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Efficient Production of Polymyxin in the Surrogate Host *Bacillus subtilis* by Introducing a Foreign *ectB* Gene and Disrupting the *abrB* Gene

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In our previous study, *Bacillus subtilis* strain BSK3S, containing a polymyxin biosynthetic gene cluster from *Paenibacillus polymyxa*, could produce polymyxin only in the presence of exogenously added L-2,4-diaminobutyric acid (Dab). The dependence of polymyxin production on exogenous Dab was removed by introducing an *ectB* gene encoding the diaminobutyrate synthase of *P. polymyxa* into BSK3S (resulting in strain BSK4). We found, by observing the complete inhibition of polymyxin synthesis when the *spo0A* gene was knocked out (strain BSK4-0A), that Spo0A is indispensable for the production of polymyxin. Interestingly, the *abrB-spo0A* double-knockout mutant, BSK4-0A-rB, and the single *abrB* mutant, BSK4-rB, showed 1.7- and 2.3-fold increases, respectively, in polymyxin production over that of BSK4. These results coincided with the transcription levels of *pmxA* in the strains observed by quantitative real-time PCR (qRT-PCR). The AbrB protein was shown to bind directly to the upstream region of *pmxA*, indicating that AbrB directly inhibits the transcription of polymyxin biosynthetic genes. The BSK4-rB strain, producing high levels of polymyxin, will be useful for the development and production of novel polymyxin derivatives.

Humans currently face a very serious threat from multiple antibiotic-resistant bacteria, commonly called superbugs (20). Multidrug resistance is an important issue for public health and is frequently found in pathogenic Gram-negative bacteria, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (20, 21).

Polymyxin, a long-known lipopeptide antibiotic having bactericidal activity against Gram-negative bacteria (1), 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 (15). However, adverse effects, such as nephrotoxicity and neurotoxicity, have been reported during the early clinical administration of polymyxin, and the clinical use of polymyxin has been limited, although it has excellent antisuperbug activity (9, 33). Therefore, it will be another challenge to develop new polymyxin derivatives having improved pharmacological and toxicological characteristics. During the last few decades, many studies have been conducted to generate polymyxin derivatives by chemical synthesis or enzymatic modifications and to analyze structure-function relationships to develop improved polymyxins having higher activity and lower toxicity (32). Although some interesting results have been obtained from these trials, the efficiency of generating derivatives using these approaches has been low (30).

We recently completed whole-genome sequencing of *Paenibacillus polymyxa* E681 (13), located nonribosomal peptide synthetase genes responsible for the synthesis of polymyxin and fusaricidin, and analyzed their functions (2, 3). We then succeeded in transferring the entire polymyxin synthetase gene cluster (*pmxABCDE*) into the chromosome of the surrogate host (*Bacillus subtilis* 168) and producing polymyxin in the recombinant strain. Molecular tools and technologies for genetic manipulation and strain improvement have been highly developed for use in *B. subtilis*, but those tools are very poor for use in *P. polymyxa*. Therefore, the *Bacillus* expression system will be very useful for conducting studies to analyze structure-function relationships, to efficiently generate polymyxin and polymyxin derivatives, and to improve polymyxin purity. Polymyxin synthesis by the recombinant *B. subtilis* strain was possible, however, only with the exogenous addition of L-2,4-diaminobutyric acid (Dab), a main substrate for polymyxin, and the production level was low (2). Therefore, it was necessary to solve these problems to increase the usefulness of the system.

In this study, we attempted to enable the recombinant *B. subtilis* strain to efficiently produce polymyxin without the addition of Dab by insertion of a gene necessary for the synthesis of Dab and to increase the production level of polymyxin by control of genes affecting the expression of the polymyxin biosynthetic gene cluster. We also attempted to reveal the mechanism for regulation of polymyxin production by analyzing the effect of null mutations of *spo0A* or *abrB* on the expression of *pmxA* and by observing the mode of interaction between AbrB and the promoter region of *pmxA*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genetic material. The bacterial strains, plasmids, and primers used in this study are described in Table 1. *Escherichia coli* DH5α was used for construction of recombinant plasmids and for monitoring of polymyxin activity. *B. subtilis* strains were grown in

