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

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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-kb 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.

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 6 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 semisynthesis or modifications of polymyxins was 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 by 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 nonribosomal 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 es-

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TABLE 1. Primers used in this study

Primer	Oligonucleotide sequence[a]
Foscm-TCF	5′-TATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATAGATACAAGAGAGGTCTCTCG-3′
Foscm-TCR	5′-GGCACCAATAACTGCCTTAAAAAAATTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATAACAAACGGGCCATATTGTTG-3′
CatF	5′-AAA*GGATCC*TCATGTTTGACAGCTTATCATCG-3′
CatR	5′-AAA*GAATTC*CCACGCCGAAACAAGCGCTC-3′
Kd4kanF	5′-CC*ATCGAT*GTGTAGGCTGGAGCTGCTTC-3′
Kd4kanR	5′-CC*ATCGAT*ATGGGAATTAGCCATGGTCC-3′
PmxEckF	5′-GCATTCAATAACAAAGATTATGCCGTTTGGCAGCATTCCCGAAGCTTACGGGCAGATGCTCCAGCCGCAGAATCATGTTTGACAGCTTATCATCG-3′
PmxEckR	5′-GCAGCACGTCCATGGAAAGGCCGTCGGAACCAATATGATGAATATCCAGCGCGAGCAGAAATTTCTCTTTCCACGCCGAAACAAGCGCTC-3′
PmxEdelF	5′-GTCTCGGATGGCATTTCGACAG-3′
PmxEdelR	5′-AGAAGTCGAGAGGCAGCTCAAG-3′
ydiO-up-F	5′-TAATGAGTTAGATGAAATACC-3′
ydiO-up-R	5′-TTT*GGATCC*TTATCATTCCTAGTATTACAC-3′
ydjA-down-F	5′-TTT*GGATCC*TGTTATTAGTCGGAATGAATG-3′
ydjA-down-R	5′-TAT*CTGCAG*GAATAAACAGAAAGGAAAGACTG-3′
1730-12DF	5′-ACTGCATGTCCCCAGTGCATCGGTCCCCATACGGATTTATACGGGTAATGTTGATAGAACAAGTGATATTGGTATGTTTCTCTTTGATGTC-3′
1730-12DR	5′-AGATTATCGGCTGAACTACCATTTAATGGCTGAATTGGGCTGGATGAATGATCCGAACGGCTTCATTCAAGAATGGCGATTTTCGTTCGTG-3′
pmxAF	TAACGTTTTCACCCCATTGG
pmxAR	GGGAGCTTGGAGCTTTGCTG
pmxBF	TCCACAACTCGAGCTAAGCC
pmxBR	ACTTACCGCTCCAGTACTGTTC
pmxCF	GAACAAGTCAAGCGGCAGATC
pmxCR	CTTTCACTTGCGAGAGCCATC
pmxDF	CAGGAATTTACCGAGTCTGCC
pmxDR	GTCGCATTCGCAAGCAGGAAG
pmxEF	GAGCGGCTGAAACGTCAGGAAGCC
pmxER	CTGCTTCGCCTGTATGATTGTC

[a] The underlined sequences indicate the targeted regions for Red recombinase, and the italicized residues indicate the synthetic restriction sites.

sential 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 *Paenibacillus 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 liquid chromatography-mass spectrometry (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 of 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). *Escherichia 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 Tryptic soy broth (Difco) for general purposes, brain heart infusion (Difco) containing 10% sucrose for transformation, and glucose-starch-$CaCO_3$ (GSC) medium (10) for analysis of polymyxin. *B. subtilis* strains were grown in LB medium for general purposes, and in GSC medium 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 and then centrifuged at 5,000 × *g* for 10 min to obtain the supernatant. LC/MS was performed with the supernatant using a high-pressure liquid chromatography system provided by Thermo Electron Co. and an ion spectrometer. The sample was injected into a reverse-phase column, YMC Pack Pro C18 (10 by 250 mm, 5 μm) or Terra MS C18 (2.1 by 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 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 reextracted with methanol. The final methanol extract was evaporated and dissolved in 2 ml water. After the concentrated sample was passed through a C18 column (Sili-Cycle 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 acetyltransferase (*cat*) gene of fosmid PP12G04 was replaced with a tetracycline resistance gene (*Tc*) using a λ Red recombination

