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 1,2,4-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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sentential 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 to date, but no polymyxin biosynthetic gene cluster has been reported to date.

During whole genome sequencing of *Paeubacinus polymyxus* 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, insertion 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 recombinase, 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-CaCO₃ (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 1,2,4-diaminobutyric acid (1-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 1-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 (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 pmxB gene was constructed using an *E. coli* fosmid clone. In brief, the fosmid DNA (P1P204) harboring truncated pmxC and complete pmxB, pmxC, pmxD, and pmxE in a 38.1-kbp chromosomal DNA fragment cloned into pCC1fos (Epicenter Biotechnologies) was introduced into *E. coli* BW25113 carrying the Red recombinase expression plasmid, pKD46 (9). The chloramphenicol acetyltransferase (*cat*) gene of fosmid P1P204 was replaced with a tetracycline resistance gene (*Tc*) using a λ Red recombinase.
system to construct fosmid pPmx-Tc. The Tc gene was amplified from pBCh16 (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 pCC110. For inactivation of the pPmxE gene, a chloramphenicol resistance gene-kanamycin resistance gene (cat-kan) cassette was introduced into the pPmxE 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 pGemTzfd+ (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 PmxEkF and PmxEkR, 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 pPdmxE 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 pPmxE 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 pPdmxE fosmid was introduced into P. polymyxa E681 to generate a polymyxin-defective mutant. The mutant was also confirmed by PCR using the pmxEEdelF and pmxEEdelR 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 Blm restriction and modification (RM) (16) were removed from B. subtilis NCIB 3610 and replaced with DNA fragments upstream of ydiO and downstream of ydiF were amplified by PCR with primers ydiO-up-F and ydiO-up-R and primers ydiF-down-F and ydiF-down-R, respectively. The DNA fragments were inserted into the EcoRI and PstI sites of plasmid pBGCS6 (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 PP12D06 containing pmxABC, pmxAR, pmxB/pmxBR, pmxC/pmxCR, pmxD/pmxDR, and pmxEF/pmxER was amplified by PCR with primers pmxEdelF and pmxEdelR, which bind to the outer regions of the homologous arm. The pPdmxE fosmid was introduced into P. polymyxa E681 to generate a polymyxin-defective mutant. The mutant was also confirmed by PCR using the pmxEEdelF and pmxEEdelR primers. Transformation of P. polymyxa was performed according to a previously reported method (7).

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 and pmxB, and pmxE encode polymyxin synthetase.

The domains of the polymyxin synthetase were analyzed based on the method of Ansari et al. (3). PmA, containing 4,953 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). PmB, a 1,102-amino-acid polypeptide, comprises one module containing A-T-TE domains. The predicted amino acid specificity of the A-domain of PmB was Thr (Table 2). Due to the presence of the TE-domain, PmB, a 6,312-amino-acid polypeptide, has five modules and a C-domain. The substrate specificities of the five PmE 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 PmE-PmA-PmB, and the last C-domains of PmE and PmA should become one module with the A-T-E domains of PmA and the A-T-TE domains of PmB, respectively. The third module of PmE 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-
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-Spα-amylEback, 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-

![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-methyl-octanoic acid.](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>A-domain</th>
<th>Active site residue at position:</th>
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<tr>
<td></td>
<td>235  236  239  278  299  301  322  330  331  517</td>
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</tr>
<tr>
<td>PmxA A1</td>
<td>D    A    W    I    V    G    A    I    V    K</td>
<td>Leu</td>
</tr>
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<td>PmxE A5</td>
<td>D    V    G    E    I    S    S    I    D    K</td>
<td>Dab</td>
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TABLE 2. Specificity-conferring amino acids of adenylation domains in the polymyxin synthetase
We found that the synthetic mechanism of an amino acid, Dab, which is a major amino acid in polymyxin, was absent in \textit{B. subtilis} 168. When Dab was added extracellularly in growth medium, the antimicrobial activity of strain BSK3S against \textit{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 \textit{P. polymyxa} E681, thus demonstrating that \textit{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 \textit{pmxC} and \textit{pmxD} genes encoding transporter-like proteins within the gene cluster. The deduced gene products, PmxC (608 amino acids) and PmxD (577 amino acids), 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 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.

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.

**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.

**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 (*pmxABCD*) in the *amyE* locus of BSK1. BSK3S was constructed by introducing a functional *sfp* into BSK3.
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-
erate structure-function study and engineering of \textit{pmx} genes for the generation of novel analogues.

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