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TABLE 1 Bacterial strains, plasmids, and primers used in the study

Strain, plasmid, or primer	Relevant genotype/characteristics/sequence (5′ to 3′)[a]	Reference/source
Strains		
E. coli		
DH5α	Strain for general DNA manipulation and monitoring of polymyxin activity	8
BL21(DE3)	Host strain for overexpression under controlled T7 promoter	29
B. subtilis		
BSK3S	Strain containing the entire polymyxin synthetase gene cluster on *amyE* locus, functional *sfp* gene	2
BSK4	*srfC*::*etcB-tet* of BSK3S	This study
BSK4-0A	*spo0A*::*erm* of BSK4	This study
BSK4-rB	*abrB*::*neo* of BSK4	This study
BSK4-0A-rB	*spo0A*::*erm* of BSK4-rB	This study
P. polymyxa E681	Wild type; produces polymyxin A	23
Plasmids		
pHPS9	*E. coli-B. subtilis* shuttle vector, cm, erm	6
pGEM-T Easy	Plasmid for cloning of PCR products, Ap	Promega
pET22b	Plasmid for expression of His-tagged protein, Ap	Novagen
pET22-bsabrB	Plasmid containing *abrB* gene of *B. subtilis* 168	This study
Primers		
ectF	CCTGTTGATAGAGTAAACTCC	
ectR	ATGGCCCGTTTGTTGATGAGCAACTAGAGCAATCC	
tetF	AACAAACGGGCCATATTGTTG	
tetR	GATACAAGAGAGGTCTCTCG	
srfC1	AGATATGTATTACCTATCGCC	
srfC2	TTACTCTATCAACAGGGATTGGCGTTCACTGCTTCCTTG	
Srf3	ACGAGAGACCTCTCTTGTAGGATTGGATGAAGGGGCTTCGCT	
Srf4	TCAACGCTTCGACATCACTTTC	
mlkneoF	TCGAGATCAGGGAATGAGTTT	
mlkneoR	AATAAATACGTAACCAACATG	
abrff	CTGTACCAGCCTTCATACTC	
abrfr-neo	AAACTCATTCCCTGATCTCGAACACGTCCTAATTCATCAAC	
abrrf-neo	CATGTTGGTTACGTATTTATTCATCAGCGAAATCCAAAACC	
abrrr	CAAATCGAGCCGAAACGTGTAC	
Bsabr-nde	TACATATGTTTATGAAATCTACTGGTATTG	
Bsabr-xho	TGCTCGAGTTTAAGGTTTTGAAGCTGG	
pmxAF/pmxAF-biotin	AGCATATTGAAGCAGAGGAG	
pmxAR/pmxAR-biotin	TCATCGGTAAAAGCAATCCGG	
BSF349	AGGCAGCAGTGGGGAAT	
16S-786R	CTACCAGGGTATCTAATC	

[a] Resistance gene or phenotype against antibiotics: tet (tetracycline), erm (erythromycin), neo (neomycin), cm (chloramphenicol), and Ap (ampicillin). The restriction sites of endonucleases in nucleotide sequences are underlined.

LB broth or LB agar medium at 37°C for general purposes and in Cal18 medium (10) with shaking at 37°C for studies of polymyxin production. A supplemental solution of antibiotics contained 5 μg/ml chloramphenicol, 1 μg/ml erythromycin, 100 μg/ml spectinomycin, 10 μg/ml tetracycline, 8 μg/ml neomycin, and 100 μg/ml ampicillin. L-2,4-diaminobutyric acid was purchased from Sigma-Aldrich (St. Louis, MO).

Construction of recombinant strains. *B. subtilis* BSK4 was constructed by the following procedures. The putative *ectB* gene (PPE_02294 in GenBank accession no. CP000154) was obtained by PCR with the primer set ectF and ectR from chromosomal DNA of *P. polymyxa* E681. The PCR product was fused to a tetracycline resistance gene amplified from plasmid pBC16 (19) with primers tetF and tetR, using a fusion PCR method to construct an *ectB-tet* cassette. The N-terminal region and the C-terminal region of *srfC* were amplified from the chromosomal DNA of *B. subtilis* with primer sets consisting of srfC1 and srfC2, and srf3 and srf4, respectively. The two PCR fragments were fused with the *ectB-tet* cassette by a second fusion PCR and then introduced into the *srfC* locus of strain BSK3S by homologous recombination to construct strain BSK4 (Fig. 1A).