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system to construct fosmid pPmx-Tc. The *Tc* gene was amplified from pBC16 (5) with the Foscm-TCF and Foscm-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) by 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 primers *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 and then screened 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^r-*amyE*$_{back}$, was constructed by integration of the Sp^r-*amyE*$_{back}$ 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 *pmxABCDE* sequence was constructed by homologous recombination between PP12D08-Sp^r-*amyE*$_{back}$ 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/pmxAR, pmxBF/pmxBR, pmxCF/pmxCR, pmxDF/pmxDR, and pmxEF/pmxER. 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 onto 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 h 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 (J. F. 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). PmxA, containing 4,953 amino acids, comprises four modules and a C-domain (Fig. 1A). The substrate specificities of the four PmxA A-domains were predicted to activate the amino acid substrates, Leu, Thr, Dab, and Dab, respectively (Table 2). PmxB, a 1,102-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. PmxE, a 6,312-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 and C). The $(M+H)^+$, $(M+2H)^{2+}$, and $(M+3H)^{3+}$ ion peaks were observed at 1,157, 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 an L-Dab is found in polymyxin M. From the results of domain analysis, we concluded that the polymyxin produced by *P. polymyxa* E681 is 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 did the parent 168 strain with large DNA frag-

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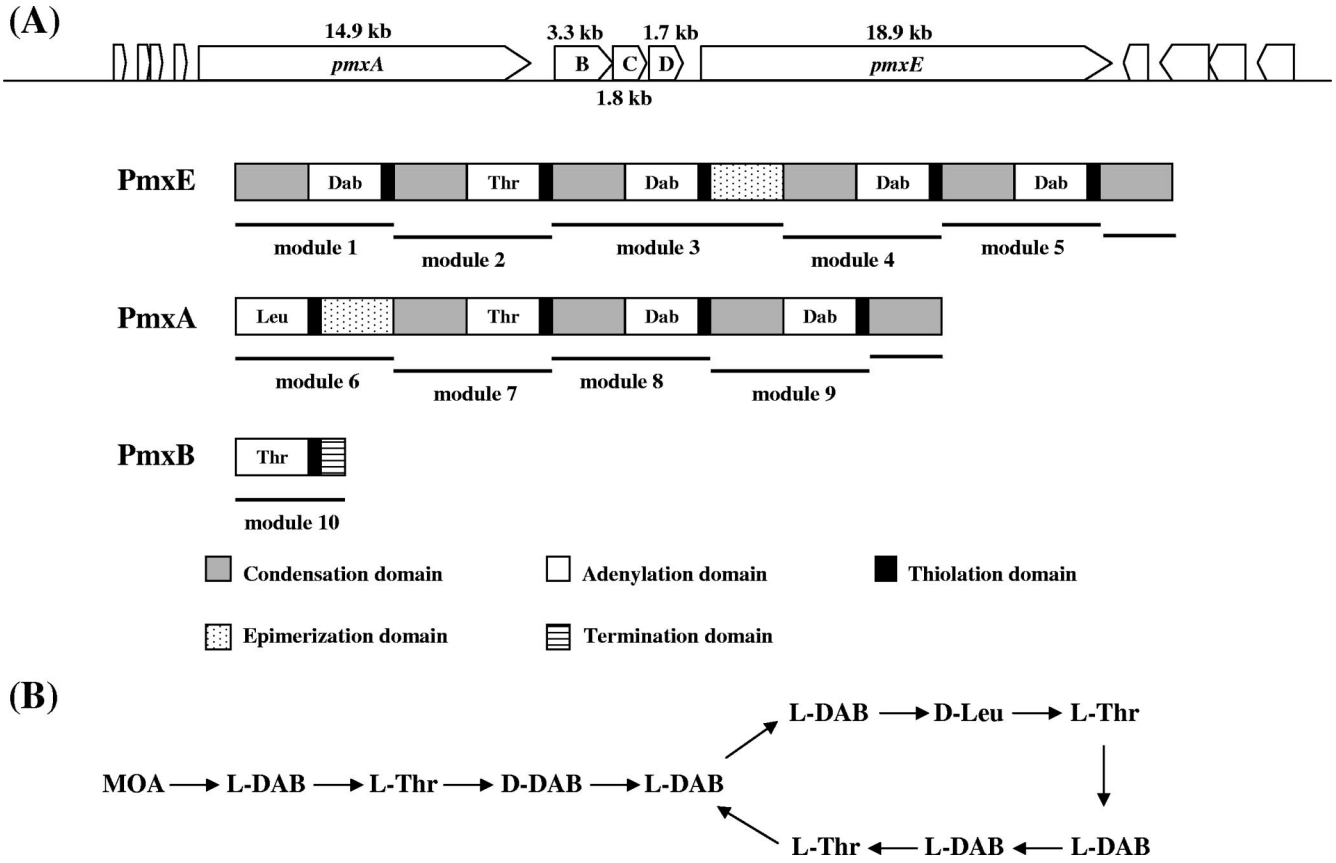

