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Paenibacillus polymyxa PKB1 Produces Variants of Polymyxin B-Type Antibiotics

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SUMMARY

Polymyxins are cationic lipopeptide antibiotics active against many species of Gram-negative bacteria. We sequenced the gene cluster for polymyxin biosynthesis from *Paenibacillus polymyxa* PKB1. The 40.8 kb gene cluster comprises three nonribosomal peptide synthetase-encoding genes and two ABC transporter-like genes. Disruption of a peptide synthetase gene abolished all antibiotic production, whereas deletion of one or both transporter genes only reduced antibiotic production. Computational analysis of the peptide synthetase modules suggested that the enzyme system produces variant forms of polymyxin B (1 and 2), with D-2,4-diaminobutyrate instead of L-2,4-diaminobutyrate in amino acid position 3. Two antibacterial metabolites were resolved by HPLC and identified by high-resolution mass spectrometry and MS/MS sequencing as the expected variants 3 and 4 of polymyxin B_1 (1) and B_2 (2). Stereochemical analysis confirmed the presence of both D-2,4-diaminobutyrate and L-2,4-diaminobutyrate residues.

INTRODUCTION

Polymyxins are a family of cationic lipopeptide antibiotics produced by Gram-positive bacteria and active against many Gram-negative bacteria. Although polymyxins are very potent antibiotics, their clinical application is limited by their inherent toxicity and the availability of other active and less toxic antibiotics. Polymyxin B (**1** and **2**) and polymyxin E (Figure 1) are the two forms of polymyxins that have been used clinically, and neurotoxicity and nephrotoxicity are major complications associated with their use (Li et al., 2006). As a result, polymyxins are typically administered to prevent, rather than to treat, infections, and are used in low concentrations in combination with other antibiotics in topical, ophthalmic, and otic formulations (Falagas and Kasiakou, 2006). Polymyxins have high affinity for lipopolysaccharide, and recently have been embedded in hemoperfusion cartridges used to remove lipopolysaccharide from blood and block initial stages of sepsis (Rachoin et al., 2010). Polymyxin-conjugated polymers have also been proposed for use as dressing materials to selectively remove Gram-negative bacteria, reduce the bacterial load in wounds, and speed healing (Shepherd et al., 2011). These new applications for polymyxins have resulted in a resurgence of interest in this family of compounds in recent years. More importantly, the emergence of multidrug-resistant Gram-negative bacteria including *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Klebsiella pneumoniae*, which are highly resistant to most antibiotics but intrinsically sensitive to polymyxin B (**1** and **2**), has led to a reassessment of these antibiotics (Gales et al., 2006).

Polymyxins consist of ten amino acids with a characteristic polycationic heptapeptide ring and an N-terminal fatty acid modification. The peptide ring is formed through cyclization of the carboxyl group of L-threonine at position 10 (C terminus) with the γ-amino-containing side chain of the L-2,4-diaminobutyrate (DAB) residue at position 4, and the α-amino group of this DAB residue is attached to the lipotripeptide of the N terminus (Storm et al., 1977). Several types of polymyxins have been identified, with molecular weights ranging from 1,129 to 1,218 Da (Govaerts et al., 2002a, 2002b; Martin et al., 2003; Orwa et al., 2001; Parker and Rathnum, 1975; Vogler and Studer, 1966). They differ in the identity of the N-terminal lipid portion as well as the amino acids at positions 3, 6, and 7. The fatty acid side chains of polymyxins are predominantly isooctanoic or methyloctanoic acids, although other variants are also seen (Govaerts et al., 2002a).

Like many low molecular weight peptide antibiotics, polymyxins are produced by a nonribosomal peptide synthetase (NRPS) mechanism (Martin et al., 2003; Marahiel, 1997; von Döhren et al., 1997). Although the chemical structures of many polymyxins have been known for years, studies on the molecular mechanisms of their biosynthesis are still very limited. Only recently, whole-genome sequences of two *Paenibacillus polymyxa* strains were made public (GenBank accession numbers CP000154.1 and NC_014622.1), and one of them was reported to contain a gene cluster encoding the NRPS genes for polymyxin biosynthesis and to produce polymyxin A (Choi et al., 2009). Here we report the cloning, sequencing, and mutational analysis of the gene cluster for polymyxin production in *P. polymyxa* PKB1, and identify two unusual forms of polymyxin that are produced by this organism.

RESULTS

Identifying the Gene Cluster

P. polymyxa PKB1 is an environmental isolate studied initially for its ability to control fungal diseases of plants due to production of the antifungal peptide fusaricidin (Beatty and Jensen, 2002).

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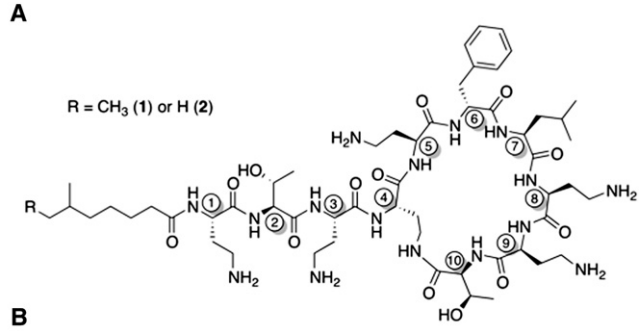


B

Polymyxin Variant	Res 3	Res 6	Res 7
Polymyxin A	D-DAB	D-Leu	L-Thr
Polymyxin B	L-DAB	D-Phe	L-Leu
Polymyxin D	D-Ser	D-Leu	L-Thr
Polymyxin E (Colistin)	L-DAB	D-Leu	L-Leu
Polymyxin M (Mattacin)	L-DAB	D-Leu	L-Thr

Figure 1. Structure of Polymyxins
(A) Structure of polymyxin B (**1** and **2**).
(B) Amino acid variations found in some naturally occurring polymyxins. DAB, 2,4-diaminobutyrate.

During characterization of the gene cluster for fusaricidin production by *P. polymyxa* PKB1, two genomic libraries were prepared (Li et al., 2007; Li and Jensen, 2008). The first contained fragments of *P. polymyxa* PKB1 genomic DNA (gDNA) in the cosmid vector, Supercos1, whereas the second library contained similar gDNA fragments inserted into pSMART-FOS, a copy control fosmid vector designed to prevent rearrangement of cloned DNA fragments. A Supercos library clone, Col-8, that hybridized with a threonine-specific adenylation domain probe was fully sequenced and found to carry a gDNA fragment encoding part of a putative polymyxin gene cluster (Figure 2A). We used the sequence information from Col-8 and related cosmids to identify fosmid clones containing overlapping DNA fragments, and then analyzed them to obtain sequence information to complete the polymyxin gene cluster. We screened the fosmid library (550 clones) with two nonradioactively labeled DNA probes, a 1.67 kb EcoRI fragment from near the 5′ end of the polymyxin gene cluster fragment in Col-8 and a 1.11 kb PCR product amplified from a related cosmid, Col-15, representing the region just beyond the 3′ end of the polymyxin gene cluster fragment in Col-8 (Figure 2A). A total of 16 fosmid clones was found to hybridize with these two probes, and the hybridization results were confirmed by PCR analysis using primers based on the sequences of the probes. Restriction digestion pattern analysis of these 16 fosmid clones identified 3 that were most likely to contain the missing ends of the gene cluster, and subcloned fragments from these were subjected to DNA sequencing. The sequence information obtained from these three fosmid clones provided 42.7 kb of contiguous *P. polymyxa* PKB1 DNA sequence encompassing the polymyxin gene cluster, which has been deposited in GenBank under accession number JN660148.