The strain BSK4-0A was constructed by homologous recombination using BS9903 chromosomal DNA (24) and then selecting erythromycin-resistant transformants. The *abrB* mutant strain, BSK4-rb, was constructed by a procedure shown in Fig. 1A. Two DNA fragments containing an N-terminal and upstream region and a C-terminal and downstream region of *abrB*, respectively, were amplified by PCR with primer sets consisting of abrff and abrfr-neo, and abrrf-neo and abrrr, respectively. The neomycin resistance gene was obtained from the pMLK83 plasmid (11) by PCR with primers mlkneoF and mlkneoR. The three PCR products were joined by a second PCR, and the resulting product was introduced into the strains BSK4 and BSK4-0A to construct BSK4-rB and BSK4-0A-rB, respectively, by homologous recombination.

Antibacterial activity assay and ESI-LC/MS analysis. Recombinant *B. subtilis* strains were grown in Cal18 medium at 37°C with vigorous shaking (220 rpm), and the cell-free supernatants were harvested at different times (18, 24, 30, and 36 h). A 5-μl aliquot of each culture was dropped onto *E. coli* plates for antibacterial assay using a previously described method (2). Electrospray ionization-liquid chromatography/mass spectrometry (ESI-LC/MS) analysis was performed using a method previously described by Choi et al. (2). For quantitative analysis of polymyxin produced by *B. subtilis* strains, high-performance liquid chromatography (HPLC) separation was performed on a Finnegan Surveyor

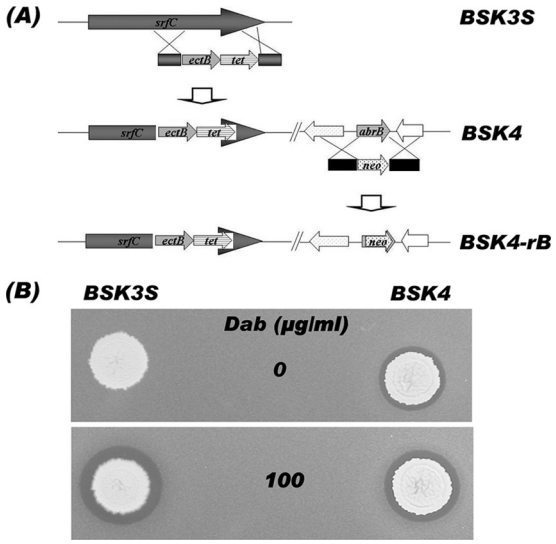

FIG 1 Schematic diagram showing the strategy for construction of BSK4 and BSK4-rB from BSK3S (A) and antibacterial activities of BSK3S and BSK4 with and without addition of exogenous Dab (B). The BSK4 strain was constructed by introducing the *ectB* gene of *P. polymyxa* E681 into the *srfC* locus of strain BSK3S. Then, the *abrB* gene was disrupted to produce the BSK4-rB strain. To analyze the antimicrobial activities of the recombinant strains, *E. coli* plates were prepared with and without Dab, as described by Choi et al. (2), and the cells of the strains were inoculated and incubated at 37°C for 24 h to observe the growth inhibition effect.

Modular HPLC System (Thermo Fisher Scientific, Inc., Waltham, MA) using an XTerra MS C_{18} column (5 μm; 2.1 by 150 mm; Waters, Milford, MA) with a BetaBasic-18 guard column (2.1 by 10 mm; Finnigan, Thermo Scientific). Mobile phase A was water, and mobile phase B was acetonitrile, both containing 0.1% formic acid. The gradient elution at a flow rate of 0.3 ml/min was performed as follows: 0 to 15 min, 15 to 80% B (linear gradient), and 15 to 20 min, 100% B (isocratic). Selected ion-monitoring (SIM) mass spectra were obtained at *m/z* 579.5 $[M + 2H]^{2+}$, with 3 microscans and a maximum ion injection time of 200 ms.

