Fusaricidin Biosynthesis Is Controlled via a KinB-Spo0A-AbrB Signal Pathway in *Paenibacillus polymyxa* WLY78

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Fusaricidins produced by *Paenibacillus polymyxa* are important lipopeptide antibiotics against fungi. The *fusGFEDCBA* (fusaricidin biosynthesis) operon is responsible for synthesis of fusaricidins. However, the regulation mechanisms of fusaricidin biosynthesis remain to be fully clarified. In this study, we revealed that fusaricidin production is controlled by a complex regulatory network including KinB-Spo0A-AbrB. Evidence suggested that the regulator AbrB represses the transcription of the *fus* gene cluster by direct binding to the *fus* promoter, in which the sequences (5'-AATTTTAAATAAATTTGTGATTT-3') located from -136 to -112 bp relative to the transcription start site is required for this repression. Spo0A binds to the *abrB* promoter that contains the Spo0A-binding sequences (5'-TGTCGAA-3', 0A box) and in turn prevents the further transcription of *abrB*. The decreasing concentration of AbrB allows for the derepression of the *fus* promoter repressed by AbrB. The genome of *P. polymyxa* WLY78 contains two orthologs (named Kin1508 and Kin4833) of *Bacillus subtilis* KinB, but only Kin4833 activates sporulation and fusaricidin production, indicating that this kinase may be involved in phosphorylating Spo0A to initiate sporulation and regulate the *abrB* transcription. Our results reveal that Kin4833 (KinB), Spo0A, and AbrB are involved in regulation of fusaricidin production and a signaling mechanism that links fusaricidin production and sporulation.

Keywords: AbrB, bacteria-plant symbiosis, biocontrol, fusaricidin, genetics and gene regulation, KinB, molecular signaling, *Paenibacillus polymyxa*, regulation, secondary metabolism, Spo0A

Fusaricidins produced by *Paenibacillus polymyxa* are antifungal lipopeptide antibiotics (Haron et al. 2019; Jeong et al. 2019; Lee et al. 2013; Li and Chen 2019). Fourteen fusaricidin members,

which contain a cyclic peptide (six amino-acids) and a common guanidinylated β -hydroxyl fatty acid chain, have been reported (Deng et al. 2011; Kajimura and Kaneda 1996; Reimann et al. 2017; Vater et al. 2015). Fusaricidins are synthesized by the products of the *fusGFEDCBA* operon (fusaricidin biosynthesis, *fus*) through a nonribosomal mechanism (Li and Chen 2019). Although fusaricidin has great potential use in medical and agricultural fields, only a few studies on the regulation mechanisms of fusaricidin biosynthesis are available. For example, mutation of AbrB resulted in increased fusaricidin levels and AbrB could directly bind to the *fus* promoter region in *P. polymyxa* SQR21 (Li et al. 2013) and the phosphoglucosyltransferase gene obviously affected fusaricidin production in *P. polymyxa* E681 (Kim et al. 2014). Therefore, it is vital to fully understand the transcriptional regulation mechanisms of fusaricidin biosynthesis for enhancing its production.

Regulation mechanisms of lipopeptide antibiotics in *Bacillus subtilis* is well-studied. It is believed that some antibiotics, such as gramicidin S, tryocidine, and surfactin were produced by *B. subtilis* around the onset of sporulation (Strauch et al. 2007). It has long been known that sporulation in *B. subtilis* is induced by nutrient limitation such as the relevant limiting growth substrates, sources of carbon, nitrogen, and phosphorus (Nandy and Venkatesh 2008). Spo0A is a global master regulator that governs the onset of stationary phase and sporulation (Stephenson and Hoch 2002). Spo0A binds to a specific DNA sequence (5'-TGTCGAA-3') known as the '0A box', where it acts as both a repressor of certain vegetatively expressed genes (e.g., *abrB*) and an activator of genes involved in sporulation (Liu et al. 2003; Strauch et al. 1990). The activity of Spo0A is governed by a multicomponent phosphorelay, and phosphorylation of its N terminal at Asp55 could switch on the transcriptional regulation function of its C terminal (Zhao et al. 2002). In *Bacillus* spp., the phosphorylation of Spo0A (Spo0A~P) is controlled by five histidine kinases (KinA, KinB, KinC, KinD, and KinE), each of which senses different signals of sporulation (Banse et al. 2011; Brunsing et al. 2005; Jiang et al. 2000; Zerihou et al. 2014). Among these histidine kinases, KinA and KinB are thought to be the major kinases for initiating sporulation (Liu et al. 2018; Tojo et al. 2013).

AbrB is a transition state regulatory protein that represses the expression of many post-exponential phase functions, such as Spo0E, Spo0H, and SpoVG necessary for normal sporulation (Strauch et al. 1990). The AbrB protein is also involved in regulation of the expression of the genes for competence, antibiotics production, and biofilm formation in *B. subtilis* (Bochmann et al. 2015). For example, in *B. subtilis* biosynthesis of gramicidin S is controlled by Spo0A, AbrB, and Spo0H, and tyrocidine production is regulated by Spo0A and AbrB (Marahier et al. 1993),

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*The e-Xtra logo stands for "electronic extra" and indicates there are supplementary materials published online.

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while surfactin biosynthesis is controlled by Spo0A and competence-related gene products (ComA, ComP, and ComQ) required for the initiation of the competence developmental pathway (Fisher et al. 1994). It was reported that, in *B. subtilis*, Spo0A~P represses AbrB and thereby AbrB decreases, allowing for the derepression of those promoters repressed by AbrB (Liu et al. 2003). Although AbrB regulates numerous genes in *B. subtilis*, it does not recognize a clear consensus DNA sequence, mainly due to the structural flexibility of AbrB (Olson et al. 2014).

Recently, we have reported that *P. polymyxa* WLY78 produces fusaricidin with strong antifungal activity against *Fusarium* species (Li and Chen 2019). Reverse transcription (RT)-PCR revealed that the seven genes (*fusG*, *fusF*, *fusE*, *fusD*, *fusC*, *fusB*, and *fusA*) within the *fus* cluster responsible for fusaricidin biosynthesis of *P. polymyxa* WLY78 are organized as an operon. *fusA* is the largest gene, with a length of 23.73 kb, which is responsible for synthesizing the cyclic polypeptide moiety of fusaricidin, while the other six genes (*fusG*, *fusF*, *fusE*, *fusD*, *fusC*, and *fusB*) are responsible for synthesizing the lipid moiety of fusaricidin (Li and Chen 2019).