FIG. 1. The *pmx* gene cluster. (A) Genetic structure of *pmx* genes and domain organization of the Pmx enzymes. (B) Primary structure of polymyxin A. MOA, 6-methyloctanoic acid.

ments 30 to 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 Fig. 3. In the first step, a DNA fragment (36.8 kb) containing *pmxABCD*, truncated *pmxE*, and an 8.2-kb upstream region was introduced into the *amyE* locus of *B. subtilis* BSK1 by homologous recombination using a recombinant plasmid, pDG-12B06, containing the *pmx* genes and a flanking region. The resulting strain, BSK2, was then transformed with a recombinant fosmid, PP12D08-Sp^r-*amyE*$_{back}$, which contained an intact *pmxE* and its 22.7-kb downstream region. Through this second step of homologous recombination, strain BSK3 containing the entire *pmxABCDE* and its flanking regions was constructed. Strain BSK3, however, did not show antibacterial activity against *E. coli* (Fig. 4). For the synthesis of nonribosomal peptide antibiotics, functional Sfp, a phosphopantetheinyl transferase, is required (24). Because Sfp in *B. subtilis* 168 is nonfunctional due to a mutation of the *sfp* gene (51), a functional *sfp* gene from *B. subtilis* CB114 (26) was introduced into BSK3 to construct strain BSK3S. However, the introduction of intact *sfp* still did not induce antibacterial ac-

TABLE 2. Specificity-conferring amino acids of adenylation domains in the polymyxin synthetase

A-domain	Active site residue at position:										Amino acid specificity
	235	236	239	278	299	301	322	330	331	517	
PmxA A1	D	A	W	I	V	G	A	I	V	K	Leu
PmxA A2	D	F	W	N	I	G	M	V	H	K	Thr
PmxA A3	D	V	G	E	I	S	A	I	D	K	Dab
PmxA A4	D	V	G	E	I	S	A	I	D	K	Dab
PmxB A1	D	F	W	N	I	G	M	V	H	K	Thr
PmxE A1	D	V	G	E	I	S	S	I	D	K	Dab
PmxE A2	D	F	W	N	I	G	M	V	H	K	Thr
PmxE A3	D	V	G	E	I	S	S	I	D	K	Dab
PmxE A4	D	V	G	E	I	S	A	I	D	K	Dab
PmxE A5	D	V	G	E	I	S	A	I	D	K	Dab