Quantitative real-time PCR (qRT-PCR) analysis. Total RNA was extracted from bacterial cells using an RNeasy minikit (Qiagen, Hilden, Germany), and 500 ng of DNA-free RNA was used for the first-strand synthesis with primers pmxAR and 16S-786R. One hundred-fold-diluted cDNA was used for amplification of the *pmxA* promoter region and the 16S rRNA gene, with the primer sets pmxAF and pmxAR, and BSF349 and 16S-786R, respectively. Real-time monitoring of amplification was performed using the iQ SYBR green Supermix kit and the CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA).

Production and purification of AbrB. The *abrB* gene of *B. subtilis* 168 was amplified with the primer set Bsabr-nde and Bsabr-xho. The amplified product was cloned into plasmid pET22b using NdeI and XhoI sites and introduced into *E. coli* BL21(DE3). The His-tagged AbrB protein was purified from the *E. coli* cells using an Ni-nitrilotriacetic acid (NTA) purification system (Qiagen, Hilden, Germany).

EMSA. An electrophoretic mobility shift assay (EMSA) was performed using biotin-labeled DNA as a probe. The promoter region of *pmxA* (P*pmxA*; 250 bp) was amplified with a 5′-biotinylated oligomer set, pmxAF-biotin and pmxAR-biotin. The PCR product was purified by agarose gel extraction. The non-biotin-labeled P*pmxA* was obtained by PCR with primers pmxAF and pmxAR and used as a specific competitor to perform EMSA. The binding reaction was performed similarly to the one described by Strauch (27), except that 50 μg of poly(dI-dC) per ml was used as the nonspecific competitor. Biotinylated P*pmxA* (10 ng) was mixed with 500 nM AbrB protein or with 5- or 10-fold amounts of unlabeled P*pmxA* (specific competitor) in a final volume of 20 μl. The mixture was incubated for 40 min at room temperature, and then, 10-μl aliquots were electrophoresed in a 5% native polyacrylamide gel. The image of electrophoretic mobility shift was detected by using a Light Shift Chemiluminescent EMSA Kit (Thermo Scientific) under a LAS 3000 Chemiluminescent Imaging Analyzer (Fujifilm, Tokyo, Japan).

RESULTS AND DISCUSSION

Production of polymyxin without external addition of Dab by introducing the *ectB* gene into *B. subtilis*. Heterologous production of polymyxin facilitated by transferring the *pmx* genes (*pmxABCDE*) responsible for the biosynthesis of polymyxin in *P. polymyxa* E681 into *B. subtilis* was dependent on the exogenous addition of Dab, because *B. subtilis* 168 was not able to produce Dab due to the lack of a gene coding for an enzyme responsible for Dab synthesis (2). The biosynthesis of Dab is mediated by 2,4-diaminobutyrate aminotransferase, encoded by *ectB*. It is known that *ectB* is composed of an operon structure with *ectA* and *ectC* and is involved in ectoine synthesis in halophilic bacteria, such as *Halobacillus halophilus* (14, 34). A gene encoding a homolog of EctB found in the genome sequence of *P. polymyxa* E681 (PPE_02294 in GenBank accession no. CP000154) showed 51% amino acid identity with that of *H. halophilus*, and interestingly, it was monocistronic.

To produce polymyxin in *B. subtilis* without an exogenous supply of Dab, we introduced the putative *ectB* gene of *P. polymyxa* into the *srfC* region of *B. subtilis* BSK3S (2) harboring entire *pmx* genes. As shown in Fig. 1B, the strain BSK4 exhibited clear antibacterial activity against *E. coli* DH5α on LB agar plates without an exogenous supply of Dab. This was in contrast with BSK3S, which could inhibit *E. coli* growth only in the presence of Dab. This result indicated that a protein encoded by the putative *ectB* gene of *P. polymyxa* was produced and successfully played the role of 2,4-diaminobutyrate aminotransferase to synthesize Dab in *B. subtilis*, which then produced polymyxin in the absence of exogenous Dab (Fig. 1B). The biosynthesis of surfactin in the strain BSK4 was completely blocked by knockout of the *srfC* gene, part of the surfactin biosynthetic gene cluster, during the construction of this strain. The deficiency of surfactin production by *B. subtilis* BSK4 in DSM-GGTris medium (4) was confirmed by measuring surface tension and the ESI-LC/MS configuration (data not shown). The elimination of surfactin production, a cyclic lipopeptide having antimicrobial activity, will be an advantage for producing polymyxin or its derivatives in pure form.

