

PmxA

Leu  Thr  Dab  Dab  Dab  Dab

module 6 module 7 module 8 module 9

PmxB

Thr

module 10

Condensation domain  Adenylation domain  Thiolation domain

Epimerization domain  Termination domain

(B) L-DAB → D-Leu → L-Thr

MOA → L-DAB → L-Thr → D-DAB → L-DAB

L-Thr ← L-DAB ← L-DAB
Fig. 2

(A) Polymyxin A

(B) Relative Abundance

(C) [M+3H] 3+

[D] Wild type pmxE

Relative Abundance

Time (min)
Fig. 4
Fig. 5

(A) E681

BSK3S (Dab+)

BSK3S (Dab-)

RT: 0.00 - 15.09 SM: 5G

Time (min)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

(B) E681

BSK3S (Dab+)

Relative Abundance