In this study, mutation and complementation analyses reveal that *kin4833* (*kinB*), *spo0A*, and *abrB* are involved in regulation of fusaricidin biosynthesis. Electrophoretic mobility shift assays (EMSA) demonstrate that AbrB binds to the *fus* promoter and Spo0A binds to the *abrB* promoter region. Furthermore, the putative AbrB-binding sequences in the *fus* promoter region is determined by truncating the *fus* promoter for promoter activity assay. To our knowledge, here is the first time that fusaricidin production has been revealed to be controlled by the KinB-Spo0A-AbrB pathway. Our results will provide guidance for improving fusaricidin production by genetically modifying the regulatory network of the *fus* gene expression.

RESULTS

Biological control of wheat *Fusarium* head blight.

Wheat *Fusarium* head blight (also known as wheat scab) is a devastating factor to wheat production and food security. As described below, fusaricidin fermentation broth of *P. polymyxa* WLY78 was applied to the aerial part of wheat seedlings in field trials. The disease index and biocontrol efficacy were investigated. The fusaricidin fermentation broth showed 64% biocontrol efficacy against *Fusarium* head blight of wheat and improved yields by 24% (Fig. 1). The data suggest that fusaricidin antibiotic plays an important role in control of *Fusarium* head blight.

Disruption of the *abrB* gene leads to overproduction of fusaricidin.

In order to enhance fusaricidin production, we investigated the transcriptional regulation mechanisms of fusaricidin biosynthesis. It is known that the AbrB protein is involved in regulation of antibiotics production in *B. subtilis* (Bochmann et al. 2015). In this study, we find that the 84-residue AbrB of *P. polymyxa* WLY78 exhibits 61% identity with AbrB (AQR84191.1) of *B. subtilis* 168 at the amino-acid sequence level and, also, that the DNA-binding domain is conserved (Supplementary Fig. S1). To clarify whether AbrB regulates the transcription of the *fus* operon, we constructed a Δ *abrB* mutant. The antifungal activity against *Fusarium asiaticum* of Δ *abrB* mutant was much stronger than that of wild-type *P. polymyxa* WLY78 (Fig. 2A). Quantitative (q)RT-PCR analysis showed that the transcript level of the *fusA* gene in the Δ *abrB* mutant was significantly higher than that in the wild-type strain, suggesting that AbrB negatively regulates transcription of the *fus* operon (Fig. 2B). To further confirm the negative regulation of AbrB on *fus* expression, a *Pfus3-gfp* plasmid carrying the entire *fus* promoter region (from -336 to +26 relative to the transcription start site) fused to the *gfp* (green fluorescent protein)

coding region was introduced into the Δ *abrB* mutant and wild-type strain, respectively. As shown in Figure 2C, the GFP expression level in Δ *abrB* mutant was almost onefold higher than that in the wild-type strain, suggesting that mutation in the *abrB* gene relieved the inhibition of AbrB on the *fus* transcription. All these results demonstrated that AbrB negatively regulates *fus* expression, consistent with the results of higher fusaricidin accumulation in Δ *abrB* mutant of *P. polymyxa* SQR-21 (Li et al. 2013).

Determination of the transcription start site and the AbrB-binding sequences in the *fus* promoter.

Our recent study has shown that the seven genes (*fusG*, *fusF*, *fusE*, *fusD*, *fusC*, *fusB*, and *fusA*) within the *fus* cluster responsible for fusaricidin biosynthesis of *P. polymyxa* WLY78 are organized as an operon (Li and Chen 2019). In this study, the transcriptional start site (TSS) of the *fus* cluster is determined by using the 5' RACE (rapid amplification of cDNA ends) method. The TSS is adenine located 26 bp upstream of the translation start codon (ATG) of *fusG* and a putative ribosome-binding site is identified 8 nt preceding the start codon. The -35 (TAGACA) and -10 (TATTTT) sequences are found in the *fus* promoter (Fig. 3A).

To ascertain whether AbrB protein binds to the *fus* promoter, the *P. polymyxa* AbrB protein with a His₆-tag was overexpressed and was purified in *Escherichia coli* BL21. The purified AbrB is a 13-kDa protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3B). The entire *fus* promoter fragment (362 bp) was synthesized and used for assaying the interaction between the *fus* promoter and AbrB. EMSA revealed the binding interaction between AbrB and the *fus* promoter (Fig. 3C). The binding is specific, as a DNA fragment of similar length derived from the *fusA* coding sequence showed no binding affinity to AbrB (Supplementary Fig. S2).

AbrB does not recognize a single consensus sequence in *B. subtilis* (Strauch et al. 1989). AbrB binds a number of DNA targets with a flexible structure, which allows a conformational change when AbrB binds (Bobay et al. 2004). Usually, DNA binding sites of transcription factors can be predicted by using the MEME/MAST program (Bailey et al. 2009). However, we failed to find consensus sequences in the AbrB-binding site of the *fus* promoter of *P. polymyxa* WLY78 by using the MEME/MAST method, suggesting that the *P. polymyxa* AbrB binds to a flexible structural DNA. Thus, we searched the AbrB-binding sequences by truncating the *fus* promoter region. Five different lengths of the *fus* promoter region (*Pfus*) fused to the *gfp*-coding region were cloned to vector pHY300PLK, respectively, yielding plasmids *Pfus3-gfp*, *Pfus2-gfp*, *Pfus1-gfp*, *Pfus0-gfp*, and *Pfus⁻-gfp*. The five *fus* promoter regions include a common downstream region (+26 bp relative to the transcription start site) and various upstream sequences from -336 bp, -236 bp, -136 bp, -36 bp, and -1 bp relative to the transcription start site, respectively. Then, the five plasmids were individually introduced into *P. polymyxa* WLY78 and the Δ *abrB* mutant to compare their expression profiles. As shown in Figure 3D, the *Pfus0-gfp* and *Pfus⁻-gfp* could not make both *P. polymyxa* WLY78 and Δ *abrB* mutant exhibit GFP activities, mainly due to no promoter region (*Pfus⁻*) or the promoter region being too short (*Pfus0*) for RNA polymerase function. In contrast, *Pfus3-gfp*, *Pfus2-gfp*, and *Pfus1-gfp* enabled both *P. polymyxa* WLY78 and Δ *abrB* mutant to exhibit GFP activities. The results suggest the possible location of an AbrB-binding site in the 136-bp promoter region of the *fus* gene.