The dependence of polymyxin production on functional Spo0A. In our preliminary study, we found that a sporulation mutant of *P. polymyxa* E681 that was obtained by knocking out *spo0A* could not produce polymyxin (data not shown). There are reports showing that spontaneous asporogenic mutants of *P. polymyxa* do not produce polymyxin, and these reports also support the dependence of polymyxin production on sporulation (12, 18, 31).

To investigate whether polymyxin production is also dependent on sporulation in *B. subtilis*, the *spo0A* mutant of BSK4 was constructed as described in Materials and Methods. The mutant strain, BSK4-0A, showed no antibacterial activity against *E. coli* (Fig. 2A) and also showed no polymyxin peak in LC analysis of the culture supernatant (data not shown). These results indicate that functional Spo0A, a master transcriptional regulator for entrance into sporulation, is also required to produce polymyxin in *B. subtilis*.

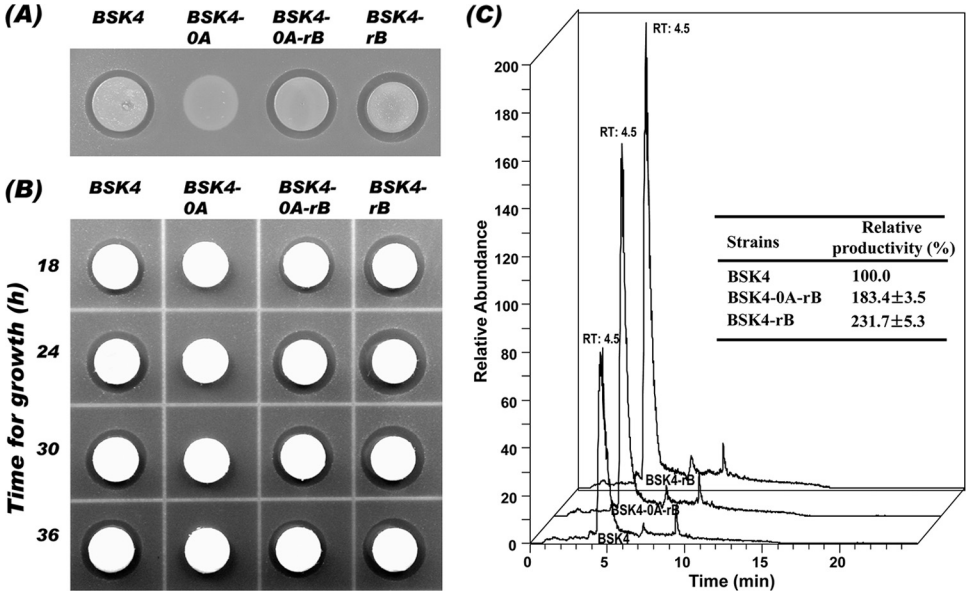

FIG 2 Antibacterial activities of *spo0A* or *abrB* mutant strains derived from BSK4 against *E. coli* (A and B) and quantitative HPLC analysis of polymyxin produced by the strains after growing for 24 h (C). The *B. subtilis* strains BSK4, BSK4-0A, BSK4-0A-rB, and BSK4-rB were grown in Cal18 medium for 18, 24, 30, and 36 h. A culture aliquot (5 μl) of each strain grown for 24 h was dropped directly onto *E. coli* plates containing *E. coli* cells (~10^6 CFU/ml) for antibacterial assay (A), and 50 μl of cell-free supernatant of each strain harvested at different growth times was loaded onto a paper disk and transferred to the *E. coli* plates (B). The growth inhibition was observed after 24 h of incubation at 37°C. (C) The 24-h culture samples were used for quantitative analysis of polymyxin by ESI-LC/MS, and the $[M + 2H]^{2+}$ ion peaks of 579.5 *m/z* were obtained. The areas of the ion peaks were determined for comparison with each other.