Analyzing the Gene Cluster

The polymyxin gene cluster is 40.8 kb long and comprises five open reading frames (ORF), *pmxA*, *B*, *C*, *D*, and *E* (Figure 2A). *pmxA*, *B*, and *E* encode three subunits of an NRPS enzyme system, and *pmxC* and *D* encode membrane permease components of ABC-type transporter systems. The contiguous DNA sequence obtained ended 8 bp upstream of the *pmxA* gene, and no cosmid or fosmid clones extending further in this direction were available, so the identity of genes upstream of *pmxA* could not be determined. Approximately 2 kb of DNA sequence from beyond the end of *pmxE* was obtained, and an ORF encoding an apparent hypothetical protein was found in this region. The protein shares very high homology with a hypothetical protein from *Geobacillus* sp. Y412MC10 (GenBank accession number 003243619), as identified by BLAST analysis, but is not apparently related to NRPS gene clusters and so it is not considered to be a part of the polymyxin gene cluster.

Each NRPS-encoding ORF was translated into an amino acid sequence, and the modular arrangement of the NRPS system and the amino acid substrate incorporated by each module was predicted using online software (PKS/NRPS Analysis; http://nrps.igs.umaryland.edu/nrps). The amino acids specified by each domain in the NRPSs are shown in Figure 2B. According to this analysis, the order and arrangement of the NRPS-encoding genes within the cluster are not colinear with the amino acids in the polymyxin end product. *pmxE* was found to encode the NRPS modules responsible for the incorporation of the first five amino acids of polymyxin, *pmxA* encodes modules for the next four amino acids, and *pmxB* encodes a single module responsible for incorporating the last amino acid of polymyxin. Because NRPS-encoding gene clusters are typically colinear with their peptide product, this observation initially led us to consider that the DNA fragment containing the cluster might be rearranged. However, the same arrangement of ORFs was recently reported for the polymyxin gene cluster in *P. polymyxa* E681, which produces polymyxin A (Choi et al., 2009).

Mutational Analysis

To demonstrate conclusively that the gene cluster is responsible for polymyxin production, the *pmxE* gene was disrupted by deleting approximately 2.0 kb from the 5′ end of the gene and replacing it with an antibiotic resistance cassette (Apra^{R}Cm^R*oriT* cassette). This cassette includes apramycin and chloramphenicol resistance genes and an *oriT* region to support interspecies conjugation, all flanked by FLP recombinase target sequences (FRT). Mutagenesis was accomplished using a PCR-targeted gene disruption protocol originally developed for mutagenesis of the fusaricidin gene cluster in this species (Li et al., 2007), and the validity of the mutants was confirmed by PCR analysis. The resulting *pmxE* mutant and wild-type strains of *P. polymyxa* PKB1 were grown in glucose starch calcium carbonate (GSC) medium, and methanol extracts of culture supernatants were assayed for bioactivity against *Escherichia coli* DH5α by agar diffusion bioassay. Supernatant from the wild-type strain gave a clear zone of inhibition against the indicator organism, but no bioactivity was observed for the extract of supernatant from the *pmxE* mutant (Figure 3A). This demonstration that the disruption of *pmxE* completely abolishes the antimicrobial activity of *P. polymyxa* PKB1 against *E. coli* strongly suggests that the gene cluster is involved in polymyxin production. It also indicates that the polymyxin product is the sole contributor to the antibiotic effect against *E. coli*.

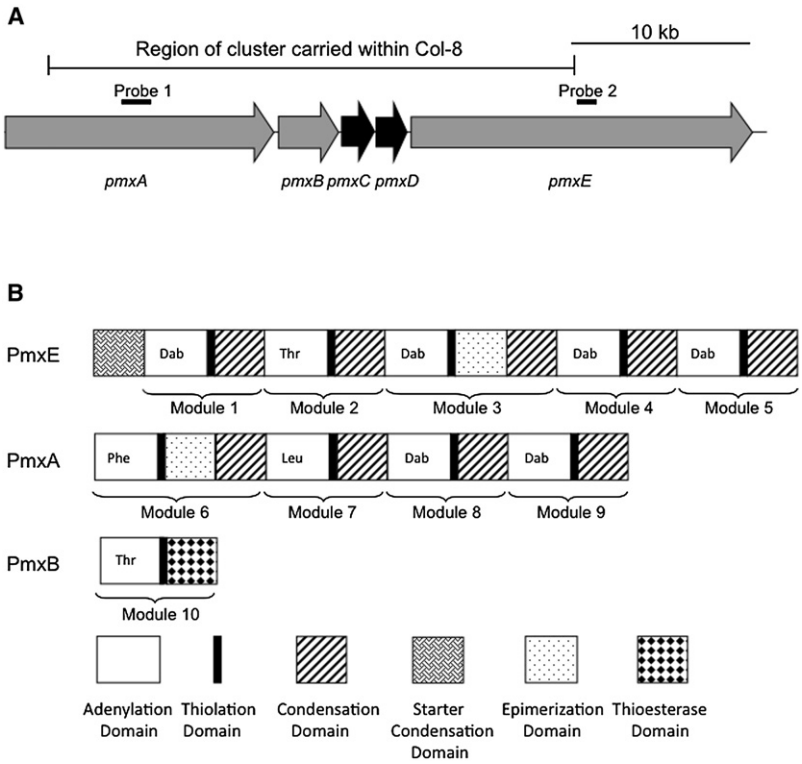


Figure 2. Gene Cluster for Polymyxin Biosynthesis in *P. polymyxa* PKB1

(A) Gray arrows represent NRPS genes whereas black arrows represent ABC transporter-like genes, and the direction of the arrows indicates the direction of transcription. Black bars, labeled probes 1 and 2, represent the probes used to screen the fosmid library. The figure is drawn to scale.

(B) Module and domain arrangement of the NRPSs for polymyxin biosynthesis.

See also Figure S3 in support of this figure.

