A CHASE3/GAF sensor hybrid histidine kinase BmsA modulates biofilm formation and motility in *Pseudomonas alkylphenolica*

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*Pseudomonas alkylphenolica* is an important strain in the biodegradation of toxic alkylphenols and mass production of bioactive polymannuronate polymers. This strain forms a diverse, 3D biofilm architecture, including mushroom-like aerial structures, circular pellicles and surface spreading, depending on culture conditions. A mutagenesis and complementation study showed that a predicted transmembrane kinase, PSAKL28_21690 (1164 aa), harbouring a periplasmic CHASE3 domain flanked by two transmembrane helices in addition to its cytoplasmic GAF, histidine kinase and three CheY-like response regulator domains, plays a positive role in the formation of the special biofilm architecture and a negative role in swimming activity. In addition, the gene, named here as *bmsA*, is co-transcribed with three genes encoding proteins with CheR (PSAKL28_21700) and CheB (PSAKL28_21710) domains and response regulator and histidine kinase domains (PSAKL28_21720). This gene cluster is thus named *bmsABCD* and is found widely distributed in pseudomonads and other bacteria. Deletion of the genes in the cluster, except for *bmsA*, did not result in changes in biofilm-related phenotypes. The RNA-seq analysis showed that the expression of genes coding for flagellar synthesis was increased when *bmsA* was mutated. In addition, the expression of *rsmZ*, which is one of final targets of the Gac regulon, was not significantly altered in the *bmsA* mutant, and overexpression of *bmsA* in the *gacA* mutant did not produce the WT phenotype. These results indicate that the sensory Bms regulon does not affect the upper cascade of the Gac signal transduction pathway for the biofilm-related phenotypes in *P. alkylphenolica*.

**INTRODUCTION**

Bacteria are exposed to physicochemically changing environments in nature where they survive and reproduce themselves. To monitor and adapt to the environmental conditions, bacteria have developed specific signal transduction systems to trigger adaptive responses (Galperin et al., 2001). Among these systems, transmembrane receptors, which possess sensing domains exposed to the outside of the cell, detect certain chemical stimuli in the environment. The input signals are then conveyed to the gene expression controls that modulate multicellularity. Two-component signal transduction systems are the most common for bacterial sensing of environmental conditions (Mascer et al., 2006). In the typical system, the transmembrane receptors are sensor histidine kinases, which have an N-terminal ligand-binding domain located outside of the cytoplasmic membrane and a C-terminal kinase domain, but other domains may also be present. Upon signal binding, the basal autophosphorylation activity in one conserved His of the kinase domain is modulated by ATP hydrolysis. As a phosphorelay event, the phosphory group on the His is transmitted to the Asp residue in the N-terminal REC.
domain (CheY-like phosphoreceptor) of the cognate response regulator, leading to binding of the transcriptional regulator to the promoter of target genes via activation of the C-terminal DNA binding domain (Laub & Goulian, 2007). As a specific example, the Gac two-component signal transduction system, which consists of a transmembrane receptor GacS His kinase and a cognate GacA response regulator, is found in Pseudomonas and known to control gene expression related to biofilm formation, antibiotic production and other traits. In most cases, this occurs through the expression of small RNA genes including rsmZ (Brencic et al., 2009; González et al., 2008). However, the precise nature of the input signals for GacS is unknown.

Pseudomonas alkylphenolica KL28 was isolated due to its ability to catabolize phenols with a linear alkyl group (C₃–C₈) at the para and meta positions via a lap catabolic pathway (Cho et al., 2009; Jeong et al., 2003). Long-chain alkylphenols are known to cause adverse effects such as developmental and reproductive toxicity in animals (Sharpe et al., 1995). Biotechnologically, the mucA mutant of this strain has significant potential for the mass production of poly-β-α-mannuronate derivatives that can be applied for development of various drugs (Veeranagouda et al., 2011a). P. alkylphenolica KL28 has been taxonomically identified as a novel species and nomenclature was correctly latinized from the name previously proposed, Pseudomonas alkylphenolica (Mulet et al., 2015). This strain forms diverse 3D biofilm architectures and displays different mobility behaviours depending on the growth medium and carbon source used (Lee & Veeranagouda, 2009). This strain forms highly branched aerial structures with a height of 2–4 mm when p-cresol is supplied in the vapour, and it forms circular pellicles (diameter 0.4–0.5 mm) on the surface of standing cultures in LB liquid. This strain spreads on semi-solid LB agar medium and swims in liquid medium (Veeranagouda et al., 2011b; Yun et al., 2007). Previously, we showed that the Gac regulatory cascade in P. alkylphenolica positively controls wrinkling colony morphology, biofilm formation, surface translocation and aerial structure formation (Choi et al., 2007; Lee & Veeranagouda, 2009). Here, a novel CHASE3/GAF multi-sensor hybrid histidine kinase, PAKL28_21690, which is widely distributed in Pseudomonas and other Gram-negative bacteria, is shown to be required for the same phenotypes controlled by the Gac regulon in P. alkylphenolica.

