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The exopolysaccharide gene cluster *pea* is transcriptionally controlled by RpoS and repressed by AmrZ in *Pseudomonas putida* KT2440

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ABSTRACT

In *Pseudomonas putida* KT2440, the exopolysaccharide Pea is associated with biofilm stability and pellicle formation; however, little is known about its regulatory pathway. In this study, we identified that the gene cluster *pea* was transcribed from 25 bp upstream of the operon and the stationary phase alternative sigma factor RpoS regulated the transcription of *pea*. When RpoS was absent, another sigma factor, likely the housekeeping sigma factor RpoD, could also mediate *pea* transcription but at a low level. The function of Pea polysaccharide was further confirmed to be necessary for full production of biofilm, formation of pellicle and c-di-GMP-dependent wrinkly colony morphology. Additionally, evidences were provided to demonstrate that the transcriptional regulator AmrZ was a negative regulator for *pea* expression. DNase I footprinting studies verified that AmrZ bound directly to the site overlapping the *pea* promoter, which might interfere with the binding of RNA polymerase to the promoter and resulted in inhibition of transcription initiation.

1. Introduction

Pseudomonas putida, a Gram-negative bacterium widely found in natural environments, is studied for multiple purposes, including quorum sensing (Arevalo-Ferro et al., 2005), degradation of aromatic compounds (Johnson et al., 2017), tolerance to heavy metals (Liu et al., 2015), bacterial motility (Xiao et al., 2017) and plant-bacterial interactions (Espinosa-Urgel et al., 2000). Since this strain possesses high metabolic diversity characteristics, it has been developed as a microbial cell factory for hosting harsh biochemical reactions to produce bioproducts, such as polyhydroxyalkanoates, muconate and adipic acid (Linger et al., 2014; Vardon et al., 2015; Nikel et al., 2016). Moreover, *P. putida* has strong ability to form biofilm which can confer resistance and tolerance to antibiotics (Tettmann et al., 2014), water-limitation (Nielsen et al., 2011) and osmotic stress (Hachicho et al., 2017), leading to improved survivability under a variety of stressful conditions. As a pWW0 plasmid-free derivative of *P. putida* mt-2, *P. putida* KT2440 is regarded as a model organism for the studies of environmental microbiology (Bagdasarian et al., 1981).

In biofilm matrix of KT2440, two adhesins (LapA and LapF) and four exopolysaccharides (Pea, Peb, alginate and Bcs) have been identified. LapA is essential for root colonization and initial attachment of biofilm formation (Espinosa-Urgel et al., 2000; Hinsa et al., 2003) while LapF is involved in cell-cell interactions and biofilm maturation (Martinez-Gil et al., 2010). The exopolysaccharides alginate and Bcs appear to be redundant in biofilm establishment under fully hydrated conditions (Nilsson et al., 2011). By contrast, Pea and Peb play important roles in biofilm stability (Nilsson et al., 2011). Multiple regulators have been identified to participate in regulation of biofilm matrix synthesis. Regulator FleQ can bind to promoters of both *lapA* and *bcs*, activating *lapA* transcription but repressing *bcs* transcription, and it can also interact with c-di-GMP which enhances the binding to *lapA* promoter while lowers the affinity to *bcs* promoter (Xiao et al., 2016). Thus, high level of intracellular c-di-GMP will raise the expression levels of both *lapA* and *bcs* (Xiao et al., 2016). Additionally, FleN participates in this process that it promotes the binding of FleQ to *lapA* and *bcs* promoters (Nie et al., 2017). In *P. aeruginosa*, FleQ also acts as both repressor and activator for expression of the exopolysaccharide gene cluster *pel* in response to c-di-GMP (Baraquet et al., 2012). Furthermore, c-di-GMP can promote polysaccharide synthesis through binding to components of polysaccharide synthase complexes, such as BcsA1 in *Acetobacter xylinum*, Alg44 and PelD in *P. aeruginosa*, which contain PilZ domain or the unidentified c-di-GMP receptor (Weinhouse et al., 1997; Lee et al., 2007; Merighi et al., 2007). The alternative sigma factor RpoS (also known as σ^S or σ^{38}), which is mainly expressed in stationary phase and under stressful conditions (Battesti et al., 2011), is another important regulator for expression of biofilm matrix genes. In this aspect, RpoS controls transcription of *lapF* in *P. putida* (Martinez-Gil et al., 2010), and

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it is also the major sigma factor for transcription of the exopolysaccharide gene cluster *psl* in *P. aeruginosa* (Irie et al., 2010) and the secondary sigma factor for *lapA* transcription in *P. putida* (Ainelo et al., 2017). There are several regulators involving in *alg* regulation that the sigma factor AlgU (σ^{22}) controls *alg* transcription and other regulators such as AlgB and AlgR play positive roles in *alg* expression through direct binding to *alg* promoter (Wozniak and Ohman, 1991; Mohr et al., 1992; Deretic et al., 1994). However, with regard to exopolysaccharide gene clusters *pea* and *peb*, we know very little about their regulation. Our previous study showed that a (p)ppGpp-negative KT2440 strain had depressed *pea* transcription but increased *peb* transcription (Liu et al., 2017). This secondary messenger is likely to regulate *pea* and *peb* expression via indirect pathways.

In recent years, the ribbon-helix-helix transcriptional factor AmrZ (alginate and motility regulator Z, previously known as AlgZ) was identified as a regulator of biofilm development and virulence in *P. aeruginosa* and *P. syringae* pv. *tomato* (Waligora et al., 2010; Jones et al., 2013; Prada-Ramirez et al., 2016). Structure analysis reveals that AmrZ binds DNA sequence as a dimer-of-dimers, and each dimmer recognizes one of the two half sites which were separated by five base pairs (bp) on DNA binding region (Pryor et al., 2012). AmrZ activates the transcription of alginate synthesis operon *alg* by directly binding to the promoter of the first gene *algD* (Xu et al., 2016). Moreover, AmrZ is required for twitching motility and biogenesis of type IV pili in *P. aeruginosa* (Baynham et al., 2006). Interestingly, the bifunctional regulator AmrZ acts more extensively as a transcriptional repressor, repressing not only its own encoding gene *amrZ*, but also the diguanylate cyclase gene *adcA*, the genes related to the pyochelin and pyoverdine siderophore systems, the regulator gene *fleQ* and the exopolysaccharide operon *psl* (Ramsey et al., 2005; Tart et al., 2006; Jones et al., 2013, 2014). As a global regulator, AmrZ is also involved in the iron uptake system in *P. fluorescens* and metal efflux system in *P. putida*, leading to improved adaption of bacteria in environments (Martinez-Granero et al., 2014; Mumm et al., 2016).

In this report, we attempted to uncover the regulation of the *pea* cluster, containing 11 ORFs termed *PP_3132* to *PP_3142*, which encode proteins participating in synthesis and transport of polysaccharides (Nilsson et al., 2011). We identified that the transcription of *pea* mainly relied on RpoS. The Pea polysaccharide appeared to be an essential component in biofilm development. Furthermore, we found that the regulator AmrZ repressed *pea* transcription by directly binding to the *pea* promoter.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. The *E. coli* S17-1/*λpir* was used for subcloning and strain construction. The *E. coli* BL21(DE3) was used for protein purification. All strains were propagated in LB medium and the *E. coli* strains were incubated at 37 °C while the *P. putida* strains were incubated at 28 °C. The antibiotics were used if needed in the following concentrations: kanamycin, 50 μg/ml; gentamicin, 20 μg/ml; tetracycline, 40 μg/ml; chloramphenicol, 25 μg/ml.