ABC transporter genes are present in the middle of the polymyxin biosynthetic gene cluster of *P. polymyxa* PKB1 and were also reported in that of *P. polymyxa* E681 (Choi et al., 2009), but the role of these genes was not examined in the previous study. To determine their role in polymyxin biosynthesis, three deletion mutants were prepared by individually deleting *pmxC* (*ΔpmxC*) or *pmxD* (*ΔpmxD*), and by deleting both *pmxC* and *pmxD* together (*ΔpmxC+D*), again using the PCR-targeted gene disruption protocol and replacing the deleted genes with $Apra^{R}Cm^{R}$*oriT* cassettes. The validity of the mutants was again demonstrated by PCR analysis. The mutant and wild-type strains were grown in GSC medium, and methanol extracts of culture supernatants were assayed for antimicrobial activity against *E. coli* DH5α by agar diffusion bioassay. Whereas the wild-type strain produced metabolites active against *E. coli* DH5α as described earlier, this bioactivity was reduced in both the *ΔpmxC* and *ΔpmxD* mutants (Figure 3B). The bioactivity of the *ΔpmxC* mutant was slightly less than that of the *ΔpmxD* mutant, but the effects of the mutations were not additive because the residual bioactivity of the *ΔpmxC+D* double mutant was the same as that of the *ΔpmxC* single mutant.

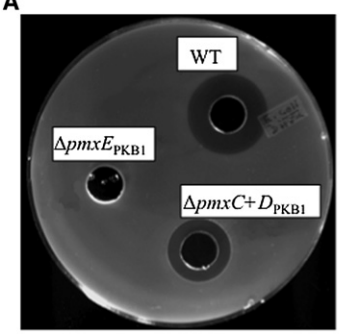
In NRPS gene clusters, ORFs are often transcribed as very large multicistronic transcripts, which leaves open the possibility that the reduction of polymyxin production seen in the *ΔpmxC* and *ΔpmxD* single mutants and in the *ΔpmxC+D* double mutant might be due to polar effects of the mutations on expression of the downstream gene, *pmxE*. To address this issue, a mutant carrying an unmarked, in-frame deletion of *pmxC* and *pmxD* (*ΔpmxC+D*-IF) was prepared. An FLP recombinase-catalyzed procedure was used to remove the *pmxC+D* genes from the wild-type strain, leaving in their place an 81 bp in-frame "scar" (Gust et al., 2003). Once again, the identity of the in-frame mutant was verified by PCR analysis. An agar diffusion bioassay of methanol extracts of the culture supernatants of the *ΔpmxC+D* and *ΔpmxC+D*-IF mutants indicated that both types of mutants had their bioactivities reduced to about the same extent as compared to the wild-type (Figure 3B), thereby discounting the possibility that the reduction in bioactivity was due to polar effects.

During a previous study of fusaricidin biosynthesis, the fusaricidin gene cluster was demonstrated to lack any potential transporter genes, leading to the hypothesis that a transporter gene from some other NRPS gene cluster might be responsible for export of fusaricidin as well as its cognate peptide products. Because both fusaricidin and polymyxin are cationic lipopeptides, the transporters from the polymyxin gene cluster seemed good candidates to fulfill this function. The culture supernatants of *ΔpmxC*, *ΔpmxD*, and *ΔpmxC+D* mutants, wild-type, and a *ΔfusA* mutant (with the NRPS for fusaricidin biosynthesis disrupted) available from a previous study (Li et al., 2007) were bioassayed for antifungal activity from fusaricidin using *Leptosphaeria maculans* as indicator organism. A pattern similar to that of polymyxin was observed. Antifungal bioactivity due to fusaricidin was not abolished, but was markedly reduced in *ΔpmxC* and *ΔpmxD* single mutants and in the *ΔpmxC+D* double mutant (Figure 3B). Culture supernatant from the *ΔfusA* mutant showed no bioactivity against *L. maculans*. This suggests that transport of both polymyxin and fusaricidin is mediated, at least in part, by the *pmxC* and *pmxD* genes of the polymyxin gene cluster. Because the fusaricidin gene cluster is not linked to the polymyxin gene cluster, this further supports our contention that the reduced bioactivity seen in transporter gene mutants is not due to polar effects.

Amino Acid Composition of Polymyxin

Although several structural types of polymyxins have been described, only one polymyxin gene cluster sequence, that of *P. polymyxa* E681, has been reported to date (Choi et al., 2009). It supports the production of polymyxin A, a rare form of polymyxin previously described only once, in 1947 (Ainsworth et al., 1947). In contrast, bioinformatic analysis of the NRPS encoded by the polymyxin gene cluster in *P. polymyxa* PKB1 predicted an amino acid composition identical to that of polymyxin B (**1** and **2**), except that the presence of an epimerization

A



B

Culture supernatant	Zone of inhibition (mm)	
	E. coli DH5a (polymyxin)	*L. maculans* (fusaricidin)
Wild type	21	21
ΔpmxE	8[a]	21
ΔpmxC	17	16
ΔpmxD	18	18
ΔpmxC+D	17	16
Δ pmxC+D-IF	17	nd

nd = not done

[a]No zone was seen surrounding the 8 mm well.

Figure 3. Production of Bioactive Metabolites by Wild-Type and Mutant Strains of *P. polymyxa* PKB1

(A) Agar diffusion bioassay of clarified methanol extracts (100 μl) of culture supernatants from wild-type, Δ*pmxC+D*, and Δ*pmxE* strains.

(B) Bioactivity due to polymyxin and fusaricidin production by various mutant strains.

domain in module 3 suggested that a D-DAB residue would be found in this position instead of the L-DAB found in polymyxin B (**1** and **2**) (Figure 2B). The overall sequence homology between the polymyxin gene clusters of *P. polymyxa* PKB1 and E681 is very high, about 92% at the nucleotide sequence level. However, in those regions of the cluster corresponding to the adenylation domains of modules 6 and 7, sequence homology dropped considerably, consistent with the prediction that different amino acids would be specified by those modules in polymyxin A from *P. polymyxa* E681, versus the polymyxin B (**1** and **2**) variants of *P. polymyxa* PKB1. Polymyxin B (**1** and **2**) is one of the more common forms of polymyxin discussed in the literature. In contrast, no polymyxin B (**1** and **2**) structural variant with D-DAB in position 3 has ever been reported, and so we considered the possibility that the epimerization domain in module 3 might be cryptic and that the gene cluster from *P. polymyxa* PKB1 might actually support production of authentic polymyxin B (**1** and **2**).