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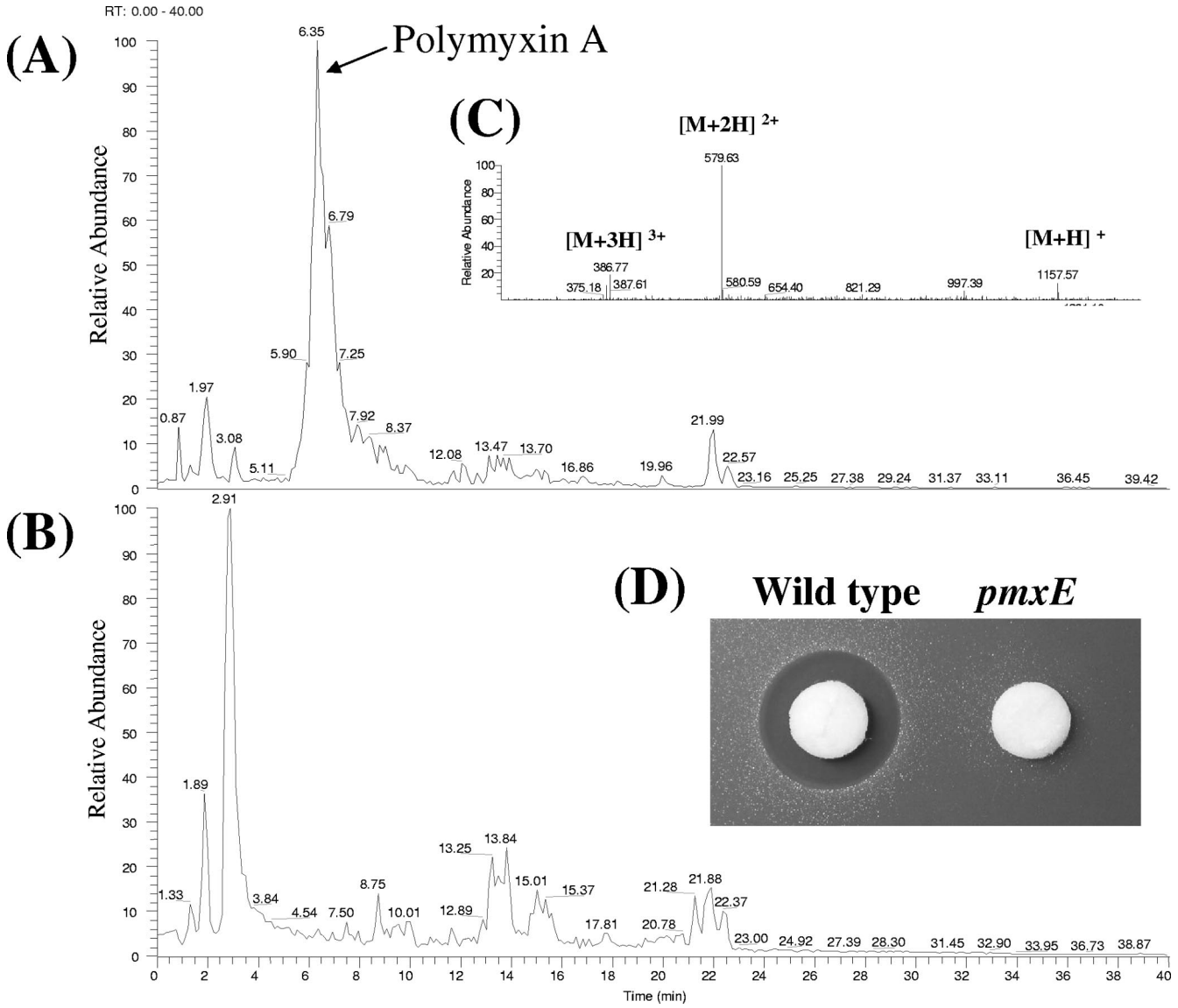

FIG. 2. Analysis of polymyxin synthesis in *P. polymyxa* E681. LC analysis of culture supernatants of E681 (A) and the *pmxE* mutant (B), respectively, using a YMC Pack Pro C18 column. (C) MS data for polymyxin A produced by *P. polymyxa* E681. The arrow indicates the peak for polymyxin A. (D) Antibacterial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

tivity (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. subtilis* 168. When Dab was added extracellularly in growth medium, the antimicrobial activity of strain BSK3S against *E. 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 amino acids) and PmxD (577 amino acids), are