In order to determine the putative AbrB-binding site in the *fus* promoter, we further constructed three additional plasmids *Pfus0.75-gfp*, *Pfus0.5-gfp*, and *Pfus0.25-gfp*, each of which individually carries a different length of the *fus* promoter region (from -111, -86, and -61 bp to +26 bp relative to the

transcription start site, respectively) fused to the *gfp* coding region. Then, these plasmids together with plasmid *Pfus1-gfp* were respectively introduced into the recombinant *E. coli* BL21, which carries the *P. polymyxa abrB* gene under the control of the T7 promoter in pET-28b vector. As shown in Figure 3E, in the absence of an isopropyl- β -D-thiogalactoside (IPTG)-induction (–) group, *Pfus1-gfp*, *Pfus0.75-gfp*, *Pfus0.5-gfp*, and *Pfus0.25-gfp* each showed high GFP activity with no significant

difference among them, indicating that these *fus* promoters were barely regulated, consistent with lack of the expressed AbrB. When IPTG was added (+), these *gfp*-fusion plasmids (*Pfus0.75-gfp*, *Pfus0.5-gfp*, and *Pfus0.25-gfp*) stably expressed high GFP activity, while *Pfus1-gfp* showed very low GFP activity. The data suggested that the 25-bp DNA region from –136 to –112 bp relative to the transcription start site is required for the AbrB binding. The regulator AbrB represses the transcription of

Fig. 1. Biological control of wheat Fusarium head blight with fusaricidin fermentation broth. **A**, Comparison of disease symptoms of wheat plants and grains in fields between treatments with fusaricidin fermentation broth of *Paenibacillus polymyxa* WLY78 (designated WLY78) and with tap water (control). **B**, Comparison of disease index and wheat yield in fields between treatments with fusaricidin fermentation broth (designated WLY78) and with tap water (control). Lower case letters indicate statistically significant difference ($P < 0.05$).

the *fus* gene cluster, likely by direct binding to the *fus* promoter, in which the sequences (5'-AATTTTAAATAAATTTTGT GATTT-3') located from -136 to -112 bp relative to the transcription start site is required for this repression (Fig. 3F). The 25-bp site in the *fus* promoter region is rich in A+T sequences. The AbrB-binding sequences that are rich in A+T content are reported in the promoter regions of *B. subtilis* *spo0H*, *spo0H*, *kinB*, *ftsA*, and *pbpE* (Frisby and Zuber 1991; Strauch 1995a). The *B. subtilis* *spo0H* promoter region has the AbrB-binding sequences (AATATGTTTACAAATAAAGTATAATCTGTA ATAATGCAC) and the *B. subtilis* *abrB* promoter region has the AbrB-binding sequences (ACAAAATGATTGACGAT TATTGGAAACCTTG) (Strauch 1995b). It is believed that A+T-rich sequences create a "bent" conformation in the DNA and the bent conformation may help to open the DNA helix, which could enhance the binding of RNA polymerase to the promoter by allowing additional polymerase-DNA contacts to occur (Frisby and Zuber 1991).

Spo0A positively regulates the *fus* expression by repressing *abrB* gene transcription.

P. polymyxa WLY78 contains a *spo0A* gene whose predicted product Spo0A (267 amino acids) exhibits 69% identity with Spo0A (CAB14353.1) of *B. subtilis*. Moreover, the N-terminal phosphorylation site Asp55 and the C-terminal helix-turn-helix DNA-binding domain are found to be conserved in both *P. polymyxa* Spo0A and *B. subtilis* Spo0A (Supplementary Fig. S3). To determine whether Spo0A of *P. polymyxa* WLY78

influences fusaricidin production, *spo0A* deletion mutant (Δ *spo0A*) was constructed. Compared with *P. polymyxa* WLY78, the Δ *spo0A* mutant completely lost the antifungal activity against *F. asiaticum* (Fig. 4A), indicating that *P. polymyxa* Spo0A is required for fusaricidin production. Also, the Δ *spo0A* mutant completely lost the ability to form spores (Fig. 4B), suggesting the correlation between sporulation and fusaricidin production. Furthermore, qRT-PCR analysis revealed that the transcription level of the *fusA* gene in the wild-type strain was 10-fold higher than that in Δ *spo0A* mutant (Fig. 4C). Meantime, the *Pfus3-gfp* plasmid, which carries the entire *fus* promoter region fused to the *gfp* coding region, was introduced into the wild-type strain and the Δ *spo0A* mutant, respectively. As shown in Figure 4D, the GFP expression level in wild-type strain was almost 15-fold higher than that in Δ *spo0A* mutant. All these data indicate that Spo0A positively affects fusaricidin production via transcriptional regulation.

To examine whether Spo0A directly binds to the promoter region of the *fus* operon, we overexpressed and purified the *P. polymyxa* Spo0A with His₆-tag in *E. coli* BL21, and the Spo0A protein has a deduced molecular weight of 33.5 kDa in SDS-PAGE (Fig. 5A). EMSA experiments revealed that Spo0A didn't bind to the *fus* promoter region (Fig. 5B), indicating that the Spo0A indirectly regulates the *fus* transcription. The results are consistent with the finding that the *fus* promoter region does not contain any O₄A boxes (TGTCGAA). Then, we searched and found there are two O₄A boxes (TGTCGAA) in the *abrB* promoter regions of *P. polymyxa* WLY78 (Fig. 5C). The EMSA