Analysis of the Bioactive Compounds

To characterize the compound responsible for the antibacterial bioactivity of *P. polymyxa* PKB1, we analyzed culture supernatants from the wild-type and mutant strains by HPLC and LC-MS. The metabolites in wild-type culture supernatant were first concentrated by adsorption to a Sep-Pak C_{18} cartridge and elution with methanol before separation by reverse-phase HPLC analysis. A 100 μl sample equivalent to 2 ml of culture supernatant was analyzed, and eluent fractions (1 ml) were collected and assayed for bioactivity against *E. coli* DH5α by agar diffusion bioassay. Two bioactive peaks were resolved in the HPLC profile of the wild-type culture supernatant sample (Figure 4A). Both peaks were missing from the *ΔpmxE* mutant and reduced in size in the *ΔpmxC+D* deletion mutants (Figure 4B).

LC-MS analysis of the wild-type culture supernatant concentrate showed that the two peaks contained ions with masses consistent with polymyxin B_1 (**1**) and B_2 (**2**) (Figure 4; see Figure S1 available online). High-resolution MS confirmed the proposed molecular formulas, and MS/MS analysis (Figure 4; Figure S1; Table S1) confirmed that the amino acid sequence is the same as for polymyxin B (**1** and **2**). However, when we analyzed a commercial polymyxin B (**1** and **2**) sample, which is a mixture of polymyxin B_1 (**1**) and B_2 (**2**) predominantly, the HPLC retention times for the bioactive peaks in a methanol extract of *P. polymyxa* PKB1 wild-type culture supernatant did not correspond to the peaks for authentic polymyxin B_1 (**1**) and B_2 (**2**) (Figure 5). This observation indicated that the bioactive compounds produced by *P. polymyxa* PKB1 are likely to be novel forms of polymyxin B_1 (**1**) and B_2 (**2**) with a D-DAB residue in position 3, as suggested by the bioinformatic analysis and, therefore, that the epimerization domain in module 3 is functional.

To confirm the stereochemistry of the amino acid residues in the polymyxins produced by *P. polymyxa* PKB1, degradation, derivatization, and chiral GC/MS analysis was carried out for material from each of the two bioactive peaks after purification by semipreparative HPLC (Figure 6; Figure S2). Each polymyxin peptide was hydrolyzed to its constituent amino acids and derivatized as the corresponding pentafluoropropanamide isopropyl ester. The derivatized peptide hydrolysates were analyzed by chiral GC/MS both individually and together with derivatized amino acid standards (Küsters et al., 1984). The two bioactive peptides each contained the same constituent amino acids: L-threonine, L-leucine, D-phenylalanine, L-DAB, and D-DAB. The relative peak areas of the DAB residues indicated a ratio of L:D DAB of 5:1, which again corresponds to the composition predicted by bioinformatic analysis.

Polymyxin antibiotics are lipopeptides, and yet no genes are found in the polymyxin gene cluster from *P. polymyxa* PKB1 to account for the lipidation of the peptide moiety. The DNA sequence available for the cluster extends just 8 bp upstream of the *pmxA* start codon, leaving open the possibility that genes associated with the activation or transfer of fatty acids might be found in this region. However, no such genes are found adjacent to the polymyxin clusters in either of the other *P. polymyxa* strains for which sequence information is available, and so it seems more likely that polymyxin synthesis resembles surfactin synthesis, and relies upon lipidation functions encoded elsewhere in the chromosome. In this regard, the N-terminal condensation domain of *pmxE*, which precedes the thiolation and adenylation domains for the first amino acid, L-DAB, shows

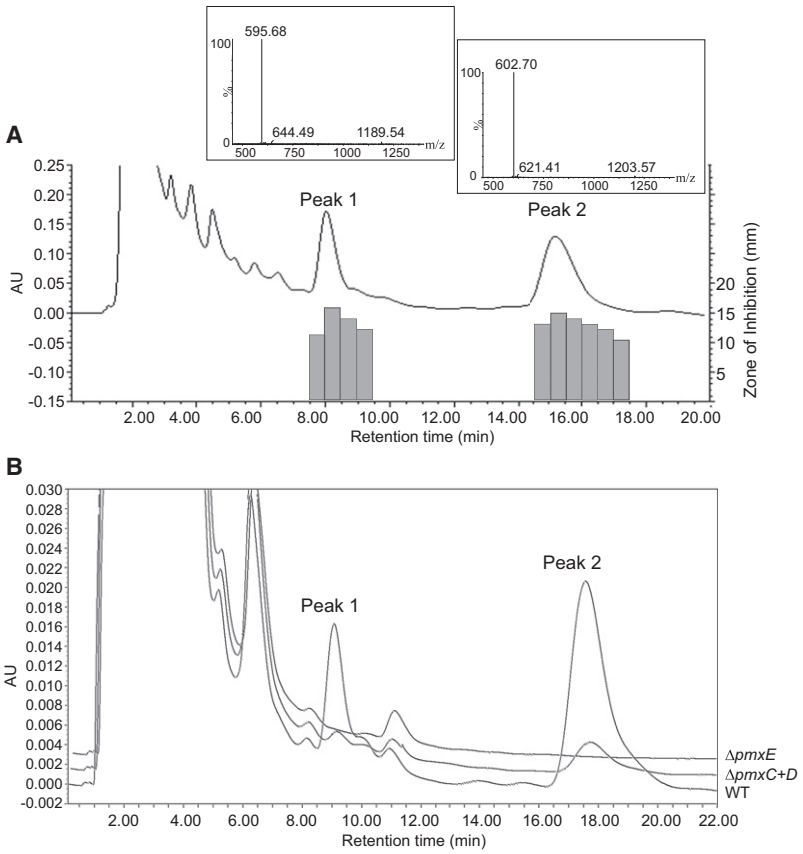


Figure 4. HPLC and Mass Spectrometry Analysis of Culture Supernatants of *P. polymyxa* PKB1 Wild-Type and Mutant Strains

(A) Methanol extract of wild-type culture supernatant was separated by HPLC, and fractions were collected and bioassayed to locate active peaks. Each active peak was analyzed by LC-MS, and the mass spectra obtained are shown (insets).

(B) HPLC profiles for methanol extracts of culture supernatants from wild-type and *ΔpmxE* and *ΔpmxC* mutant strains.

See also Figure S1 and Table S1 in support of this figure.

DISCUSSION

The gene cluster responsible for the production of polymyxin in *P. polymyxa* PKB1 was identified and subjected to DNA sequence analysis. The cluster shows extensive similarity to the polymyxin gene cluster from *P. polymyxa* E681, the only other polymyxin cluster reported to date, but notable differences are also evident. Overall, the sequences of the two clusters are highly similar except for the first two modules of *pmxA*, which correspond to amino acid residues 6 and 7 in the polymyxins, the amino acids that differ between polymyxin A and polymyxin B (**1** and **2**). *P. polymyxa* E681 produces only one form of polymyxin, polymyxin A_1, which is consistent with the sequence of its gene cluster.

similarity to so-called starter condensation domains that attach lipids to amino acids in other lipopeptides, rather than catalyzing the formation of a peptide bond between two amino acids. Two alternative systems have been described for lipid attachment, typified by the surfactin, plipstatin, lichenysin system in *Bacillus* spp. and the ramoplanin, daptomycin system in actinomycetes. The starter domain for polymyxin formation in *P. polymyxa* PKB1 clearly shows a closer relationship to the surfactin-type system than to the actinomycete system (Figure 2B; Figure S3).