2.2. Strain and plasmid construction

All primers used in this study are listed in Table A1. For construction of the reporter plasmid pBRTZ-*pea*P, the fragment from -633 to +72 relative to *PP_3132* start codon was amplified from KT2440 genome DNA by primers 3132PS and 3132PA, and then the fragment was ligated into the vector pBRTZ digested with XbaI and PstI. The fragments which were amplified by primers 3132PS/3132PmA and 3132PA/3132PmS were linked by overlap extension PCR and then inserted into XbaI-PstI sites of pBRTZ, yielding pBRTZ-*pea*Pm. The complete *rpoS* and *amrZ* amplified by primers *rpoS*s-oe/*rpoS*a-oe and *amrZ*s-oe/*amrZ*a-oe were inserted into the EcoRI-HindIII site of pBBR1-403, generating pB403-*rpoS* and pB403-*amrZ*, respectively. All cloning plasmids were verified by sequencing.

In order to generate the knockout vectors for mutant constructions, the regions upstream and downstream of *rpoS*, *pea* cluster, *lapF* and *amrZ* were amplified from KT2440 genome DNA using the primers listed in Table A1. The upstream and downstream fragments of each gene were inserted into SacI-digested pDS3.0 (Gao et al., 2006) by recombination using the ClonExpress II One Step Cloning Kit (Vazyme), yielding pDS-*rpoS*, pDS-*pea*, pDS-*lapF* and pDS-*amrZ*, respectively. The knockout vectors in *E. coli* S17-1 were transferred into KT2440 by two-parental mating. The cells met with single crossover event were selected on LB agar supplemented with gentamicin, and then these strains were streaked on M9 agar contained 13% (w/v) sucrose for purpose to isolate the bacteria met with the second crossover event. The knockout mutants were identified by PCR analysis. Finally, the unmarked mutants of *rpoS* (deleted 229–810 bp of 1008 bp), *pea* operon (deleted 630–11,672 bp of 12,068 bp), *lapF* (deleted 1288–16,470 bp of 18,933 bp), *pea-lapF* and *amrZ* (deleted 67–264 bp of 327 bp) were obtained.

2.3. 5′ Rapid amplification of cDNA ends (5′ RACE) assay

Total RNA was isolated from KT2440 grown in LB to an optical density at 600 nm (OD_{600}) of 2.0 by using RNAiso Plus (Takara). RACE cDNA amplification Kit (Clontech) was used to determine the transcription start site of *pea* cluster, as described by manufacturer's instruction. The adaptor primer 5′-adaptor and gene-specific primer 3132-5RACE (Table A1) were used to amplify the target fragment and then the product was ligated into pMD18-T vector (Takara) for sequencing.

2.4. Biofilm formation assay

The overnight cultures were adjusted to OD_{600} of 0.005 with fresh LB medium, and then 1 ml of the dilution culture was added to each well of 24-well polystyrene microtiter plates. After 10 h or 24 h of incubation under shaking at 180 rpm, the planktonic cultures were discarded and the amount of biofilm attached on the wells were quantified by crystal violet staining (O'Toole and Kolter, 1998). The crystal violet bound to the biofilms was dissolved into 2 ml of 95% (v/v) ethanol for 30 min. The absorbance at 590 nm (A_{590}) of the dissolved crystal violet was measured to evaluate the relative amount of biofilm formed by each strain.

For pellicle formation assay, 2.4 ml of the diluted culture described above was added to 12-well plates and incubated statically for 24 h. The images of floating pellicles were taken after removal of all liquid cultures.

2.5. β-Galactosidase activity assay

The β-galactosidase activity assays were performed as previously described with minor modification (Griffith and Wolf, 2002) Overnight cultures of the strains harboring the reporter plasmid were diluted to an OD_{600} of 0.005 with LB medium and incubated under shaking condition. At the appointed time, cells were collected and resuspended in isometric volume of Z buffer, which contained 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol. The reaction system contained 100 μl of resuspended cells, 900 μl of Z buffer, 50 μl of 1 mg/ml sodium dodecyl sulfate (SDS) and 100 μl of chloroform. Time was recorded after adding 200 μl of 4 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG). The reactions were stopped by adding 500 μl of 1 M Na_2CO_3. After the biomass was precipitated off by centrifugation, A_{420} of the supernatant was measured. Meanwhile, A_{600} of the resuspended cells was measured. The β-galactosidase activity was calculated as: Miller units = $1000 \times (A_{420}/A_{600}/\text{volume}/\text{time})$.

Table 1
The strains and plasmids used in this study.

Strains	Description	Reference/source
E. coli		
S17-1/*λpir*	RP4-*mob*+, *λpir*, host for R6K-based suicide plasmids	Invitrogen Corp
BL21(DE3)	F-, *ompT*, *hsdS (rBB-mB-)*, *gal*, *dcm*	Invitrogen Corp
P. putida		
KT2440	Cm^r, *P. putida* mt-2 derivative cured of pWW0	(Bagdasarian et al., 1981)
Δ*rpoS*	*rpoS* deletion mutant derived from KT2440	This study
Δ*pea*	*pea* deletion mutant derived from KT2440	This study
Δ*lapF*	*lapF* deletion mutant derived from KT2440	This study
Δ*pea*Δ*lapF*	*lapF* deletion mutant derived from Δ*pea*	This study
Δ*relA*Δ*rpoS*	*relA*::Km, *spoT*::Gm derived of from KT2440	(Liu et al., 2017)
Δ*amrZ*	*amrZ* deletion mutant derived from KT2440	This study
Plasmids		
pBBR1MCS5	Gm^r, *mob*+, expression vector	(Kovach et al., 1995)
pBBR1-*wspR*	pBBR1MCS5 carrying *wspR* of KT2440	(Xiao et al., 2016)
pBRTZ	Tet^r, reporter vector with promoter-less *lacZ*	(Liu et al., 2017)
pBRTZ-*pea*P	pBRTZ carrying *pea* promoter region	This study
pBRTZ-*pea*Pm	pBRTZ carrying mutated *pea* promoter	This study
pBBR1-403	Gm^r, expression vector with *tac* promoter	(Xiao et al., 2016)
pB403-*rpoS*	pBBR1-403 carrying the complete *rpoS*	This study
pB403-*amrZ*	pBBR1-403 carrying the complete *amrZ*	This study
pET28a	Km^r, protein expression vector	Invitrogen Corp
pET28a-*amrZ*	pET28a carrying *amrZ,* C-terminal His tag	This study
pET28a-*amrZ*R20A	pET28a carrying R20 A mutated *amrZ,* C-terminal His tag	This study
pDS3.0	Gm^r, suicide plasmid, *ori R6K*, *sacB*	(Gao et al., 2006)
pDS-*rpoS*	*rpoS* knockout vector	This study
pDS-*pea*	*pea* knockout vector	This study
pDS-*lapF*	*lapF* knockout vector	This study
pDS-*amrZ*	*amrZ* knockout vector	This study

2.6. Quantification real-time PCR (qRT-PCR)

In colony assay, 3 μl of each overnight culture was pointed on LB agar and incubated for 3 days. The colonies were collected for RNA isolation. The KT2440 wild-type and Δ*amrZ* strains harboring pBBR1-403 or pB403-*amrZ* were incubated to an OD_{600} of 2.0, and then added with 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG). Cells were collected when cultures were grown to an OD_{600} of 3.0. Total RNA was isolated using the RNAiso Plus (Takara), and then the cDNA was synthesized by treating with the PrimeScript RT reagent Kit with gDNA Eraser (Takara). The SYBR Green RT-PCR kit (Takara) was used for qRT-PCR analysis. The housekeeping gene *gyrA* (*PP_1767*) was used as an internal control for normalization (Prada-Ramirez et al., 2016). Relative expression values were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Purification of AmrZ

The *amrZ* fragment was amplified from KT2440 genome DNA by PCR using primers *amrZ*s and *amrZ*a. This fragment was cloned into linear pET28a digested with NcoI and XhoI by recombination using the ClonExpress II One Step Cloning Kit (Vazyme), yielding pET28a-*amrZ*. Two fragments amplified respectively by primers *amrZ*s/*amrZ*-R20Aa and *amrZ*-R20As/*amrZ*a were linked together by overlap extension PCR using primers *amrZ*s and *amrZ*a, and then the fragment was inserted into pET28a as described above, yielding pET28a-*amrZ*R20 A. *E. coli* BL21(DE3) harboring the pET28a-*amrZ* or pET28a-*amrZ*R20 A was grown in LB medium at 37 °C to an OD_{600} of 0.5, and induced with 1 mM IPTG at 16 °C for 6 h, and then the cells were collected and re-suspended in lysis buffer (10 mM Tris − HCl, pH 7.5, 300 mM KCl, 10% (v/v) glycerol, 4 M urea). After cells were lysed by high-pressure