Fig. 2. Comparison of the antifungal abilities, transcription levels of the *fusA* gene, and fluorescence intensities of the *Pfus3-gfp* fusion among *Paenibacillus polymyxa* WLY78, Δ *abrB* mutant, and complementation strain Δ *abrB* (*abrB*). **A**, The inhibition activities against *Fusarium asiaticum* by *P. polymyxa* WLY78, Δ *abrB* mutant, and complementation strain Δ *abrB* (*abrB*), with sterilized water as control. **B**, Transcription levels of the *fusA* gene in *P. polymyxa* WLY78 and Δ *abrB* mutant determined by quantitative reverse transcription PCR. **C**, Fluorescence microscope observation and fluorescence intensities of in *P. polymyxa* WLY78 and Δ *abrB* mutant carrying the *Pfus3-gfp* fusion. Lower case letters indicate statistically significant difference ($P < 0.05$).

experiments demonstrated that Spo0A directly bind to the *abrB* promoter region (Fig. 5D). qRT-PCR revealed that the transcript levels of the *abrB* gene in $\Delta spo0A$ mutant were onefold higher than that in wild-type strain (Fig. 5E). The results suggest that *P. polymyxa* Spo0A represses *abrB* transcription, consistent with the findings that Spo0A negatively controlled *abrB* expression in *B. subtilis* 168 (Fujita et al. 2005; Strauch et al. 2007). Therefore, *P. polymyxa* Spo0A indirectly activates fusaricidin production by directly repressing *abrB* expression. Our findings support the reports that spontaneous asporogenic mutants of *P. polymyxa* do not produce antibiotics (polymyxin) and support the dependence of polymyxin production on sporulation (Kaur et al. 1978; Nefelova et al. 1980; Park et al. 2012).

Kin4833 (KinB) functions upstream of Spo0A and activates sporulation and fusaricidin production.

It was reported that, in *B. subtilis*, either KinA or KinB is the sensor kinase that carries out auto-phosphorylation at a histidine residue and then transfers a phosphoryl group to an aspartyl residue in Spo0F, and the resulting Spo0F~P, in turn, transfers the phosphoryl group to Spo0B. Finally, Spo0B~P transfers the phosphoryl group to Spo0A and the resulting Spo0A~P activates or represses gene expression (Perego et al. 1989; Tojo et al. 2013).

In this study, we screened the *P. polymyxa* WLY78 genome with the conserved histidine kinase A phosphoacceptor (HisKA)

domain of *B. subtilis* histidine kinases and found two putative histidine kinases, named Kin1508 and Kin4833. Comparison of *P. polymyxa* Kin1508 and Kin4833 with *B. subtilis* KinA, KinB, KinC, KinD, and KinE at the amino-acid sequence level showed that the conserved residues, especially a histidine residue for auto-phosphorylation, exist among all the proteins (Fig. 6A). Further, protein domain analysis showed that each of the Kin1508 and Kin4833 proteins of *P. polymyxa* WLY78 has three domains, transmembrane, HisKA, and ATPase. The phylogenetic tree shows that Kin1508 and Kin4833 of *P. polymyxa* WLY78 have relate closely with KinB of *B. subtilis* 168 (Fig. 6B). Moreover, phylogenetic analysis of Kin1508 and Kin4833 demonstrates their high homology to KinB by aligning them with all the published histidine kinase nucleotide sequences from *Bacillus* spp. (Supplementary Fig. S4). The identity of the histidine kinase nucleotide sequences is 41.23% (Supplementary Fig. S5). Similar analysis shows that *P. polymyxa* Kin1508 and Kin4833 exhibit 23 and 31% identity, respectively, with KinB of *B. subtilis* 168, at the amino-acid sequence level (Supplementary Fig. S6). *B. subtilis* KinB was reported to phosphorylate Spo0A to initiate sporulation (Liu et al. 2018). Thus, we deduce that *P. polymyxa* WLY78 Kin1508 and Kin4833 may phosphorylate Spo0A just as *B. subtilis* KinB did.

To further determine whether or not Kin1508 and Kin4833 are involved in phosphorylation of Spo0A, two deletion mutants,

Fig. 4. Comparison of the antifungal abilities, endospore formation, transcription levels of the *fusA* gene and fluorescence intensity of the *Pfus3-gfp* fusion among *Paenibacillus polymyxa* WLY78, $\Delta spo0A$ mutant, and complementation strain $\Delta spo0A$ (*spo0A*). **A**, The inhibition activities against *Fusarium asiaticum* by *P. polymyxa* WLY78, $\Delta spo0A$ mutant, and its complementation strain $\Delta spo0A$ (*spo0A*). **B**, Sporulation frequencies of *P. polymyxa* WLY78, $\Delta spo0A$ mutant, and complementation strain $\Delta spo0A$ (*spo0A*). Rod cell in dark red is vegetative cell and spheroid cell in blue is spore. **C**, Transcription levels of the *fusA* gene in *P. polymyxa* WLY78 and $\Delta spo0A$ mutant determined by quantitative reverse transcription-PCR. **D**, Fluorescence microscope observation and fluorescence intensity of *P. polymyxa* WLY78 and $\Delta spo0A$ mutant carrying the *Pfus3-gfp* fusion. Lower case letters indicate statistically significant difference ($P < 0.05$).

$\Delta kin1508$ and $\Delta kin4833$, as well as their complementary expression $\Delta kin1508$ (pHY-Pkin1508) and $\Delta kin4833$ (pHY-Pkin4833) were constructed. Here, the sporulation frequencies are used as an indicator of the phosphorylation levels of Spo0A (Spo0A~P), based on reports that *B. subtilis* initiated sporulation only if a threshold concentration of phosphorylated Spo0A was reached (Kiehler et al. 2017). Compared with wild-type *P. polymyxa* WLY78, $\Delta kin1508$ mutant showed no obvious difference in sporulation efficiency, but the $\Delta kin4833$ mutant showed significant reduction in sporulation ability. Complementation of the $\Delta kin4833$ mutant with *kin4833* restored the sporulation defect, indicating that Kin4833 may be involved in phosphorylation of Spo0A to initiate sporulation (Fig. 6C).

To elucidate the effect of Kin4833 on fusaricidin production, the antifungal activities against *F. asiaticum* of $\Delta kin4833$ mutant, complementation strain $\Delta kin4833$ (pHY-kin4833), and the wild-type strain were detected. The inhibition zone of $\Delta kin4833$ is obviously smaller than that of *P. polymyxa* WLY78, while complementation strain $\Delta kin4833$ (pHY-kin4833) rescues the antifungal effect of mutant $\Delta kin4833$ (Fig. 6D). These results indicate, for the first time, that *kin4833* positively regulates fusaricidin production. To examine the effect of $\Delta kin4833$ mutation on the transcription of the *fus* operon of *P. polymyxa* WLY78, the transcription levels of the *fusA* gene were determined by qRT-PCR. The transcription level of the *fusA* gene in the wild-type strain exhibited threefold higher than that in $\Delta kin4833$ mutant (Fig. 6E). This result is consistent with an antifungal phenotype of $\Delta kin4833$ mutant, indicating that Kin4833 (KinB) positively regulates the *fus* transcription.