The regulation of polymyxin production by AbrB. The DNA-binding protein Spo0A regulates, directly or indirectly, the expression of >500 genes in *B. subtilis*. Among them, 40 genes are directly activated and 81 are directly repressed by phosphorylated Spo0A (17). Spo0A also regulates many genes indirectly through repression of *abrB* (5, 7, 26), and when Spo0A is nonfunctional in a cell, it leads to an increase in *abrB* expression. It was reported that AbrB repressed the expression of *tycA*, a *Bacillus brevis* gene that encodes tyrocidine synthetase I, which is an enzyme involved in the biosynthesis of the cyclic decapeptide tyrocidine (22). In the case of *Bacillus cereus*, production of cereulide, which is a cyclic dodecadepsipeptide synthesized by a nonribosomal peptide synthetase encoded by the *ces* genes, was regulated by Spo0A and AbrB (16). It was also reported that deletion of the *abrB* gene strongly increased the production of the lantibiotic subtilin in *B. subtilis* (25). In this context, it was also expected that the necessity for functional Spo0A in polymyxin production might be related to repression of *abrB*. To test this hypothesis, we constructed *abrB* single and *spo0A abrB* double mutants of BSK4. As shown in Fig. 2A, BSK4-0A-rB with *spo0A abrB* double mutations recovered the antibacterial activity against *E. coli* in an agar plate assay. The antibacterial activity seemed to be even higher than that of BSK4. Another mutant strain, BSK4-rB, with the *abrB* single mutation, also showed higher antibacterial activity than BSK4 (Fig. 2A). For the quantitative analysis of polymyxin produced by the recombinant *B. subtilis* strains, Cal18 medium was selected for growing bacterial cells in a preliminary test for comparing 20 kinds of media (data not shown), and the cells grown in Cal18 medium for 24 h, which showed the highest antibacterial activity among samples harvested at different times, was used for the quantitative analysis of polymyxin (Fig. 2B). LC/MS results revealed that strains BSK4-rB and BSK4-0A-rB produced 2.3- and 1.8-fold more polymyxin, respectively, than BSK4 (Fig. 2C). This suggests that polymyxin production is repressed by AbrB and that Spo0A regulates polymyxin production in a positive manner by repression of *abrB*.

Effect of *spo0A* and/or *abrB* mutation on expression of *pmxA*. As in the case of Spo0A, AbrB, a transition state regulator,

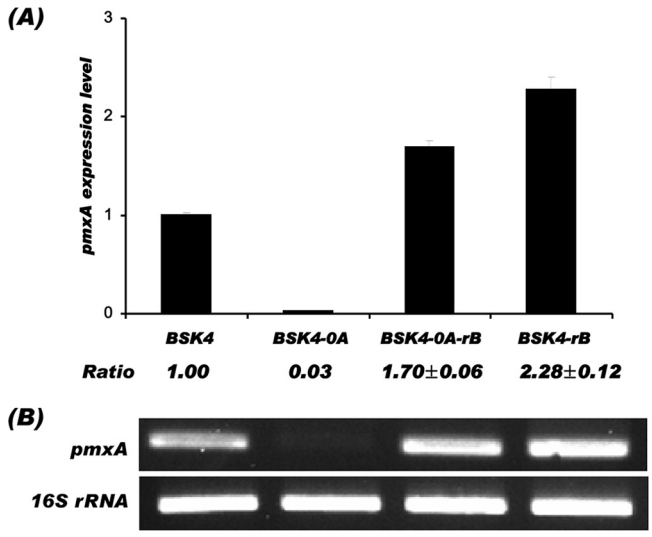

FIG 3 Effects of *spo0A* or *abrB* mutations on expression of *pmxA* in *B. subtilis* analyzed by qRT-PCR (A) and the gel electrophoresis image (B). The relative expression ratios of *pmxA* for the three mutant strains, BSK4-0A, BSK4-0A-rB, and BSK4-rB, to their parent strain, BSK4, were calculated from the real-time PCR efficiencies. The error bars indicate standard errors of the mean (SEM); $n = 3$ in all RT-PCR analyses; $P < 0.05$.