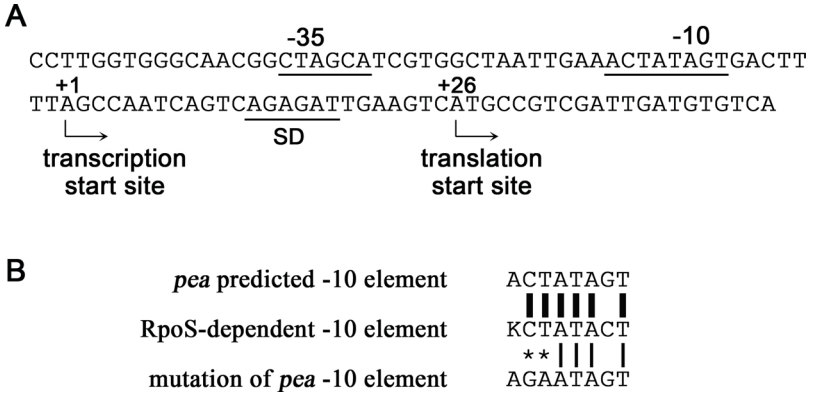

Fig. 1. The promoter region of *pea* operon. (A) Transcription start site of *pea* was identified by 5′ RACE assay. The arrows indicate the transcription start site (+1) and the translation start site (+26). The predicted RpoS-like -10 and -35 elements and Shine-Dalgarno (SD) sequence are underlined. (B) The -10 element of the *pea* promoter shares high similarity with the consensus sequence of RpoS-dependent -10 elements. The second and the third nucleotides in the *pea* -10 element were replaced by guanine and adenine, respectively, as indicated by asterisks, to generate the mutated *pea* promoter in pBRTZ-*pea*Pm. (K = T/G).

homogenizer, the soluble expressed protein was purified by Ni-NTA spin columns (BBI life sciences) followed the protocol. The eluted protein was dialyzed and stored in storage buffer (20 mM KCl, 100 mM NaCl, 20% (v/v) glycerol) at −20 °C.

2.8. Electrophoretic mobility shift assay (EMSA) and DNase I footprinting assay

DNA fragment for EMSA was produced from two PCR processes that the first process amplified the target region by using primers *pea*PS300FAM/*pea*PA55FAM and the second process added the 6-FAM label at the ends of the fragment by PCR using primer PFAM5U1 which contained a 6-FAM label at 5′-end. The product in the first PCR process was also used as unlabeled specific probes in EMSA. For fragment with mutation in AmrZ binding site, two fragments amplified respectively by primers *pea*PS300FAM/*pea*P50mA and *pea*P50mS/*pea*PA55FAM were linked together by overlap extension PCR, and then added with 6-FAM label as described above. Unless otherwise noted, 6 nM FAM-labeled DNA was mixed with increasing concentration of AmrZ or R20 A AmrZ in binding buffer (25 mM Tris − HCl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 5% (v/v) glycerol, 50 ng/μl bovine serum albumin (BSA), pH 8.0) with a total reaction volume of 20 μl, and allowed to bind for 20 min at 25 °C. 15 μl of reaction mixtures were loaded in a 5% (w/v) acrylamide gel containing TGE buffer (12.5 mM Tris − HCl, 95 mM Glycine, 2.5 mM EDTA, pH 8.0) and electrophoresed at 100 V on ice for 90 min. The gels imaged by the fluorescent and radioisotope science imaging systems (FLA-5100, FUJIFILM).

For DNase I footprinting assays, two fragments with one end-labeled were obtained as described above but using the primer pairs *pea*PS300FAM/*pea*PA55 and *pea*PS300/*pea*PA55FAM in the first PCR process, and PFAM5U1/*pea*PA55 and *pea*PS300/ PFAM5U1 in the second PCR process, respectively. In a 200 μl reaction system, 1 μg of labeled DNA was mixed with 60 μg AmrZ (BSA was used instead of AmrZ in the control group) in binding buffer for EMSA assay, and the system was incubated for 20 min at 25 °C. Subsequently, 0.3 U of DNase I was added and allowed to react for 3 min at 25 °C. The reaction was terminated by adding 100 μl of phenol-chloroform (1:1, v/v). After centrifugation, the supernatant was mixed with 500 μl ethanol and 70 μl of 3 M sodium acetate. The DNA was precipitated by centrifugation at 4 °C and the supernatant was discarded. After drying, the precipitates were dissolved in ddH_2O. The final samples were analyzed in a 3730XL DNA Sequencer (Applied Biosystems) and the electropherograms were aligned with Peak Scanner Software v1.0 (Applied Biosystems).

2.9. Statistical analyses

Error bars correspond to the standard deviation of at least three biological replicates. Data were analyzed using Student's *t* test. Statistical significance was considered when *P* value less than or equal to 0.05.

3. Results and discussion

3.1. Transcription of the pea operon is RpoS-dependent

The first step of this study to explore the expression pattern of the *pea* operon is to identify the transcription start site. The 5′ RACE assay had determined alone transcription start site, which was located on 25 bp upstream of the first codon (ATG) of *PP_3132* (Fig. 1A). As the -10 and -35 promoter regions of *pea* highly resembled consensus recognition sequences for the sigma factor RpoS (Fig. 1A and B) (Weber et al., 2005; Typas et al., 2007), we speculated that transcription of the *pea* operon was dependent on RpoS. To confirm this hypothesis, we constructed a *P. putida* KT2440 *rpoS* deletion mutant (Δ*rpoS*) and a reporter plasmid pBRTZ-*pea*P, in which the promoter-less *lacZ* was fused with a fragment containing 633 bp upstream of *pea* operon and the first 72 bp of *PP_3132*. The wild-type KT2440 strain harboring the *lacZ* fusion reporter showed very low β-galactosidase activity in 10-hour-old cells but had significantly increased activity in cultures grown more than 22 h (Fig. 2A). Conversely, when RpoS was absent, β-galactosidase activity was too low to be quantified through the entire incubation period (Fig. 2A). This result indicates that RpoS is necessary for the normal expression of *pea*. Furthermore, we noticed that the Δ*rpoS* strain