To further confirm the effect of Kin4833 on regulation of the *fus* expression, a *Pfus3-gfp* plasmid carrying the entire *fus* promoter region fused to the *gfp* coding region was introduced into wild type and $\Delta kin4833$ mutant, respectively. As shown in Figure 6F, the GFP expression level in the wild-type strain was almost fourfold higher than that of $\Delta kin4833$ mutant, consistent with the antifungal activity and the *fusA* transcription level. Overall, our results demonstrate that the fusaricidin production and sporulation are both activated by Kin4833 (KinB). Our results are consistent with the reports that high amounts of fusaricidin was produced only when cells entered into sporulation (Beatty and Jensen 2002).

DISCUSSION

Fusarium head blight has caused serious damage to wheat production in China. Multidisciplinary efforts have been made to fight against this disease for a long time (Ma et al. 2020). Our previous studies revealed that the fusaricidin antibiotic produced by *P. polymyxa* WLY78 plays an important role in inhibition against *Fusarium* species, *Verticillium albo-atrum*, and other plant-pathogenic fungi (Li and Chen 2019). In this study, we have found that the fusaricidin fermentation broth of *P. polymyxa* WLY78 showed 64% biocontrol efficacy against *Fusarium* head blight and improved wheat yields by 24%. Our study suggests that fusaricidin has potential to be used in the control of *Fusarium* head blight.

In order to enhance fusaricidin production, we attempt to reveal the regulatory network of the *fus* gene transcription of

Fig. 5. Determination of Spo0A-binding site in the *abrB* promoter by electrophoretic mobility shift assay (EMSA) and effect of *spo0A* mutation on the *abrB* transcription. **A**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the expressed and purified *Paenibacillus polymyxa* His₆-Spo0A protein in *Escherichia coli*. **B**, EMSA analysis of the binding ability of Spo0A protein to the *fus* promoter region. Lanes 1 through 5 indicate increasing concentrations of Spo0A protein (0, 5, 20, 50, 200 ng) and 0.1 pmol-labeled promoter DNA was used for each lane. **C**, Spo0A-binding sites in the promoter region of the *abrB* gene. **D**, Transcription levels of the *abrB* gene in *P. polymyxa* WLY78 and $\Delta spo0A$ mutant determined by quantitative reverse transcription-PCR. **E**, EMSA analysis of the binding ability of Spo0A protein to the *abrB* promoter region. Lanes 1 through 5 indicate increasing concentrations of Spo0A protein (0, 5, 20, 50, 200 ng) and 0.1 pmol-labeled promoter DNA was used for each lane. Lower case letters indicate statistically significant difference ($P < 0.05$).

P. polymyxa WLY78. Our studies have shown that AbrB as a transcription protein represses fusaricidin biosynthesis in *P. polymyxa* WLY78 by direct binding on the promoter region of the *fus* gene cluster, consistent with the results obtained in *P. polymyxa* SQR-21 (Li et al. 2013). We provided evidence suggesting that the regulator AbrB represses the transcription of the *fus* gene cluster by direct binding to the *fus* promoter, in which the sequences (5'-AATTTTAAATAAATTTTGTGATTT-3') located from -136 to -112 bp relative to the transcription start site are required for this repression. Previous studies on the transcriptional properties of *P. polymyxa* SQR-21 inferred that the *fus* promoter region between -383 and -282 bp (relative to transcription start codon) is a binding region for some negative regulators (Li et al. 2013). By comparing the AbrB-binding sequences in the *fus* promoter of *P. polymyxa* WLY78 with those in *B. subtilis* AbrB-dependent promoter region, A/T-rich sequences are evident. A relevant

TGGNA motif connected by A/T-rich sequences in AbrB-binding sites is identified in some genes of *B. subtilis* (Chumsakul et al. 2011; Xu and Strauch 1996). But neither the TGGNA motif nor a clear consensus DNA sequence is detected in the *fus* promoter region of *P. polymyxa* WLY78. Our results are consistent with the previous reports that AbrB does not recognize a clear consensus DNA sequence in *B. subtilis* (Olson et al. 2014). AbrB functions as a tetramer or dimer in DNA regulation scale, which means that multiple binding motifs for AbrB are possible (Sullivan et al. 2008).

Our mutation analyses have revealed that *P. polymyxa* Spo0A is required for efficient fusaricidin production of *P. polymyxa* WLY78. There is no OA box (TGTCGAA) for binding of Spo0A in the *fus* promoter region, and EMSA also has shown that Spo0A does not bind to the *fus* promoter region, suggesting that the Spo0A regulates the *fus* transcription indirectly. However,

Fig. 6. Homology analysis of histidine kinases of *Paenibacillus polymyxa* WLY78 with those of *Bacillus subtilis* 168 and comparison of sporulation, antifungal activities, *fusA* transcription levels, fluorescence intensity of the *Pfus3-gfp* fusion among *P. polymyxa* WLY78, $\Delta kin1508$, $\Delta kin4833$, and complementation strains. **A**, Alignment of histidine kinases (Kin1508 and Kin4833) of *P. polymyxa* WLY78 and histidine kinases (KinA, KinB, KinC, and KinD) of *B. subtilis* 168 by using the conserved sequence of HisKA domains. **B**, Phylogenetic tree and domain organization of *P. polymyxa* kinases (Kin1508 and Kin4833) and *B. subtilis* 168 kinases (KinA, KinB, KinC, KinD, and KinE). **C**, Sporulation frequencies of *P. polymyxa* WLY78, $\Delta kin1508$, and $\Delta kin4833$ and complementation strains $\Delta kin1508$ (kin1508) and $\Delta kin4833$ (kin4833). Rod cell in dark red is vegetative cell and spheroid cell in blue is spore. **D**, The inhibition activities against *Fusarium asiaticum* by *P. polymyxa* WLY78, $\Delta kin4833$ mutant, and complementation strain $\Delta kin4833$ (kin4833). **E**, Transcription levels of the *fusA* gene in *P. polymyxa* WLY78 and $\Delta kin4833$ mutant determined by quantitative reverse transcription-PCR. **F**, Fluorescence microscope observation and fluorescence intensity of *P. polymyxa* WLY78 and $\Delta kin4833$ carrying the *Pfus3-gfp* fusion. Lower case letters indicate statistically significant difference ($P < 0.05$).