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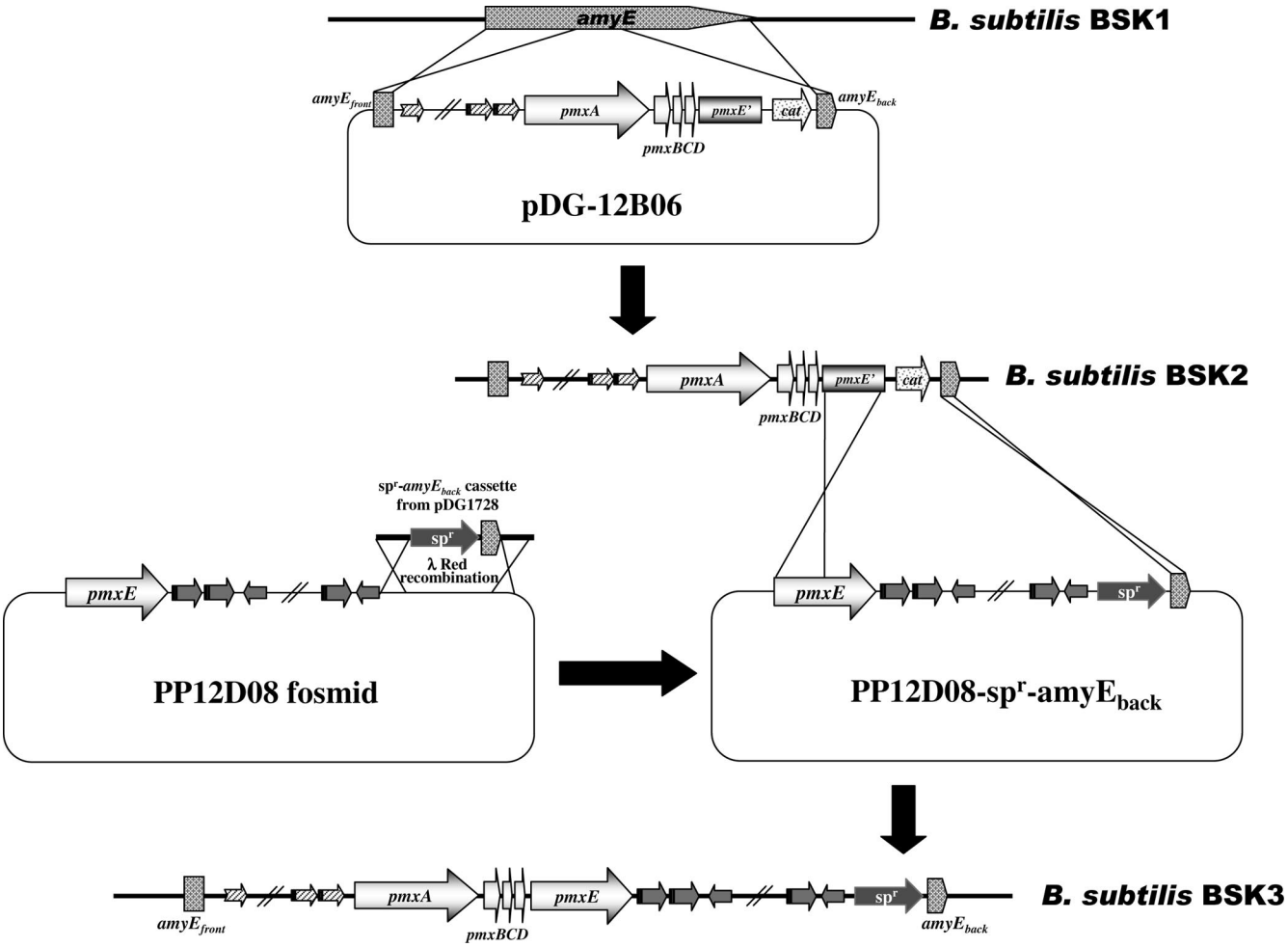

FIG. 3. Scheme for the transfer of *pmx* genes into *B. subtilis*. The *pmx* gene cluster was integrated into the *amyE* locus of *B. subtilis* BSK1 containing a deleted *BsuM* RM system from *B. subtilis* 168. The detailed protocol is described in Materials and Methods.

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 with the Transporter Classification 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.

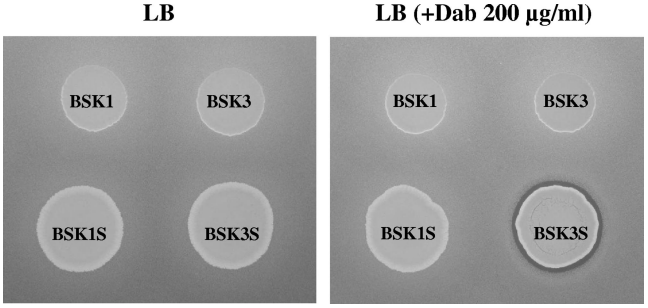

FIG. 4. Antibacterial activities of recombinant *B. subtilis* strains against *E. coli* under conditions with or without L-Dab. *B. subtilis* BSK1 derived from *B. subtilis* 168 contains a deleted *BsuM* RM system. Strain BSK1S was constructed by introducing a functional *sfp* from *B. subtilis* CB114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

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). 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