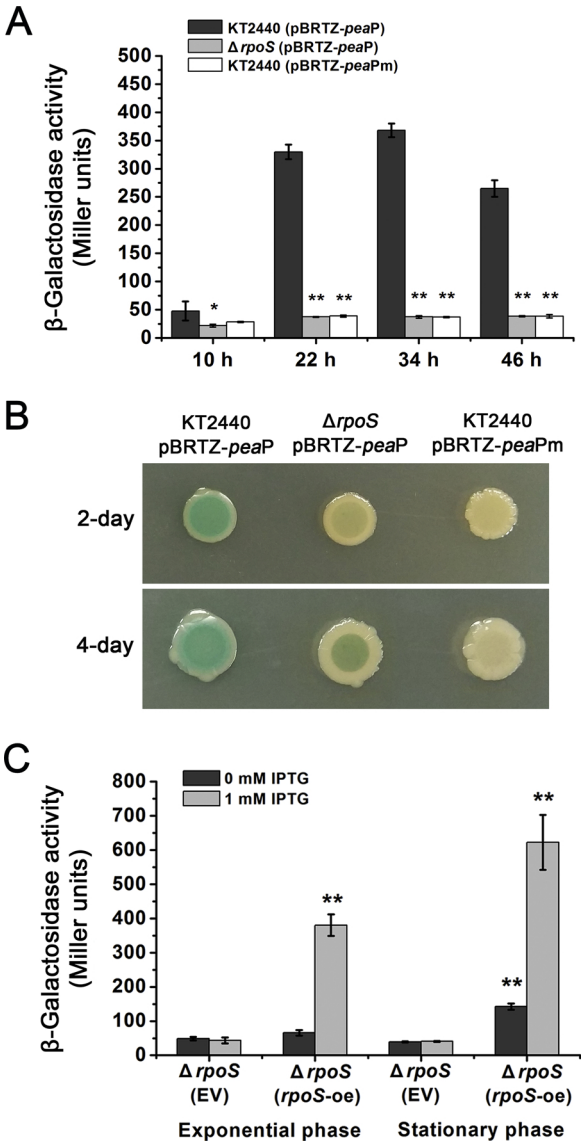

Fig. 2. Transcription of *pea* relies on the sigma factor RpoS. (A) Expression levels of the *lacZ* transcriptional fusions with either the native *pea* promoter (pBRTZ-*pea*P) or a *pea* promoter with mutated -10 element (pBRTZ-*pea*Pm). β-Galactosidase activity was measured to evaluate the activity of the promoters in the indicated strains. (B) Wild-type KT2440 and Δ*rpoS* harboring the indicated reporter plasmids were incubated on LB agar with supplement of 40 μg/ml X-gal. The blue product indicated the expression of *lacZ* fusion. (C) The empty vector pBBR1-403 (EV) or overexpression vector pB403-*rpoS* (*rpoS*-oe) were transferred into Δ*rpoS* harboring pBRTZ-*pea*P, and the strains were incubated with or without 1 mM IPTG. The β-galactosidase activity was measured when cultures were incubated to exponential phase (10 h) and stationary phase (24 h). All assays were performed in triplicate. Asterisks indicate significant differences from the wild-type (*$P < 0.05$, **$P < 0.01$, determined by Student's *t* test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

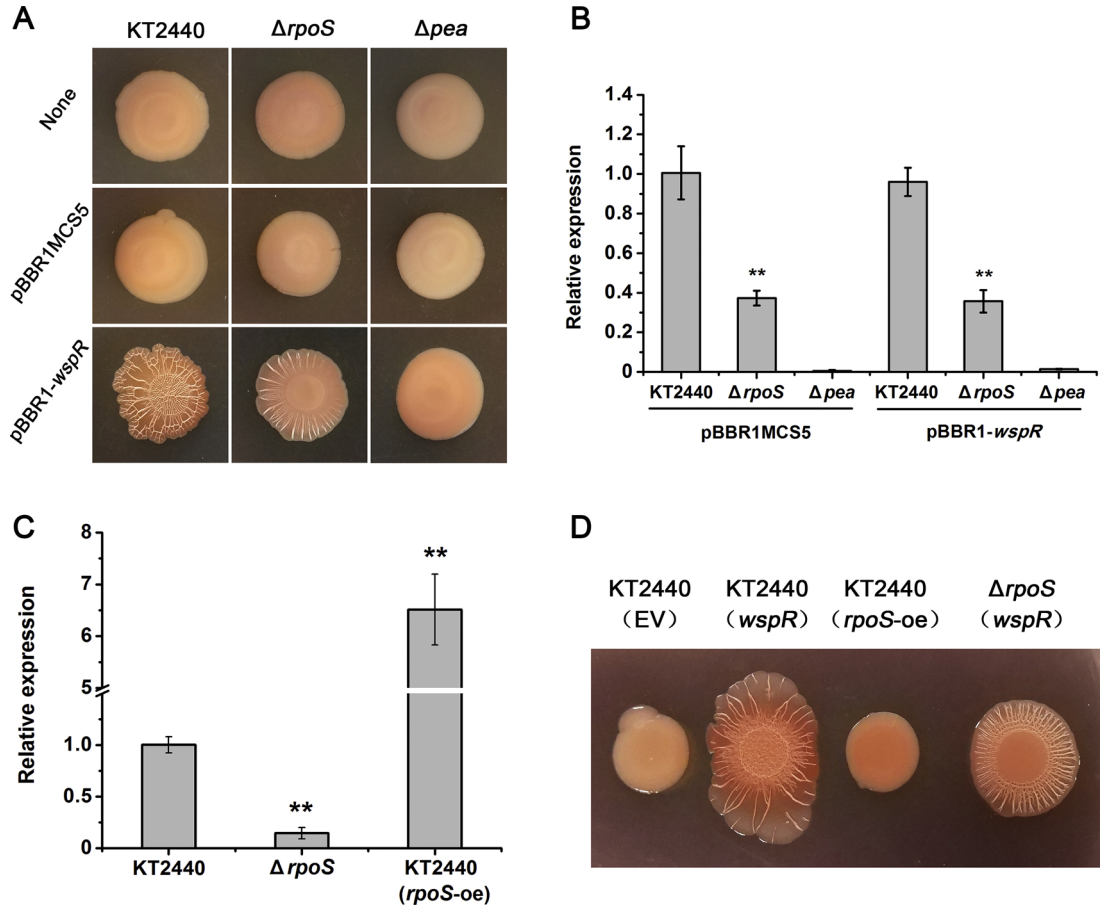

Fig. 3. Pea involves in formation of c-di-GMP-dependent wrinkly colony. (A) Colony morphology of KT2440, Δ*rpoS* and Δ*pea* with elevated c-di-GMP levels. The strains containing the indicated plasmid were incubated on LB agar supplemented with Congo red (40 μg/ml) for 5 days. (B) Expression of *pea* in colonies formed by KT2440, Δ*rpoS* and Δ*pea* strains with elevated levels of c-di-GMP. The expression levels of *pea* (*PP_3140*) in 3-day-old colonies were measured using qRT-PCR. (C) Overexpressed RpoS from pB403-*rpoS* increased transcription of *pea*. Relative expression level of *pea* (*PP_3140*) was detected using qRT-PCR when cultures were incubated with 0.5 mM IPTG for 24 h. (D) Colony morphology of KT2440 harboring pBBR1-403 (EV), pBBR1-*wspR* (*wspR*) or pB403-*rpoS* (*rpoS*-oe) and Δ*rpoS* containing pBBR-*wspR*. The strains containing the indicated plasmid were incubated on LB agar supplemented with 40 μg/ml Congo red and 0.5 mM IPTG for 5 days. All assays were performed in triplicate. Asterisks indicate significant differences from the wild-type (*$P < 0.05$, **$P < 0.01$, determined by Student's *t* test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

harboring the reporter plasmid showed slight β-galactosidase activity when grown on LB agar containing X-gal for more than 2 days (Fig. 2B). The blue product produced from hydrolyzation of X-gal by LacZ could not be further degraded by KT2440, thus the blue substance would accumulate in colonies. Even though incubated for 4 days, the colony of Δ*rpoS* harboring pBRTZ-*pea*P still showed weak blue color (Fig. 2B), suggesting that the activity of *pea* promoter was very low but still able to switch on gene transcription when RpoS was absent. Among the other 23 sigma factors in KT2440 (Martinez-Bueno et al., 2002), the housekeeping sigma factor RpoD (known as σ^{70} or σ^{D}) was the most possible element for the residual expression of *pea* promoter since only one transcription start site was identified and the -10 region of *pea* promoter contained a motif (TATAGT) which corresponds to that of RpoD-dependent promoter (TATAAT) (Typas et al., 2007). In fact, RpoD is able to target multiple promoters that are also recognized by RpoS, as an example provided by *osmE* that it could be transcriptionally mediated by both RpoS and RpoD, but the transcription level was intrinsically low when controlled by RpoD rather than by RpoS (Bordes et al., 2000). This is also supported by the work from Gaal and colleagues that the promoter sequences selected by the RNA polymerase holoenzyme $E\sigma^{S}$ contained the known consensus elements (-10 and -35 elements) for recognition by $E\sigma^{D}$ and the different deviations from the consensus sequences would lead to relative DNA recognition preferences of the two holoenzymes (Gaal et al., 2001). In spite of this, it still requires to be further verified that whether the residual expression of *pea* is transcribed from this identified transcription start site.