there are two 0A boxes in the *abrB* promoter region of *P. polymyxa* WLY78, and EMSA has demonstrated that Spo0A directly binds to the *abrB* promoter region. qRT-PCR has shown that *abrB* expression is inhibited in the $\Delta spo0A$ mutant. The results suggest that *P. polymyxa* Spo0A indirectly activates fusaricidin production by directly repressing *abrB* expression, consistent with the findings that Spo0A negatively controlled *abrB* expression in *B. subtilis* 168 (Fujita et al. 2005; Strauch et al. 2007).

The activity of Spo0A is governed by a multicomponent phosphorylation in *Bacillus* spp. The phosphorylation of Spo0A (Spo0A~P) is controlled by five histidine kinases (KinA, KinB, KinC, KinD, and KinE), each of which senses different signals of sporulation (Banse et al. 2011; Brunsing et al. 2005; Jiang et al. 2000; Zerihouh et al. 2014). Among these histidine kinases, KinA and KinB are thought to be the major kinases for initiating sporulation (Liu et al. 2018; Tojo et al. 2013). In this study, we screened the *P. polymyxa* WLY78 genome and found two putative histidine kinases, named Kin1508 and Kin4833. The *P. polymyxa* Kin1508 and Kin4833 exhibit 23 and 31% identity, respectively, with KinB of *B. subtilis* 168 at the amino-acid sequence level. Protein domain analysis has shown that each of the Kin1508 and Kin4833 proteins of *P. polymyxa* WLY78 has three domains, transmembrane, HisKA, and ATPase. A conserved histidine residue for auto-phosphorylation is found in the N-terminal domain of both Kin1508 and Kin4833 proteins. The structure characteristics of the Kin1508 and Kin4833 proteins suggest that they are the sensor kinases that carry out autophosphorylation and then transfer a phosphoryl group to a receptor. To investigate whether both *P. polymyxa* Kin1508 and Kin4833 are involved in phosphorylation of Spo0A (Spo0A~P), the sporulation frequencies are used as an indicator of the phosphorylation levels (Kiehler et al. 2017). The $\Delta kin4833$ mutant showed significant reduction in the sporulation ability compared with *P. polymyxa* WLY78, and the complementation strain restored the sporulation defect, indicating that Kin4833 may be involved in phosphorylation of Spo0A to initiate sporulation. Further, the antifungal ability and the transcription levels of *fusA* gene in the $\Delta kin4833$ mutant are much lower than those in wild type, suggesting that Kin4833 is positively correlated with *fus* transcription and is consistent with the results obtained in $\Delta spo0A$ mutant.

In *B. subtilis*, KinB or KinA, as the sensor kinase, carries out autophosphorylation and then transfers a phosphoryl group to Spo0F, the resulting Spo0F~P, in turn, transfers the phosphoryl group to Spo0B. Finally, Spo0B~P transfers the phosphoryl group to Spo0A, and the resulting Spo0A~P activates or represses gene expression (Tojo et al. 2013). However, there are no Spo0F and Spo0B and only the Spo0A protein is found in *Clostridial* genomes, suggesting that *Clostridial* species, the anaerobic relatives of *Bacillus* genus, do not use a multicomponent phosphorylation system but use a classical two-component module in which Spo0A is activated directly by a sensor kinase (Stephenson and Hoch 2002). We have found that, in addition to the *spo0A* gene, there are a *spo0F* and a *spo0B* in the genome of *P. polymyxa* WLY78. Thus, we deduce that *P. polymyxa* Spo0A may be phosphorylated and activated by a multicomponent phosphorylation system composed of Kin4833 (KinB), Spo0B, and Spo0F, as *B. subtilis* did.

On the basis of our results and the previous findings obtained in *B. subtilis*, we propose a regulation mode of fusaricidin production of *P. polymyxa* WLY78. During nutrient limitation, Kin4833 (KinB) senses the specific signals and is auto-phosphorylated, yielding KinB~P. Then, KinB~P transfers its phosphoryl group to Spo0F, the resulting Spo0F~P, in turn, transfers the phosphoryl group to Spo0B, and finally, Spo0B~P transfers the phosphoryl group to Spo0A. The resulting Spo0A~P negatively regulates the expression of *AbrB* that represses the *fus* expression by binding to

the *fus* promoter, in which the sequences (5'-AATTTTAAAA TAAATTTTGTGATTT-3') located from -136 to -112 bp relative to the transcription start site is required for this repression. Therefore, fusaricidin is produced with spore formation under nutrient limitation (Fig. 7). Under excess nutrient conditions, Kin4833 (KinB) is not auto-phosphorylated, and thus Spo0A is not phosphorylated to repress the *abrB* expression. Thus, *AbrB* binds to the *fus* promoter to inhibit fusaricidin biosynthesis. The signal transduction pathway by phosphorylation among KinB, Spo0F, Spo0B, and Spo0A in fusaricidin synthesis of *P. polymyxa* WLY78 needs to be deeply studied by using the ($r\text{-}^{32}\text{P}$) ATP method described by Haydel et al. (1999) and Cui et al. (2011).

MATERIALS AND METHODS

Microorganisms, plasmids, and culture conditions.