We now show that *P. polymyxa* PKB1 produces two unusual polymyxins, and we propose that these two new metabolites are stereochemical variants **3** and **4** of polymyxin B_1 (**1**) and B_2 (**2**). Both of these variant metabolites have the same amino acid composition, and this composition is consistent with that predicted by bioinformatic analysis of the NRPS-encoding genes of the gene cluster. They differ from one another in the nature of the lipid side chain, which mass spectrometric analysis suggests is isooctanoate in the case of peak 1 and methyloctanoate in the

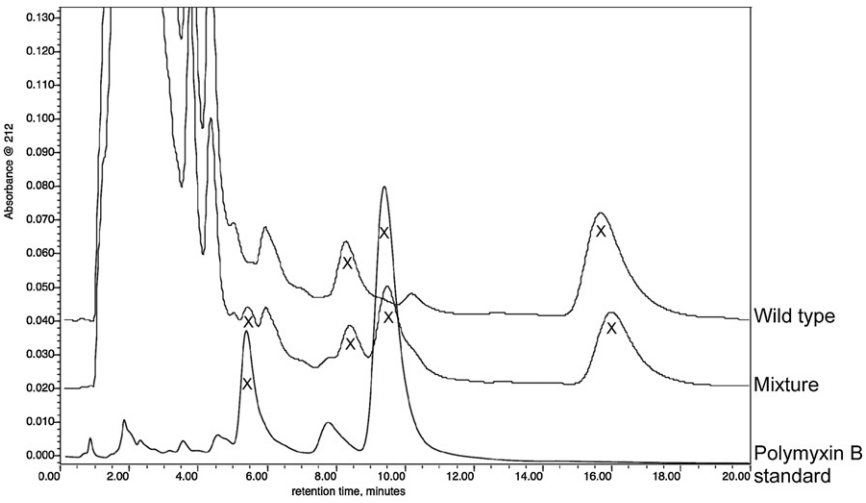


Figure 5. HPLC Analysis of Culture Supernatant from Wild-Type *P. polymyxa* PKB1 in Comparison to Authentic Polymyxin B Standard

Samples of methanol extracts of culture supernatant alone, polymyxin B standard alone, and an equal mixture of the two were subjected to HPLC analysis. Fractions were collected and analyzed for bioactivity. Bioactive peaks are marked with an "×."

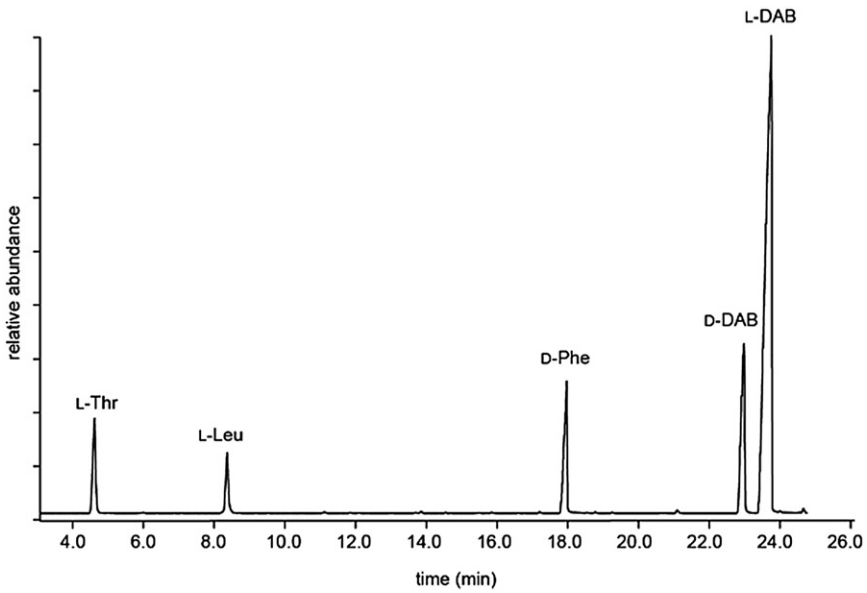


Figure 6. Chiral GC/MS Chromatogram Showing the Constituent Amino Acids of D-DAB$_3$-Polymyxin B$_1$ (3) after Acidic Hydrolysis and Subsequent Derivatization as Pentafluoropropanamide Isopropyl Esters

The amino acids of the peptides are labeled on the trace. See also Figure S2 in support of this figure.

case of peak 2. We designate these variant forms D-DAB$_3$-polymyxin B$_1$ (**3**) and D-DAB$_3$-polymyxin B$_2$ (**4**) (Figure 7) to indicate that they differ from normal polymyxin B$_1$ (**1**) and polymyxin B$_2$ (**2**) only in the stereochemistry of the DAB residue in position 3, which is L-DAB in normal polymyxin B (**1** and **2**) but D-DAB in these new compounds. Because the biological properties of peptide antibiotics can be markedly affected by the presence of D-amino acids (Hong et al., 1999; Lee et al., 2004), these new variants of polymyxin B (**1** and **2**) may have novel activity or spectrum properties worth evaluation. In particular, the location of the D-DAB$_3$ residue as the first amino acid in the lipotripeptide side chain adjacent to the heptapeptide ring of the polymyxins may make the structural consequences of the variation even more pronounced (Velkov et al., 2010).

Secondary metabolites are often produced as families of compounds closely related in structure, and the particular array of metabolites produced varies depending on the composition of the growth medium and cultural conditions. This variability in the nature of the end products is due to flexibility in the substrate specificity of the enzymes involved, such that a single suite of biosynthetic enzymes gives rise to all of the various structural forms (Kajimura and Kaneda, 1997; Schimana et al., 2001). In contrast, we now see that the polymyxin family of antibiotics apparently arises, not from a single NRPS system with relaxed substrate specificity, but rather from an array of closely related NRPSs, each carrying a different complement of modules specifying the amino acids particular to its polymyxin product. Thus, the collection of polymyxin NRPSs found in the various strains of *P. polymyxa* that have been isolated over the years represents a naturally occurring example of combinatorial biochemistry driven by evolution rather than by in vitro manipulation. Examination of the junctions that have evolved naturally between modules in these various forms may provide insights that can inform the attempts of researchers seeking to create novel peptide antibiotics artificially.