To block RpoS recognition of the *pea* promoter, we mutated the *pea* -10 element 5′-ACTATAGT-3′ to 5′-AGAATAGT-3′ in pBRTZ-*pea*P, yielding pBRTZ-*pea*Pm (Fig. 1B). The second nucleotide cytosine in the -10 element is a hallmark of RpoS-dependent promoters and it contributes to RpoS selectivity for a promoter (Weber et al., 2005; Typas et al., 2007). Replacement of the cytosine by other nucleotides would result in severe reduction of RpoS-mediated transcription (Bordes et al., 2000). As expected, the mutated reporter plasmid did not produce detectable β-galactosidase in wild-type (Fig. 2A and B). The mutation in the -10 element likely eliminated the affinity to both RpoS and RpoD, thus preventing the binding of RNA polymerase to the promoter. As a result, the mutated *pea* promoter lost the ability to interact with RNA polymerase and switch on gene transcription. According to these results, there is probably no other transcription start site exists in the *PP_3132* promoter. To further confirm the involvement of RpoS in *pea* expression, we overexpressed *rpoS* in Δ*rpoS* by putting the gene under the control of IPTG-inducible LacI/*Ptac* cassette on plasmid pBBR1-403. The reporter plasmid pBRTZ-*pea*P was used to estimate the activity of

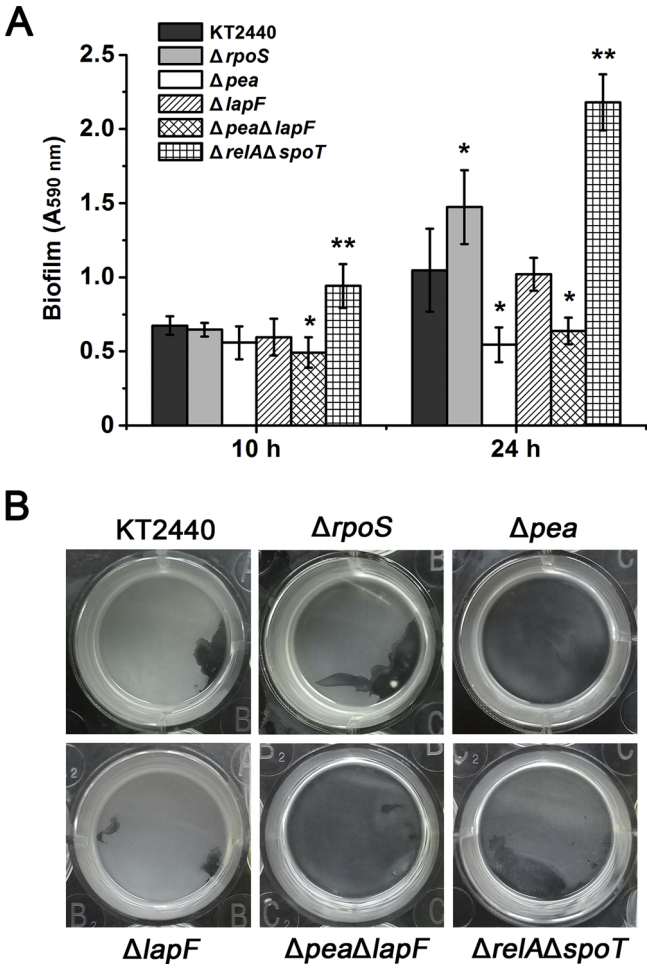

Fig. 4. Biofilm formation of KT2440 wild-type and mutants. (A) The amount of biofilm at 10 h and 24 h was quantified by crystal violet staining. The assay was performed in quadruplicate. Asterisks indicate significant differences from the wild-type (*$P < 0.05$, **$P < 0.01$, determined by Student's *t* test). (B) Pellicle morphology of KT2440 wild-type and derivatives. Each strain was incubated statically for 24 h.

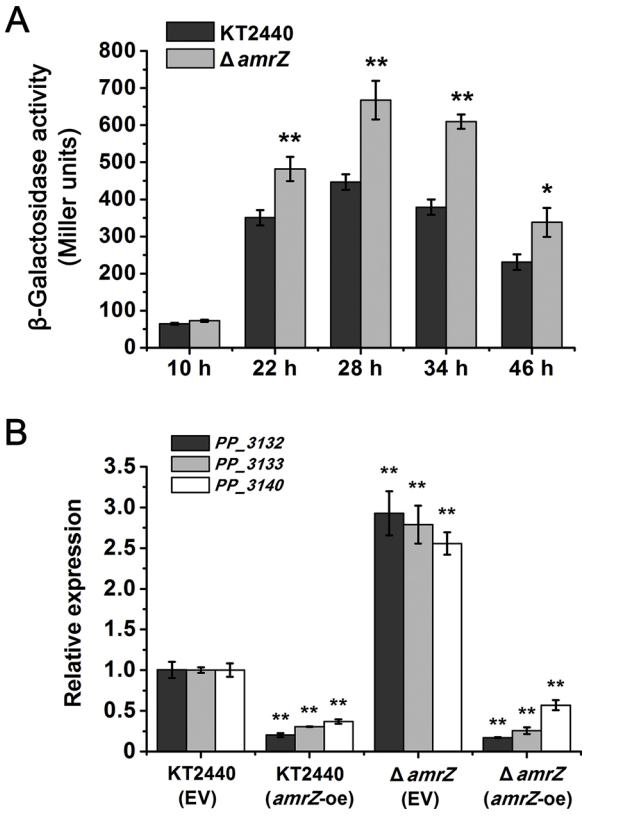

Fig. 5. Expression of *pea* is repressed by AmrZ. (A) The reporter plasmid pBRTZ-*pea*P was used to measure *pea* expression levels in wild-type and Δ*amrZ*. (B) The empty vector pBBR1-403 (EV) and the *amrZ* overexpression vector pB403-*amrZ* (*amrZ*-oe) were introduced into wild-type or Δ*amrZ*. The expression levels of genes in the *pea* operon were measured using qRT-PCR. All assays were performed in triplicate. Asterisks indicate significant differences from the wild-type (*$P < 0.05$, **$P < 0.01$, determined by Student's *t* test).

pea promoter as above. When *rpoS* was overexpressed in Δ*rpoS*, the activity of *pea* promoter was significantly increased in exponential phase cells and much higher in stationary phase cells, as compared to Δ*rpoS* with empty vector (Fig. 2C). The Δ*rpoS* containing the overexpression plasmid but without IPTG-induction displayed slight activity of *pea* promoter in stationary phase (Fig. 2C). Leaky expression of the *tac* promoter could account for this result.

Overall, these results verify that the sigma factor RpoS is necessary for full transcription of the *pea* operon, while RpoD may provide slight expression when RpoS is absent. This is not the only example of a regulatory role for RpoS and RpoD in polysaccharide gene expression. In *P. aeruginosa* PAO1, transcription of the polysaccharide operon *psl* is also controlled by both RpoS and RpoD, while RpoS can lead to a much higher transcription level than RpoD (Irie et al., 2010). What is different from our finding is that RpoS and RpoD recognize the different regions in *psl* promoter, which the -10 elements are separated by about 80 bp segment of DNA (Irie et al., 2010).