The source of strains and plasmids are summarized in Supplementary Table S1. *E. coli* DH5 α was cultivated at 37°C in Luria-Bertani (LB) broth for cloning of plasmids. *E. coli* BL21 (DE3) was cultivated at 30°C in LB broth for protein expression. *P. polymyxa* WLY78 was routinely cultivated at 30°C in LB medium. The synthetic medium, which was described by Katznelson and Lochhead (1944) and used for biosynthesis of polymyxin b by (Paulus and Gray 1964), was used for fusaricidin production. Pathogen *F. asiaticum* was cultivated at 28°C in potato dextrose agar (PDA) medium. Thermo-sensitive vector pRN5101 was used for gene deletion in *P. polymyxa*. Shuttle vector pHY300PLK was used for the complementation experiment and *gfp* fusion. Vector pET-28b was used for expressing recombinant His₆-tagged protein in *E. coli* BL21. When necessary, LB broth was solidified by adding 1.5% agar, and the antibiotics were added in the following concentrations: ampicillin, 100 $\mu\text{g/ml}$; erythromycin, 5 $\mu\text{g/ml}$; and tetracycline, 12.5 $\mu\text{g/ml}$.

Preparation of fusaricidin fermentation broth.

P. polymyxa WLY78 was grown in LB liquid medium for overnight at 30°C with shaking at 200 rpm. Then, 500 ml of liquid culture was transferred into a 7.5-liter fermenter filled with 5 liters of the synthetic medium (Paulus and Gray 1964).

Fig. 7. The predicted regulatory network that controls fusaricidin production of *Paenibacillus polymyxa* WLY78. Kin4833 (KinB) senses the specific signals and is auto-phosphorylated, yielding KinB~P. Then, KinB~P transfers its phosphoryl group to Spo0F, the resulting Spo0F~P, in turn, transfers the phosphoryl group to Spo0B, and finally, Spo0B~P transfers the phosphoryl group to Spo0A. The resulting Spo0A~P negatively regulates the expression of *AbrB* that represses the *fus* expression by binding to the *fus* promoter, in which the sequences (5'-AATTTTAAAA TAAATTTTGTGATTT-3') located from -136 to -112 bp relative to transcription start site is required for this repression. Dashed arrow indicates the signal transduction by phosphorylation that needs to be determined by experiments and the T bar indicates repressed gene expression that has been confirmed by our experiments.

During the fermentation process, temperature was maintained at 30°C, pH was controlled at 6.8 by the addition of 5 M KOH, and culture was continuously stirred at 200 rpm. The liquid culture was harvested after 3 days of fermentation and was then used for control of wheat *Fusarium* head blight. The bacterial cell density was about 1×10^9 .

Biological control of wheat *Fusarium* head blight in field trials.

The field trials were conducted at the Wujiao Experimental Station of China Agricultural University at Cangzhou, one of the most important wheat production areas in Hebei Province, China. This area had been cultivated with winter wheat–summer maize for many years. The areas were naturally infested by *F. asiaticum*. Winter wheat was sown in October and was harvested the following June. A randomized block design was adopted for experiments. Six plots, each of which was 40 square meters, were used for our experiment. Three plots were treated with fusaricidin fermentation broth, and another three plots were treated with tap water as control. In the early period of May, when wheat scab did not occur, 33 ml of fusaricidin fermentation broth was diluted with tap water (vol/vol) 1:10 and was then applied to the aerial part of wheat seedlings via a sprayer in each plot. Tap water was used instead of fusaricidin fermentation broth in the control group. Crop management followed routine commercial practices. A total of 100 wheat seedlings from each plot were collected to investigate the disease severity at 28 days after treatment. The disease severity was scored using a grade value, denoting proportions of disease over the whole wheat ear area, where grade 0: 0%, grade 1: <25%, grade 2: 25 to 50%, grade 3: 50 to 75%, grade 4: 75 to 100%, grade 5: 100% (Landschoot et al. 2012). Then the disease index (DI) and control efficacy (CE) was computed, using the formula

$$DI = \frac{\sum (gN_g)}{5N_t},$$

where g is the grade value, N_g is the number of the plants of the corresponding grade, and N_t is the total number of plants in each group. Then, the CE was determined by the following formula.

$$CE(\%) = \left[1 - \frac{DI_{\text{treatment}}}{DI_{\text{control}}} \right] \times 100\%$$

To calculate the grain yield per hectare, the grains in three plots were harvest and weighed.

Bioinformatics analysis of histidine kinases in *P. polymyxa*.

A functional domain and homology search was performed, using the BLAST program. ClustalW was used for sequence alignment. The domain structures were predicted using HMMER.

Construction of $\Delta kin1508$, $\Delta kin4833$, $\Delta spo0A$, and $\Delta abrB$ mutants and their complementation strains.

Four in-frame deletion mutants, $\Delta kin1508$, $\Delta kin4833$, $\Delta spo0A$, and $\Delta abrB$, were constructed by a homologous recombination method. Briefly, the upstream (1 kb) and downstream fragments (1 kb) flanking the coding region of *kin1508*, *kin4833*, *spo0A*, and *abrB* were PCR-amplified from the genomic DNA of *P. polymyxa* WLY78. Briefly, the two fragments flanking each coding region of *kin1508*, *kin4833*, *spo0A*, and *abrB* were assembled into the *Bam*HI-digested pRN5101 vector, yielding the four recombinant plasmids pRN-*kin1508*, pRN-*kin4833*, pRN-*spo0A*, and pRN-*abrB*, respectively. Subsequently, each recombinant plasmid was transformed into *P. polymyxa* WLY78. For selecting

the $\Delta kin1508$, $\Delta kin4833$, $\Delta spo0A$, and $\Delta abrB$ mutants, the transformants were cultivated at 39°C and were identified by PCR according to the methods described by Li and Chen (2019). The primers for PCR are listed in Supplementary Table S2.

For complementation of $\Delta kin1508$, $\Delta kin4833$, $\Delta spo0A$, and $\Delta abrB$ mutants, four DNA fragments carrying the gene open reading frames and their own promoters, *Pkin1508* (1,478 bp), *Pkin4833* (2,022 bp), *Pspo0A* (1,020 bp), and *PabrB* (515 bp) were PCR-amplified, respectively. Then, each fragment was assembled into the *Eco*RI-digested pHY300PLK vector and then transformed into *P. polymyxa* WLY78, respectively. The PCR primers are listed in Supplementary Table S2. The nucleotide sequence of *kin1508*, *kin4833*, *spo0A*, and *abrB* in *P. polymyxa* WLY78 were deposited in GenBank under accession numbers MT096381, MT096382, MT096380, and MT096379, respectively.

Antifungal activity assays.