The polymyxin gene cluster from *P. polymyxa* E681 became available upon analysis of the complete genome sequence for this strain, and a second *P. polymyxa* complete genome sequence, for strain SC2, has recently become available. No analysis of the polymyxin gene cluster has yet been reported for the SC2 strain, but evidence of some elements of a polymyxin gene cluster is present. However, many of the polymyxin-producing genes are listed as pseudogenes and appear to be truncated or rearranged. Another possibility is that this region of the genome sequence has not yet been fully verified, and that an intact polymyxin gene cluster does exist in the strain. The nature of the NRPS-encoding genes for polymyxin biosynthesis makes it very difficult to assemble fragments of DNA sequence correctly. NRPS modules, regardless of the amino acids that they specify, show a considerable degree of sequence similarity. The six DAB-specifying modules found in the polymyxin NRPSs have long stretches of sequence that are nearly 100% identical, and so assembling DNA sequence information, as well as performing any experimental techniques that rely upon hybridization of probes or primers to a DNA template such as Southern hybridization and PCR, is exceptionally challenging. With this in mind, we examined the SC2 *pmxA*, *B*, and *E* genes as identified in the genome sequence deposit using the PKS/NRPS Analysis website (http://nrps.igs.umaryland.edu/nrps), and were able to identify several modules, including module 3 from the *pmxA* gene, which specifies that there would be a D-DAB residue in this position if a polymyxin product is produced by this strain.

It is striking that the first polymyxin gene cluster, that from *P. polymyxa* E681, supported the production of polymyxin A,

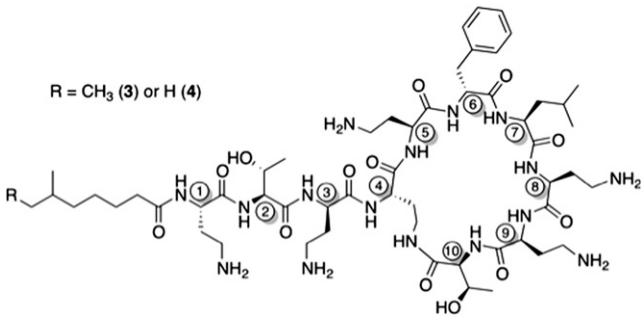


Figure 7. Chemical Structure of D-DAB$_3$-Polymyxin B$_1$ (3) and D-DAB$_3$-Polymyxin B$_2$ (4)

B$_1$ when R = CH$_3$ and B$_2$ when R = H.

a rare form of polymyxin containing a D-DAB residue in position 3, and bioinformatic examination of the genome sequence of *P. polymyxa* SC2 suggests that it may also produce a polymyxin containing a D-DAB residue in position 3. We now report the polymyxin gene cluster of a third *P. polymyxa* strain, PKB1, and, again, it specifies a polymyxin with D-DAB in position 3. This stands in marked contrast to the literature, which would suggest that these forms of polymyxins are rare. Production of peptide metabolites is not a uniform property of all members of this species, with production of various peptide products reported for only certain strains of the species. Perhaps the fact that all three of the polymyxin gene clusters examined to date are from strains of *P. polymyxa* isolated and studied for their potential as agents of biocontrol of plant fungal diseases is relevant to this observation. All three strains are also producers of fusaricidin, and this may mean that these strains are a closely related subgroup of *P. polymyxa* strains, in evolutionary terms.

SIGNIFICANCE

The polymyxin gene cluster from *Paenibacillus polymyxa* PKB1 has been sequenced and the two major polymyxin products (3 and 4) produced by this strain have been identified as unusual forms of polymyxin B (1 and 2), with D-DAB residues in place of L-DAB in amino acid position 3. The substitution of L for D forms of amino acids in peptide antibiotics can markedly influence both the spectrum and degree of bioactivity. Polymyxins are considered to be drugs of last resort to combat infections by multidrug-resistant Gram-negative bacteria, and so this report of variant forms of polymyxins may be of therapeutic interest if the products have altered bioactive properties or reduced toxicity. To date, only one gene cluster for polymyxin biosynthesis has been described in the literature. This work provides the sequence of a second cluster and indicates that the different forms of polymyxins arise from nonribosomal peptide synthetases (NRPSs) that have evolved to contain modules with different specificity codes, rather than arising from a single flexible-substrate NRPS system. It also raises interesting questions about the evolutionary relationships between polymyxin-producing organisms, and provides data potentially useful for making new forms of polymyxin through site-directed mutation of NRPS gene sequences.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions

Bacterial strains and plasmid, cosmid, and fosmid vectors used in this study are listed in Supplemental Experimental Procedures. *E. coli* strains were routinely grown in Lennox broth (LB) at 37°C for 16 hr on a shaker at 250 rpm or a tube roller. Antibiotics were added to the medium when appropriate at the following concentrations: 100 μg/ml ampicillin, 50 μg/ml apramycin, 25 μg/ml chloramphenicol, and 50 μg/ml kanamycin. LB agar medium was prepared by adding 15 g/l agar to the LB. *E. cloni* Replicator (Lucigen) strains containing fosmid clones were grown in tryptone broth (TB; 11.8 g/l Bacto tryptone, 23.6 g/l yeast extract, 9.4 g/l K_2HPO_4, 2.2 g/l KH_2PO_4, 2 g/l glycerol) containing 12.5 μg/ml chloramphenicol, for 16 hr at 37°C with shaking at 225 rpm. When increased fosmid copy number was desired, 1× Replicator induction solution (Lucigen) was included to activate the medium-copy number origin of replication (*oriV*) of the pSmart vector to increase its number to 20–50 copies per cell.

P. polymyxa PKB1 strains were routinely grown in glucose broth (GB; 10 g/l glucose, 10 g/l peptone, 2 g/l beef extract, 1 g/l yeast extract, 5 g/l NaCl) or Brain Heart Infusion (BHI; Difco) at 37°C in a tube roller or rotary shaker for 16 hr at 28°C. When required, 15 g/l agar was added to GB or BHI, and when appropriate, chloramphenicol was added to 5 μg/ml.

L. maculans was grown on potato dextrose agar (PDA) at 28°C for 3 d to produce spores for use as an indicator strain for antifungal bioassay.

DNA Isolation, Manipulation, Cloning, and Sequencing

Plasmid isolation and cloning were carried out according to standard methods (Birnboim and Doly, 1979). A salting-out protocol originally described for the isolation of genomic DNA from *Streptomyces* (Kieser et al., 2000) was used to prepare gDNA from *P. polymyxa* PKB1. Restriction endonucleases and other DNA manipulating and cloning products were manufactured by Roche, New England BioLabs, or Fermentas Life Sciences. All of the oligonucleotide primers used were prepared by Integrated DNA Technologies. DNA fragments separated by agarose gel electrophoresis were isolated using QIAquick gel extraction kits (QIAGEN). Sequencing of DNA was routinely carried out using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Screening of Fosmid Library

All fosmid clones from the PKB1 genomic library were patched with sterile toothpicks on LB agar plates (12 × 12 in) containing ampicillin at 100 μg/ml. The plates were incubated at 37°C for 16 hr and then colonies were transferred and lysed, and the DNA was fixed to Hybond-N nylon membranes using a standard colony-lift procedure (Sambrook et al., 1989).