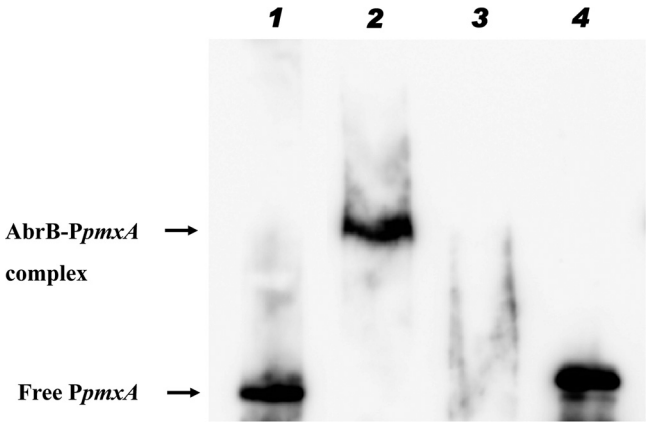

FIG 4 Binding of AbrB to the promoter region of *pmxA*. The promoter region of *pmxA* (P*pmxA*; 250 bp) was labeled with biotin. The biotin-labeled P*pmxA* (10 ng) interacted with 500 nM AbrB in a binding buffer containing 50 μg of poly(dI-dC) per ml as the nonspecific competitor. Lanes: 1, biotin-labeled P*pmxA* only; 2, mixture of biotin-labeled P*pmxA* and AbrB; 3 and 4, mixture of biotin-labeled PmxA, AbrB, and non-biotin-labeled P*pmxA* (5- and 10-fold for each lane).

binds to DNA and regulates the expression of downstream genes (28). In this study, we investigated the effects of null mutations of *spo0A* or *abrB* on the expression of *pmxA*, and the transcription level of *pmxA* was analyzed by qRT-PCR. As shown in Fig. 3, the expression of *pmxA* was completely repressed in BSK4-0A cells; however, it was recovered in BSK-0A-rB, and the expression level reached 1.7 times that of BSK4. This result suggests that AbrB is a negative regulator of *pmxA* expression, and null mutation of *spo0A*, whose product is a repressor of the transcription of *abrB*, caused overexpression of *abrB* and then repression of *pmxA*. In BSK4-0A-rB with double mutations in *spo0A* and *abrB*, the repression of *pmxA* by AbrB could be relieved. In BSK4-rB with an *abrB* single mutation, the expression of *pmxA* was increased and reached a 2.3-times-higher level than in BSK4 (Fig. 3). These qRT-PCR results coincided well with the results of quantitative analysis of polymyxin production in the strains (Fig. 2C).

Direct binding of AbrB to the promoter region of *pmxA*. To find out whether AbrB represses the expression of polymyxin biosynthetic genes directly or indirectly, we investigated the binding of AbrB to the upstream region of the *pmxA* gene. As shown in Fig. 4, the movement of biotin-labeled P*pmxA* was retarded in the presence of *B. subtilis* AbrB. However, the addition of 5- or 10-fold excess of unlabeled P*pmxA*, the specific competitor, restored the movement of biotin-labeled P*pmxA*, possibly by hindering the interaction between the biotin-labeled DNA and AbrB. From these results, it was found that *pmxA* gene expression is controlled by direct binding of AbrB to the *pmxA* promoter in *B. subtilis*.

Conclusions. A *B. subtilis* strain, BSK4, that can produce polymyxin without addition of exogenous Dab was constructed in this study by introducing the *ectB* gene of *P. polymyxa*. We found that two important transcription factors, Spo0A and AbrB, are involved in the regulation of biosynthesis of polymyxin in *B. subtilis*. AbrB was shown to bind directly to the upstream region of *pmxA*, and it functions as a negative transcriptional regulator. A functional Spo0A was found to be indispensable for polymyxin production. The polymyxin productivity of BSK4 was improved 2.3-fold by null mutation in *abrB*. This improved system for the production of polymyxin, using the surrogate host *B. subtilis*, may accelerate structure-function studies and engineering of *pmx* genes for the generation of novel polymyxin derivatives.

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