To test the antifungal activity of *P. polymyxa* WLY78 and its mutant strains, the inhibition zone of the strain against the fungi *F. asiaticum* was recorded. Briefly, the *F. asiaticum* was inoculated onto the center of PDA medium. Then, 1 μ l of *P. polymyxa* cell suspensions (10^7 CFU/ml) and mutant cell suspensions (10^7 CFU/ml) were respectively inoculated around the fungi at a distance of 2 cm, with 1 μ l of sterilized water as a negative control (Li and Chen 2019). All the plates were cultured at 28°C for 4 days. Then the inhibition effect on fungi hyphae growth was recorded.

Spore assays.

To evaluate the sporulation efficiency of *P. polymyxa* WLY78 and kinase mutant, the ratio of spores was calculated as follows. One milliliter of overnight bacterial culture grown in LB broth was inoculated into 20 ml of the synthetic medium (Paulus and Gray, 1964) and was grown at 37°C with shaking at 220 rpm for 3 days. Then the cells were collected and were stained using Schaeffer-Fulton's method, after which they were observed under an optical microscope (Mormak and Casida 1985).

RNA preparation and qRT-PCR assay.

To compare the relative expression level of *fusA* between *P. polymyxa* WLY78 and each mutant, qRT-PCR analysis was performed as follows. After cultivation for 3 days, 50 ml of culture were harvested and rapidly frozen in liquid nitrogen. Total RNA was extracted by RNAiso Plus (TaKaRa) and 1 μ g of total RNA was converted into cDNA with a reverse transcription reagent kit (TaKaRa). 16S RNA was used as an internal reference. The qRT-PCR procedure was conducted as described previously (Li et al. 2015). The relative transcription level was calculated using the $\Delta^{\Delta CT}$ method (Livak and Schmittgen 2001). The primers are listed in Supplementary Table S3.

Expression and purification of Spo0A and AbrB proteins in *E. coli*.

The *spo0A* and *abrB* genes were PCR-amplified from the genomic DNA of *P. polymyxa* WLY78. These PCR products were cloned into pET-28b to construct tagged proteins with His₆-tag at the N terminal and were then transformed into *E. coli* BL21 (DE3). The recombinant *E. coli* strains were cultivated in LB broth with 50 μ g of kanamycin per milliliter until the optical density at 600 nm (OD_{600}) = 0.5, when 0.2 mM IPTG was added and the cells were incubated at 30°C for 4 h. Then the cells were collected in a lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole) and sonication on ice. Recombinant His₆-tagged proteins in the supernatant were purified on Ni₂-NTA resin (Qiagen), according to the manufacturer instructions. Proteins eluted with 250 mM imidazole were dialyzed into binding buffer

(20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM dithiothreitol, 0.2% Tween 20, and 30 mM KCl) for EMSAs. The primers are listed in Supplementary Table S4.

EMSA.

EMSAs were performed using the DIG gel shift kit (Roche), according to the manufacture instructions. The promoter fragments of the *fus* operon were synthesized by Sangon Biotech Co., Ltd. Two DNA fragments corresponding to the sequences of the first strand and the complementary DNA strand were synthesized. The two strands were annealed and then labeled at the 3' end with digoxigenin using terminal transferase and were used as probes. Each binding reaction (20 µl) consisted of 1 µg poly [d(A-T)], 0.3 nM labeled probe, and various concentration of purified His₆-protein in the binding buffer. Binding reaction was performed at 25°C for 30 min and was then analyzed by electrophoresis, using native 5% polyacrylamide gel run with 0.5× Tris-borate-EDTA as running buffer and electrophoretically transferred to a positively charged nylon membrane at 4°C (GE Healthcare). Labeled DNAs were detected by chemiluminescence, according to manufacturer protocol, and recorded on X-ray film. The primers are listed in Supplementary Table S5.

Transcription start site identification.

The 5' RACE assay was performed, using the SMARTer RACE cDNA amplification kit (Clontech). Gene-specific primers are listed in Supplementary Table S5. The PCR fragment obtained from 5' RACE was inserted into the pMD18-T vector and individual clones were selected for DNA sequencing.

Pfus-gfp fusion plasmid construction and GFP activity assay.

To evaluate the *fus* expression level, various different lengths of the *fus* promoter region were fused to the *gfp* coding region (*Pfus-gfp* fusions) (Fig. 3A). Briefly, eight *fus* promoter DNA fragments (from -336, -236, -136, -111, -86, -61, -36, and -1 to +26 relative to the transcription start site) were respectively amplified from the genomic DNA of *P. polymyxa* WLY78, using the primers listed in Supplementary Table S6. The coding region of *gfp* was PCR-amplified with primers from the plasmid pGFP4412. Each *fus* promoter fragment and *gfp* fragment were assembled into the vector pHY300PLK digested by *Bam*HI, yielding eight *Pfus-gfp* fusion plasmids. Then, each of *Pfus-gfp* fusion plasmid was transformed into *P. polymyxa* WLY78, $\Delta kin4833$, $\Delta spo0A$, $\Delta abrB$, and the recombinant *E. coli* BL21 carrying the *abrB* gene in pET-28b vector that could overexpress *AbrB* when induced by 0.2 mM IPTG. The transformants were cultured overnight in LB broth with 12.5 µg of tetracycline per milliliter at 37°C with shaking at 200 rpm. The OD₆₀₀ = 1 of the bacterial suspension was adjusted. A drop of the bacterial suspension was taken to observe the green fluorescence of these transformants carrying the *Pfus-gfp* fusion plasmids by fluorescence microscope (Olympus BX60). Meanwhile, 200 µl of suspension was filled into the 96-well cell plate for relative fluorescence unit assay by the multidetection microplate fluorescence reader (BioTek FLX800). The fluorescence intensity (FI) was calculated by the following formula:

$$FI = \frac{\text{Relative fluorescence unit (RFU)}}{OD_{600}}$$

Statistical analysis.

All the experiments were repeated three times with a similar result. The significant difference ($P < 0.01$) of data were analyzed by one-way analysis of variance with Duncan's multiple-range test, using SPSS version 22 statistical software.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Basic Local Alignment Search Tool (BLAST):

<https://blast.ncbi.nlm.nih.gov/Blast.cgi>

HMMERweb server: <https://www.ebi.ac.uk/Tools/hmmer>

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