The probes used to screen the fosmid library were prepared based on the polymyxin gene sequences obtained from Supercos-1 cosmid clones. Probe 1 was a 1.67 kb EcoRI fragment isolated from an area near the 5′ end of the polymyxin gene cluster fragment in cosmid Col-8. Probe 2 was a 1.11 kb DNA fragment specific to an area just beyond the 3′ end of the polymyxin gene cluster and generated with primers pSHA09 and pSHA10 using cosmid Col-15 as a template. Potential polymyxin gene cluster-containing clones in the fosmid library were identified by hybridization with nonradioactively labeled DNA probes prepared using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science) according to the manufacturer's instructions.

Generation of Deletion and Disruption Mutants

All *P. polymyxa* mutants were generated using REDIRECT technology, a PCR-targeting gene disruption system originally developed for use in *Streptomyces* (Gust et al., 2003) and adapted for use in *Paenibacillus* (Li et al., 2007). Disruption mutants were generated by replacing all (*ΔpmxC*, *ΔpmxD*, and *ΔpmxC+D*) or part (*ΔpmxE*) of the genes with a PCR-amplified $Apra^RCm^R$*oriT* cassette by taking advantage of the λ-red-mediated recombination functions carried on plasmid pIJ790. Cosmid Col-9 was used as the platform for creation of all of the mutants. In each case, the mutation was targeted by incorporating sequences specific for the gene(s) to be deleted onto the 5′ ends of the PCR primers used to amplify the $Apra^RCm^R$*oriT* cassette. PCR reaction mixtures used to amplify the $Apra^RCm^R$*oriT* cassette contained 50 ng of template DNA, 50 pmol of each primer, 50 μmol of each dNTP, and 0.5 U of Taq DNA polymerase in Taq buffer (200 mM Tris-HCl [pH 8.3], 500 mM KCl, 25 mM $MgCl_2$). PCR reaction mixtures were incubated at 95°C for 3 min, then 10 cycles of 95°C for 45 s, 50°C for 45 s, and 72°C for 2 min, followed by 20 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 2 min, with a final elongation at 72°C for 5 min.

Oligonucleotide primers used in this study are listed in Supplemental Experimental Procedures.

Generation of In-Frame Deletion Mutants

In-frame deletion mutants of *P. polymyxa*, free of the $Apra^RCm^R$*oriT* cassette, were generated according to Gust et al. (2003) with some modifications. The Col-9 *ΔpmxC+D* mutant cosmid that was used to create the *ΔpmxC+D* double mutant was transformed into *E. coli* BT340, an *E. coli* DH5α derivative strain carrying a plasmid that expresses FLP recombinase. FLP recombinase recognizes the FRT sites flanking the $Apra^RCm^R$*oriT* cassette and catalyzes site-specific recombination to remove the intervening sequence between the FRT sites, leaving an 81 bp in-frame "scar." Once recombination had taken

place, the temperature-sensitive FLP recombinase plasmid was removed by growing the cells at 42°C. Cosmids isolated from colonies showing apramycin and chloramphenicol sensitivity (indicating loss of the Apra^{R}Cm^R*oriT* cassette) were screened by restriction endonuclease digestion to confirm that the recombination had occurred.

Because the Cm^R gene was the only antibiotic resistance marker found to be useful for selecting *P. polymyxa* PKB1 exconjugants, the newly constructed Col-9 *ΔpmxC+D*-IF cosmid lacked a usable selectable marker. Therefore, it was subjected to a second round of PCR-targeted gene replacement to replace the resident kanamycin resistance gene of Supercos-1 with the Apra^{R}Cm^R*oriT* cassette. The Apra^{R}Cm^R version of Col-9 *ΔpmxC+D*-IF was then conjugated to wild-type *P. polymyxa* PKB1, and Cm^R-resistant exconjugants, resulting from integration of the Apra^{R}Cm^R version of Col-9 *ΔpmxC+D*-IF into the chromosome through homologous recombination, were cultivated in the absence of Cm to allow loss of the integrated plasmid by a second recombination event. Genomic DNA from Cm^S colonies was analyzed by PCR to identify those in which a second recombination event resulted in replacement of the wild-type *pmxC* and *pmxD* genes by the unmarked in-frame deletion.

The authenticity of all mutants was confirmed by PCR analysis of gDNA.

Bioassays for Antibacterial and Antifungal Activity

The wild-type and mutant strains of *P. polymyxa* PKB1 were grown in GSC medium (20 g/l glucose, 20 g/l starch, 20 g/l $(NH_4)_2SO_4$, 10 g/l yeast extract, 2.6 g/l K_2HPO_4, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.25 g/l NaCl, 9.0 g/l $CaCO_3$) on a rotary shaker for 72 hr at 30°C and 250 rpm, for antibiotic production. Cultures were harvested by centrifugation, and the culture supernatants were mixed with methanol to give a final concentration of 20% (v/v) and held at 4°C for 30 min. The methanol-culture supernatant mixtures were centrifuged at 15,000 × *g*, and the clarified extracts were analyzed for antibacterial activity by agar diffusion bioassay. Molten LB agar inoculated with *E. coli* DH5α culture to 0.1% (v/v) was poured into plates, and 100 μl samples were applied to wells cut with an 8 mm sterilized cork borer. The plates were then incubated at 37°C for 16 hr and the zones of inhibition were measured.

In some cases, the metabolites in culture supernatants were first concentrated by adsorption to a Sep-Pak C_{18} cartridge (Waters) and elution with methanol before analysis.

Whole-culture supernatants were assayed for antifungal activity because of the production of fusaricidin by agar diffusion bioassay against *L. maculans* as an indicator fungus. PDA plates were spread with a spore stock of *L. maculans*, wells were punched with a sterile 10 mm borer, and 150 μl amounts of culture supernatants were added to each well. The plates were incubated at 28°C for 3 days and the zones of inhibition were measured.

HPLC Analysis of Culture Supernatants

HPLC analysis was carried out using an Alliance 2695 separation module controlled by Waters Millenium^{32} software (version 3.20). Twenty-five microliter samples of clarified methanol extracts were analyzed on a reverse-phase Bondclone C_{18} column (8 mm × 100 mm, 10 μm; Phenomenex) with a mobile phase consisting of 0.1 M Na_2HPO_4 (adjusted to pH 3.0 with H_3PO_4) and acetonitrile (77:23) at a flow rate of 2.0 ml/min for 25 min with eluent absorbance monitored at 212 nm. Alternatively, when eluent was to be assayed for bioactivity, 100 μl of Sep-Pak-concentrated sample, equivalent to 2 ml of culture supernatant, was analyzed, and eluent fractions (1 ml) were collected and assayed for bioactivity against *E. coli* DH5α by agar diffusion bioassay.