3.2. Pea is essential for a c-di-GMP-dependent wrinkly colony morphology

When the multi-copy plasmid carrying *rup4959*, which could lead to increased c-di-GMP level and was transcriptionally controlled by RpoS, was transferred into KT2440, the strain would form a colony with wrinkly morphology, however, strains with mutation in *pea* operon did not exhibit this phenotype (Matilla et al., 2011). We were interested in the role of Pea polysaccharide in formation of wrinkly colony. Therefore, we employed a c-di-GMP synthase WspR, which could lead to elevated intracellular c-di-GMP levels in KT2440 strains (Xiao et al., 2016). After transferred with the multi-copy vector pBBR1-*wspR*, wild-type, Δ*rpoS* and Δ*pea* could form significantly increased biofilm (Fig. A1), implying that *wspR* expressed in a multi-copy plasmid was able to raise the c-di-GMP levels in these strains. As shown in Fig. 3A, the empty vector pBBR1MCS5 had no effect on the colony morphology since the strains with or without the empty vector formed similar smooth colonies. When *wspR* was overexpressed through multi-copy, wild-type displayed the typical wrinkly colony phenotype, but Δ*pea* did not have any visible change in colony morphology (Fig. 3A), indicating that Pea is necessary for the development of c-di-GMP-dependent wrinkly colonies. Interestingly, the Δ*rpoS* strain containing pBBR1-*wspR* also exhibited wrinkly morphology, even though it was much weaker than that of wild-type (Fig. 3A). We then isolated the RNA from the colonies grown on LB agar for 3 d and detected the levels of *pea* mRNA using qRT-PCR analysis. The transcription of *pea* gene cluster was decreased by 64% in Δ*rpoS* (pBBR1MCS5) as compared to wild-type harboring pBBR1MCS5, the same in Δ*rpoS* (pBBR1-*wspR*) (Fig. 3B). This result reminds us that transcription of *pea* could also be mediated by RpoD and that the low expression level of *pea* may also contribute to developing a colony with wrinkly morphology. We also noticed that no difference in *pea* transcription level was detected when c-di-GMP level

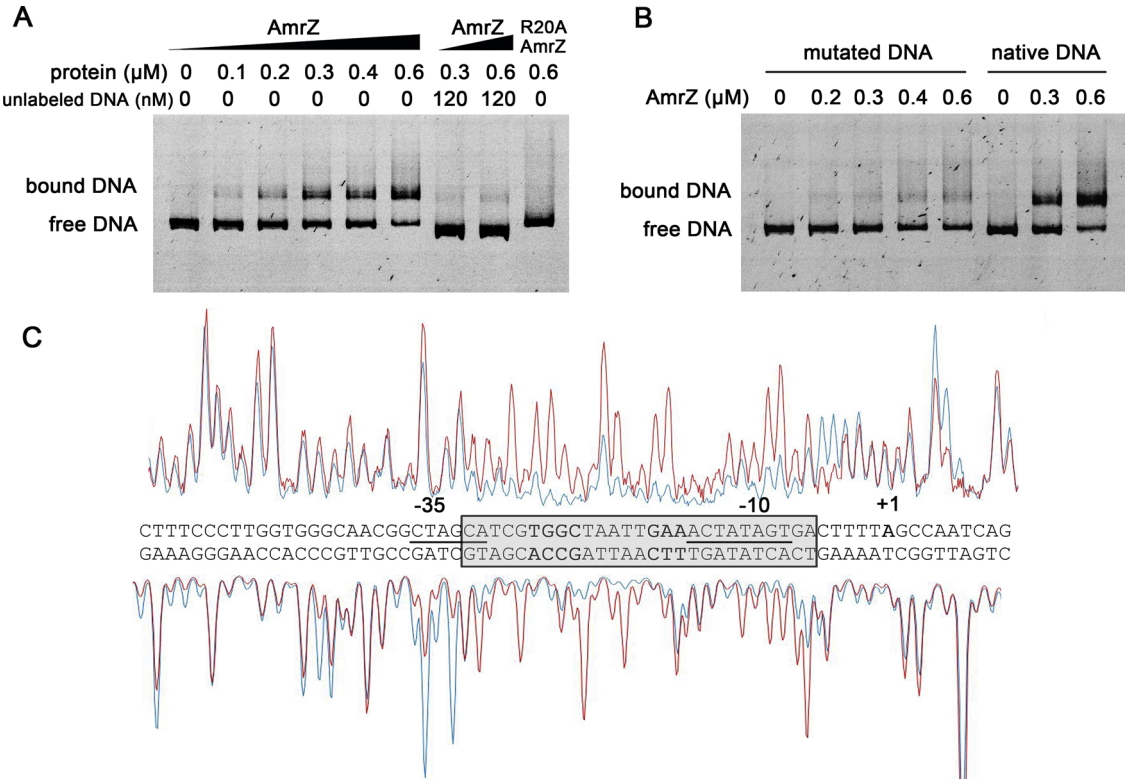

Fig. 6. AmrZ binds to *pea* promoter directly. (A) EMSA used 6 nM of the FAM-labeled fragment containing *pea* promoter in each reaction with a volume of 20 μl. The purified AmrZ, R20 A AmrZ (DNA binding deficient AmrZ) and unlabeled cold probe were added at the indicated concentrations. (B) The FAM-labeled *pea* promoter with mutation in the AmrZ binding site which identified by DNase I footprinting assay was used in the EMSA. Each reaction contained 6 nM of FAM-labeled mutated DNA or FAM-labeled native DNA, and different concentrations of AmrZ as indicated. (C) DNase I footprinting assay identified the AmrZ binding site in *pea* promoter. FAM-label was added to the 5′ end of the coding strand (peaks above the promoter sequence) or to the non-coding strand (peaks below the promoter sequence) of the DNA fragments containing the *pea* promoter. AmrZ was present (blue peaks) or absent (red peaks) in each reaction. The protected nucleotides were boxed in gray. The -10 and -35 elements were underlined, and the transcription start site was marked as "+1". (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was elevated in this case. Actually, c-di-GMP was reported to directly participate in process of polysaccharide synthesis, for example, it bound to components of polysaccharide synthase complexes and promoted the synthesis of polysaccharides alginate and Pel in *P. aeruginosa* (Lee et al., 2007; Merighi et al., 2007). However, the analysis of *pea* loci based on comparisons with protein sequences showed that it did not contain any PilZ, GGDEF, EAL or HD-GYP domains which were relevant to c-di-GMP interaction, synthesis or degradation (Nielsen et al., 2011). As *pea* transcription level in Δ*rpoS* strain with pBBR1-*wspR* was far below that in wild-type strain with pBBR1MCS5 (Fig. 3B), we inferred that the Pea polysaccharide was necessary but not sufficient for formation of c-di-GMP-dependent wrinkly colony. Therefore, we tried to raise the expression level of *pea* via overexpressing RpoS to test whether overexpressed *pea* could lead a wrinkly colony. Although the KT2440 strain with overexpressed RpoS had about 6.51-fold increased *pea* expression level (Fig. 3C), it could not form a wrinkly colony (Fig. 3D). This further suggests overproduced Pea is not enough for development of wrinkly colony in *P. putida*. By contrast, in *P. aeruginosa*, increased production of Psl polysaccharide, as a result of overexpression of RpoS, was able to lead a rugose colony variant (Irie et al., 2010).

3.3. Pea contributes to biofilm development and pellicle formation

Generally, the exopolysaccharides are implicated in development of biofilm, for instance in *P. aeruginosa*, Psl polysaccharide is required for initiation and maintenance of biofilm structure (Ma et al., 2006) and Pel polysaccharide contributes to formation of air-liquid interface pellicle (Friedman and Kolter, 2003). With the aim of determining the role of Pea in biofilm formation, we carried out the biofilm assay in LB medium. The strains were incubated in 24-well polystyrene microtiter plates under shaking condition and the amount of biofilm was quantified by crystal violet staining. As shown in Fig. 4A, Δ*pea* formed similar amount of biofilm as wild-type at 10 h, but when the incubation period was increased to 24 h, biofilm production of Δ*pea* did not increase whereas that of wild-type had risen by 55% in comparison to that of wild-type at 10 h. This result, along with that Pea acts a significant role in biofilm stability in KT2440 (Nilsson et al., 2011), implies Pea may be important for the subsequent development of biofilm under shaking condition. It is interesting that Δ*rpoS* formed significantly more biofilm than wild-type (Fig. 4A) and this phenotype was also observed in *P. aeruginosa* PAO1 (Whiteley et al., 2001). Psl is essential for biofilm formation in PAO1 (Jackson et al., 2004), and RpoS is the major sigma factor for transcription of the gene cluster *psl* (Irie et al., 2010). However, the *rpoS* mutant of PAO1 has much stronger ability to form biofilms that it attached to and covered much more of the glass surface than wild-type (Whiteley et al., 2001). It seems that the positive effect of *rpoS* deletion on biofilm development is stronger than the negative effect in *Pseudomonas* species. As a global regulator, RpoS probably regulates other factors that involve in biofilm development. In the pellicle assay (observation of the biofilm formed at air-liquid interface), it showed that Pea was essential for pellicle formation since the Δ*pea* strain was completely defective in pellicle formation (Fig. 4B), in accordance with the finding from a previous study (Matilla et al., 2011). The Δ*rpoS* strain had considerable ability to form pellicle, but it was much weaker than wild-type (Fig. 4B), implying that full expression of *pea*, which relies on RpoS, is necessary for forming a robust pellicle.