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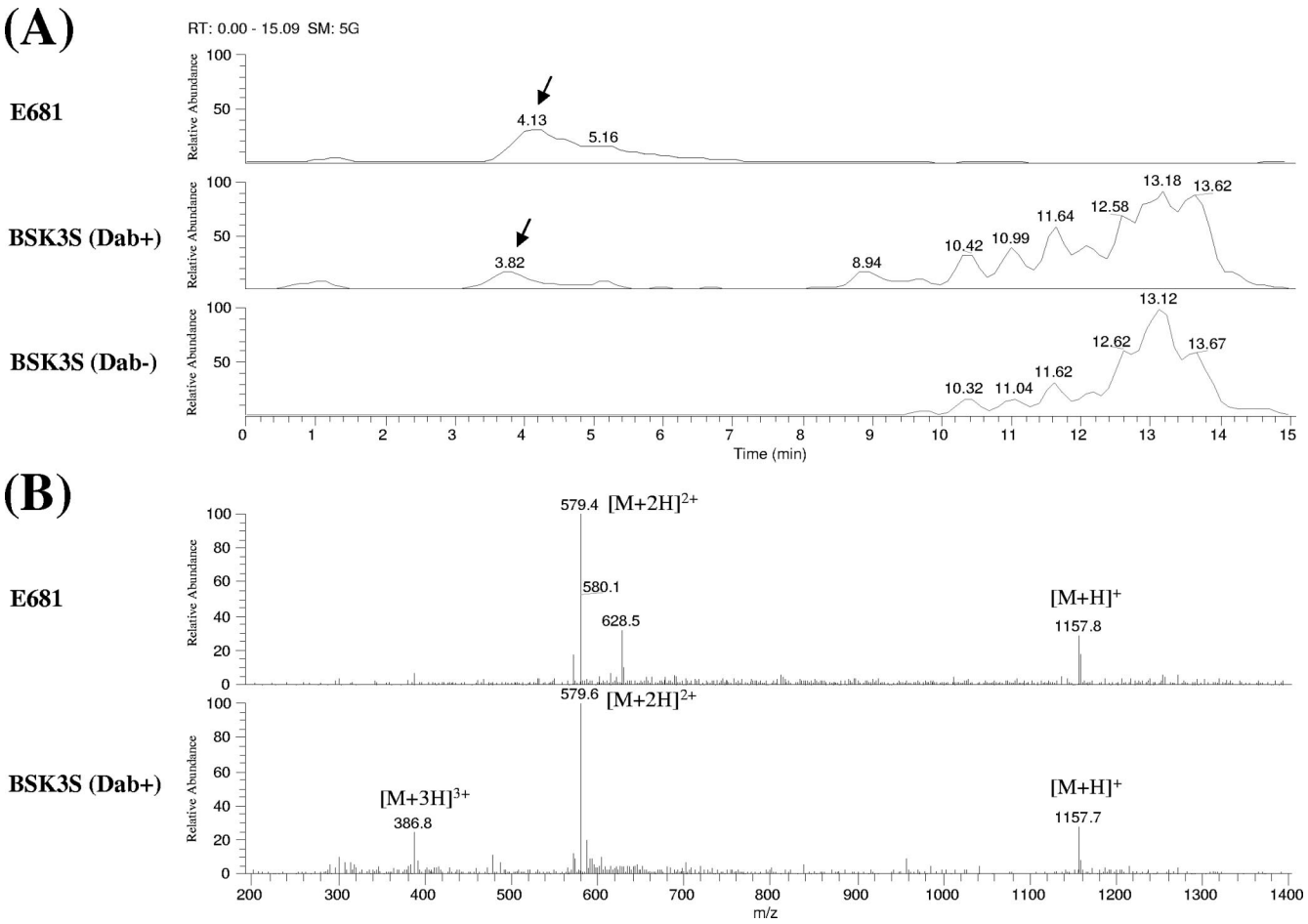

FIG. 5. Biosynthesis of polymyxin in *B. subtilis*. (A) LC analysis of culture supernatants of *P. polymyxa* E681 and *B. subtilis* BSK3S grown in GSC medium with or without L-Dab, using a Terra MS C18 column. Arrows indicate the peaks to be analyzed by MS. (B) MS data for polymyxins produced by *P. polymyxa* E681 and *B. subtilis* BSK3S.

region. The upstream and downstream flanking regions contain 7 and 24 putative open reading frames, respectively (see Table S1 in the supplemental material). 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 homologue 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* homologue 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 homologue of *ectA* or *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 in 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 of the production of natural products and development of novel analogues 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* knockout 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 accel-

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erate structure-function study and engineering of *pmx* genes for the generation of novel analogues.

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