LC-MS Analysis of Culture Supernatants

LC-MS analysis of culture supernatants of wild-type and mutant *P. polymyxa* strains was carried out using a single-quadrupole mass spectrometer (Micromass ZMD-2; Waters) controlled by MassLynx software. Samples were analyzed by injecting 5–10 μl of Sep-Pak-concentrated culture supernatant onto a reverse-phase XTerraMS C_{18} column (2.1 mm × 150 mm, 5 μm; Waters) with a mobile phase consisting of 0.1% formic acid (adjusted to pH 3.0 with ammonium hydroxide) and acetonitrile (77:23) at a flow rate of 0.2 ml/min for 20 min. The column was kept at a temperature of 25°C and eluent absorbance was monitored at 212 nm. Mass spectrometric data were acquired for the total ion current of the samples ionized over time with electrospray ionization operated in a positive ion mode. The other MS parameters were source temperature 150°C, desolvation temperature 300°C, cone voltage 15V, and nitrogen gas flow 15 l/min. Full mass spectra were acquired over a mass range of m/z 450–1,400 with a scan time of 1.0 s and interscan time of 0.1 s.

Material representing each bioactive peak was purified for subsequent analysis by semipreparative HPLC. One hundred microliter samples of Sep-Pak-concentrated culture supernatant were repeatedly injected onto the Bondclone C_{18} column and eluted using a mobile phase of 0.1% formic acid (adjusted to pH 3.0 with ammonium hydroxide) and acetonitrile (77:23) at a flow rate of 2 ml/min. Bioactive fractions were pooled and concentrated on a rotary evaporator.

High-Resolution MS and MS/MS Sequencing

After HPLC purification, the high-resolution matrix-assisted laser desorption ionization-Fourier transform ion cyclotron resonance-mass spectrometry (MALDI-FTICR-MS) spectrum of the peptides (**3** and **4**) was obtained using a Bruker 9.4T Apex-Qe FTICR (Bruker Daltonics). For D-DAB$_3$-polymyxin B_1 (**3**), an elemental composition of $C_{56}H_{99}N_{16}O_{13}$ for the $(M+H)^+$ ion was obtained with a mass accuracy of 0.63 ppm. For D-DAB$_3$-polymyxin B_2 (**4**), an elemental composition of $C_{55}H_{97}N_{16}O_{13}$ for the $(M+H)^+$ ion was obtained with a mass accuracy of 0.81 ppm. The proposed peptide sequences were confirmed by tandem MALDI-MS/MS analysis (Figure 4; Figure S1; Table S1). To do this, HPLC-purified and concentrated peptide solutions were directly spotted onto a Bruker Daltonics MTP AnchorChip 800/384 target and air dried. Then 0.42 μl of α-cyano-4-hydroxycinnamic acid matrix solution was spotted on top and air dried. The matrix solution was prepared as follows. A 36 μl aliquot of a saturated matrix solution prepared in 0.1% TFA in 90:10 ACN:H_2O was diluted to a final volume of 800 μl using 0.1% TFA in 85:15 ACN:H_2O, containing 1 mM ammonium phosphate. Mass spectra were obtained in the positive reflectron mode of ionization using a Bruker Daltonics ultrafleXtreme MALDI TOF/TOF mass spectrometer. The MS/MS spectra were obtained manually with collision-induced dissociation gas turned off. DataAnalysis, BioTools, and Sequence Editor software packages provided by the manufacturer were used for analysis of the mass spectra.

MS/MS sequencing of D-DAB polymyxin B_1 (**3**) and B_2 (**4**) is made more complex by the cyclic nature of the peptides, as the C terminus of each peptide is connected to the amino side chain of residue 4 by an amide bond. This means that the formation of smaller ions is often the product of two fragmentations, one to open the ring and a second to shorten the resulting linear peptide. When the peptide fragments in the spectrometer and the ring is opened, either the bond between the C terminus and the side chain of residue 4 or the backbone amide bond between residues 4 and 5 breaks. When the side chain to C-terminal bond breaks, the resulting linear peptide follows the sequence residues 1–10. However, if the backbone bond between residues 4 and 5 breaks, the resulting linear peptide has the sequence 1, 2, 3, 4, 10, 9, 8, 7, 6, 5. Using both sets of linear fragments along with larger fragments containing an intact ring, the sequences of **3** and **4** were confirmed.

Hydrolysis and Derivatization of Polymyxin Samples

Each peptide sample (1 mg) was heated to 110°C in a sealed tube in 6 M HCl (3 ml) for 18 hr. The solution was allowed to cool to room temperature and the solvent was removed in vacuo. Acetyl chloride (1.5 ml) was added dropwise to isopropyl alcohol (5 ml) at 0°C. This solution was added to the peptide residue, and the mixture was heated to 110°C in a sealed tube for 45 min. The solution was allowed to cool and the solvent was removed in vacuo. A solution of pentafluoropropionic anhydride (1 ml) in dichloromethane (3 ml) was added to the peptide residue, and the mixture was heated to 110°C in a sealed tube for 15 min. The mixture was allowed to cool to room temperature and then the solvent was evaporated under a stream of argon. The resulting residue was dissolved in dichloromethane (1 ml) and transferred to a vial for storage. Derivatization of standard amino acids was carried out in the above manner, except the first step used 0.2 M HCl as solvent and was only heated for 15 min.

Chiral GC/MS Analysis of Polymyxin Samples

The hydrolyzed and derivatized samples of **3** and **4** were analyzed using chiral GC/MS. All samples were analyzed using a Varian 25 m × 0.22 mm fused silica wall-coated open tubular Chirasil-L-Val (0.12 μm) column on an Agilent Technologies 7890 GC with 5975C MSD and 7683B series injector. Each sample was introduced into the machine dissolved in dichloromethane via a pulsed

split-less injection. The temperature method used was 90°C (5 min) raised to 180°C by 3°C/min.

Each peptide was analyzed independently, and then the two peptides were mixed together and analyzed (Figure 6; Figure S2). It was found that **3** and **4** contained the same amino acids. The identity of each amino acid in the peptide was confirmed by sequential addition of derivatized standard amino acids. This confirmed that the amino acids present were indeed L-threonine, L-leucine, D-phenylalanine, L-2,4-diaminobutyric acid, and D-2,4-diaminobutyric acid. The integration supports that the ratio of L:D diaminobutyric acid is 5:1.

ACCESSION NUMBERS

The GenBank accession number for the *P. polymyxa* PKB1 DNA sequence encompassing the polymyxin gene cluster reported in this paper is JN660148.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.09.017.

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