We previously found that the expression levels of *rpoS*, *lapF* and *pea* were decreased and those of *lapA* and *bcs* were increased in KT2440 (p)ppGpp-negative mutant. In comparison to wild-type, the mutant formed increased biofilm production but a frail pellicle (Liu et al., 2017). It was indeterminate whether the low expression of *lapF* or *pea* led the failure of forming a healthy pellicle in (p)ppGpp-negative mutant. Here, we employed the *lapF* mutant (Δ*lapF*), the *pea-lapF* mutant (Δ*pea*Δ*lapF*) and the (p)ppGpp-negative strain (Δ*relA*Δ*spoT*) in biofilm and pellicle assays. As shown in Fig. 4A and B, Δ*relA*Δ*spoT* had 1.40-fold (10 h) and 2.08-fold (24 h) increased biofilm production but it was defective in forming a pellicle with perfect structure, in accordance with the previous results (Liu et al., 2017). The Δ*lapF* strain displayed similar biofilm production and pellicle morphology compared to wild-type, and the Δ*pea*Δ*lapF* strain was not different from the Δ*pea* strain in terms of biofilm phenotypes (Fig. 4A and B), indicating that LapF is redundant in biofilm development in this case. Furthermore, the defective pellicle in Δ*relA*Δ*spoT* is probably caused by decreased expression of *pea* but not *lapF,* even though LapF also has a role in the establishment of cell-cell interactions (Martinez-Gil et al., 2010).

3.4. AmrZ represses transcription of the pea operon

For purpose to identify the regulator involved in *pea* expression, we inspected the sequence from -300 to +55 relative to the *PP_3132* start codon and found several motifs that displayed similarity to the AmrZ consensus binding sequences of 5′-TGGCN5GCC-3′ (Prada-Ramirez et al., 2016) or 5′-CARATTGCCATCA-3′ (Jones et al., 2014), as shown in Fig. A2. The KT2440 AmrZ (encoded by *PP_4470*) shares 93.4% amino acid sequence similarity with the AmrZ from *P. aeruginosa* PAO1 (Fig. A3). It has been demonstrated that AmrZ acts as a functional regulator in *P. putida* PaW85 which is isogenic to KT2440 (Mumm et al., 2016).

To verify the regulatory role of AmrZ in *pea* expression, we constructed an *amrZ* deletion mutant (Δ*amrZ*) and then evaluated the activity of the *pea* promoter by utilizing the *lacZ* fusion reporter plasmid. With exception of the first 10 h in which *pea* kept extremely low expression, *pea* expression levels in Δ*amrZ* increased by 37% (22 h), 50% (28 h), 61% (34 h), 47% (46 h) compared to that in wild-type (Fig. 5A). Furthermore, we attempted to test the effect of overexpressed *amrZ* on *pea* expression. Since AmrZ would repress its own promoter (Ramsey et al., 2005), we accomplished this by employing the LacI/*Ptac* system. The qRT-PCR analysis was performed to assess the transcription levels of three genes (*PP_3132*, *PP_3133* and *PP_3140*) that belonged to the *pea* operon. Transcription levels of all these genes decreased by 43%–83% in both wild-type and Δ*amrZ* strains with overexpressed *amrZ* in comparison to the wild-type with empty vector (Fig. 5B). Furthermore, the *pea* transcription level in Δ*amrZ* containing the empty vector increased by more than 1.55-fold compared to that in wild-type with empty vector (Fig. 5B), which was consistent with the result from the β-galactosidase assay (Fig. 5A). Based on these results, we consider that AmrZ acts as a repressor in *pea* regulation. The role of AmrZ as a repressor of polysaccharide genes was also found in other *Pseudomonas* species, for instance, AmrZ repressed the transcription of the *psl* operon in *P. aeruginosa* (Jones et al., 2013) and the cellulose operon *wssABCDEFGHI* in *P. syringae* pv. *tomato* DC3000 (Prada-Ramirez et al., 2016).

3.5. AmrZ regulates pea by directly binding to promoter

Plenty of evidences suggest that AmrZ plays its regulatory role in gene expression by directly binding to promoters (Pryor et al., 2012; Jones et al., 2013, 2014; Xu et al., 2016). To determine whether AmrZ repressed *pea* promoter activity through direct binding, we performed the EMSA with purified AmrZ. As shown in Fig. 6A, purified AmrZ could specifically bind to the fragment from -300 to +55 relative to *PP_3132* start codon. Binding activity of PAO1 AmrZ requires the arginine at residue 22 (R22) (Jones et al., 2014), which corresponds to the 20th residue (R20) of KT2440 AmrZ (Fig. A3). In this experiment, the ability of AmrZ to bind to the *pea* promoter fragment also depended on the R20 residue since substitution of alanine for the arginine (R20 A) eliminated AmrZ binding activity to the *pea* promoter (Fig. 6A). To further define the precise AmrZ binding site in the *pea* promoter, DNase I footprinting assay was employed. AmrZ protected the region from -32 to -6 relative to the transcription start site (or from -57 to -31 relative to *PP_3132* start codon) (Fig. 6C). Subsequently, we mutated the AmrZ binding site in the *pea* promoter by replacing “TGGCT” (from -27 to -23 relative to the transcription start site) with “TTTTT”, which was identified as being able to block the affinity between DNA and AmrZ (Prada-Ramirez et al., 2016). As expected, AmrZ failed to bind the mutated fragment (Fig. 6B), indicating that this site was essential for AmrZ recognition. The position of the AmrZ binding site in the *pea* promoter is in accordance with the consensus of AmrZ-repressed promoters, in which AmrZ binding sites locate in region from -100 to +15 relative to transcription start site (Jones et al., 2014). The AmrZ binding site covers the -10 and -35 elements in the *pea* promoter, implying that AmrZ interferes with the RNA polymerase to bind to the *pea* promoter and results in depressed transcription level.

4. Conclusion

This study focuses on the regulation of the exopolysaccharide gene cluster *pea* and the role of Pea polysaccharide in *P. putida* KT2440. In the course of the analysis, we identified that the transcription start site of the *pea* operon located on 25 bp upstream of *PP_3132*, the first gene of the operon. The transcription of *pea* is mainly controlled by the stationary phase sigma factor RpoS, and RpoD may also be able to mediate *pea* transcription but has much less contribution than RpoS. As a component of extracellular matrix, Pea plays a significant role in biofilm development, such as in full production of biofilm, formation of air-liquid interface pellicle and establishment of c-di-GMP-dependent wrinkly colony. Moreover, transcription of *pea* is under the regulation of AmrZ, which can directly bind to the region between the -10 and -35 elements of the *pea* promoter, leading to inhibition of transcription initiation. Apart from these, a previous finding should be noticed that expression of *pea* was highly induced when KT2440 grew under water-limiting condition (Nielsen et al., 2011), but the mechanism of how environmental factors affected *pea* expression had not been characterized. Additional regulatory pathways for *pea* expression may exist and wait for exploration.

Acknowledgements

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Appendix A

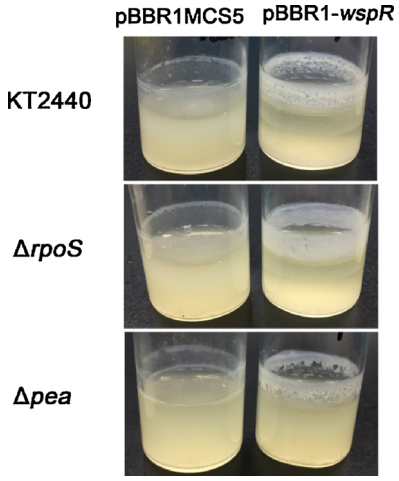

Fig. A1. Biofilm formation of KT2440 wild-type, Δ*rpoS* and Δ*pea* harboring pBBR1MCS5 or pBBR1-*wspR*. The cultures were incubated for 16 h under shaking at 180 rpm.

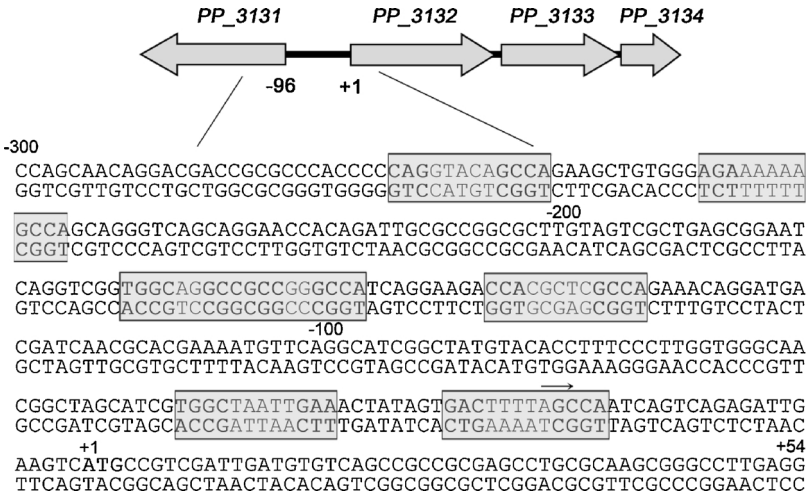

Fig. A2. The potential AmrZ binding sites in region upstream of the *pea* operon. The motifs resembled to AmrZ binding sequence 5′-TGGCN5GCC-3′ were boxed. The start codon of *PP_3132* is marked as "+1" and the arrow indicates the transcription start site (N = A/T/C/G).

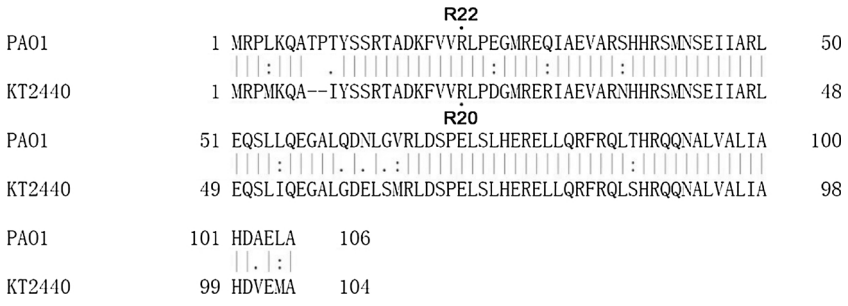

Fig. A3. Alignment of AmrZ from *P. aeruginosa* PAO1 and *P. putida* KT2440. The similarity between two sequences was calculated by the online tool EMBOSS Matcher (https://www.ebi.ac.uk/Tools/psa/emboss_matcher/).

Table A1
Primers used in this study.

Primer	Sequence
For mutant constructions	
*rpoS*upS	TGTGGAATTCCCGGGAGAGCTC GCCAACGGTGTACTGATT
*rpoS*upA	CAGCGGCGAGAAACCAATC
*rpoS*dnS	GATTGGTTTCTCGCCGCTG GAGTTGACCGACAAGCAGCG
*rpoS*dnA	TACCGCATGCGATATCGAGCTC AACGAGACGGCGACGCCTA
*pea*upS	TGTGGAATTCCCGGGAGAGCTC GCCAGCAGTTTATCAGCCCA
*pea*upA	TG GTATGCCCCAGCACGGTCC
*pea*dnS	GGACCGTGCTGGGGCATAC CAACCGGAAGCAGGTGTGG
*pea*dnA	TACCGCATGCGATATCGAGCTC GCCTACGGCAGTATCGAAGC
*lapF*upS	GTGGAATTCCCGGGAGAGCTC ATCAATCTAAACGCACCAAGC
*lapF*upA	GCCGTCAGCACCAACTGTAAT
*lapF*dnS	ATTACAGTTGGTGCTGACGGC GCAACGGTGGGCAGCAACGG
*lapF*dnA	ACCGCATGCGATATCGAGCTC TCACTGGCAGGTTCAGCGAG
*amrZ*upS	ACCGCATGCGATATCGAGCTC TCACCAGCACTGCCCTCCCC
*amrZ*upA	GGAATTCCAGGCAGGCGGACGACGAACT
*amrZ*dnS	TCGTCCGCCTGCCTGGAATTC CAGCAGAACGCCTTGGTCGC
*amrZ*dnA	GTGGAATTCCCGGGAGAGCTC CCGTGAGGAAAGCGAGAGCG
For plasmid constructions	
3132PS	GGTCTAGA CCACGTAATACGGCAGGAG
3132 PA	AGCTGCAG CAGGTTCCAAGACCCGGCCC
3132PmS	GTGGCTAATTGAAAGAATAGTGACTTTTAG
3132PmA	CTAAAAGTCACTATTCTTTCAATTAGCCAC
rpoSs-oe	TGAATTC ATGGCTCTCAGTAAAGAAGTG
rpoSa-oe	GCAAAGCTT TGCCTCGGGCTTCCTTCAG
amrZs-oe	AGAATTC ATGCGCCCAATGAAACAGG
amrZa-oe	GACAAGCTT GGCCTTGCTGCCAGTCAGAC
amrZs	TTTAAGAAGGAGATATACC ATGCGCCCAATGAAACAGGC
amrZa	GTGGTGGTGGTGGTGCTCGAG GGAGGCGTCTGCGGCCATTT
amrZ-R20As	CTGACAAGTTCGTCGTCGCCCTGCCTGACGGAATGCGT
amrZ-R20Aa	ACGCATTCCGTCAGGCAGGGCGACGACGAACTTGTCAG
For EMSA and DNase I footprinting assay	
*pea*PS300FAM	TGTCAAGCTACCTGCTGA CCAGCAACAGGACGACCGC
*pea*PS300	CCAGCAACAGGACGACCGC
*pea*PA55FAM	TGTCAAGCTACCTGCTGA CCCTCAAGGCCCGCTTGC
*pea*PA55	CCCTCAAGGCCCGCTTGC
PFAM5U1	6FAM-TGTCAAGCTACCTGCTGA
*pea*P50mS	CAACGGCTAGCATCGTTTTTAATTGAAACTATAG
*pea*P50mA	CTATAGTTTCAATTAAAAACGATGCTAGCCGTTG
For qRT-PCR	
gyrA-RTS	ATGGTGGTTATGCCAAGA
gyrA-RTA	CGTAGGTCTTCTTCCAGTA
3132-RTS	GCTATACCTTGGCTCATC
3132-RTA	TGCAGTTCACATACACAA
3133-RTS	TGGTTTTGTCTGACTATGAG
3133-RTA	CGATATTCAGGTGTTCCAT
3140-RT4F	GCGGCAAAGGTTACGCATTGGATTTC
3140-RT4R	TGCACGCATCAGATACAGCGAC
alg-RTF	ATGATCTTCCTCTACGGCGTGTACC
alg-RTR	AGCAGTTCATCGGACATTTCCACC
For 5' RACE assay	
3132-5RACE	TGCGAGACCAGGTTCCAA
5'-adapter	GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGG

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