**METHODS**

**Strains and culture conditions.** The P. alkylphenolica KL28 WT strain (KCTC 2206 and JCM 16553) was cultured in LB medium (Bertani & Bertani, 1970) or minimal salts basal medium (Stanier et al., 1966) with appropriate carbon source(s) at 30 °C. Escherichia coli cells were grown on LB medium at 37 °C. For maintenance of the plasmids in cells, an appropriate antibiotic was added to the medium in amounts as previously described (Yun et al., 2007). The experimental details for the measurement of surface spreading motility, pellicle and aerial structure formation by cells were previously described (Choi et al., 2007; Veeranagouda et al., 2011b).

**Construction and genetic analysis of a P. alkylphenolica transposon mutant library.** A P. alkylphenolica mutant transposon library was generated using the transposon delivery vector pRL27, a plasmid carrying a hyperactive Tn5 transposase gene (tnp) (Larsen et al., 2002), and the mutation sites in genes were identified by a plasmid rescue procedure as previously described (Yun et al., 2007). More than 2000 transposon mutants were screened as previously described (Lee et al., 2014). The gene locus tags were identified from the genome annotation, and the deduced amino acid sequences were compared to the protein sequence database (GenBank) using the BLASTP algorithm (http://www.ncbi.nlm.nih.gov). The sequence homology between proteins was calculated using the BioEdit program with the BLOSUM62 score matrix (http://www.mbio.ncsu.edu/BioEdit/page2.html). The domains in proteins were searched using the SMART algorithm (http://smart.embl-heidelberg.de). Orthologue, paralogue and gene clusters in other bacteria were searched via the KEGG database (http://www.genome.jp/kegg). The complete genome sequence of P. alkylphenolica KL28 is available in the NCBI Reference Sequence: NZ_CP009048.1.

**Measurement of pellicle biofilm growth relative to suspension growth.** The strains were grown in LB medium with shaking at 160 r.p.m. for 12 h (SI-900R model, Jeio Tech Co. Korea). The cell suspensions (10 μl) from the seed cultures were inoculated into 20 ml LB liquid in Petri dishes by gentle swirling with PIPETMAN tips. At cultivation at 25 °C for 48 h without shaking, the cell suspension liquids were carefully removed and the Petri dishes were rinsed twice with 10 ml of saline. The pellicles that remained during the washing step were suspended in 20 ml of 0.5 % SDS. The solutions were then transferred to 50 ml Falcon tubes and incubated for 40 min at 37 °C with rocking at 60 r.p.m. (Compact Rocker CR95, Finchp Co, Korea) in a horizontal position. Following incubation, the suspensions were centrifuged to remove cell debris, and the absorbance of the supernatants was measured at 260 nm to determine the amount of nucleic acids released. This method is based on the assumption that the nucleic acids released are proportional to the biomass in the biofilms as applied previously (Lee et al., 2014). The specific amounts of biofilms formed were determined by dividing the absorbance values obtained at 260 nm by those obtained at 660 nm of the cell suspension in the Petri dishes of the initial harvests.

**Construction of the bmsA expression vector, pBmsA.** The 5' end of the bmsA gene, including the EcoRI site (Fig. 1), was PCR amplified with P. alkylphenolica chromosomal DNA. The primers used were C21-KpnF and C21-EcoR (Table 1). The 0.4 kbp PCR product was first cloned into the pEGM-T easy vector (Promega), and after the nucleotide sequence was confirmed, it was cloned into the KpnI and EcoRI sites in pBR1MCS-5 resulting in pB1MCS-5::NterBmsA. The remainder of the bmsA gene was cloned into pB1MCS-5::NterBmsA with a 3.1 kb EcoRI/BamHI fragment (Fig. 1) of pYVS23-C. The latter vector was created by self-ligation of the BamHI-digested fragment of the YVS23 mutant chromosome as previously described (Yun et al., 2007). YVS23 is a pRL27 transposon mutant of P. alkylphenolica, and the mutation site is indicated in Fig. 1. The final construct, the bmsA expression vector, was named pBmsA. The plasmid pB1MCS-5 vector contains the lac promoter that is expressed constitutively in pseudomonads (Kovach et al., 1995).
screened, and the mutation sites were confirmed by sequencing the PCR products.

**Construction of the bmsBC mutant.** The PCR product (1.3 kb) containing part of the 3’ end of bmsB and the 5’ end of bmsC was obtained with the primers CheR_BamHI and CheB_EcoRI (Table 1) and *P. alkylphenolica* chromosomal DNA as the template. The fragment was cloned into the pGEM-T Easy vector, and the nucleotide sequence was confirmed. The resulting vector was digested with EcoRI/BamHI and the fragment containing the PCR product was cloned at the same sites of pK18mobSacB. The resulting vector was digested with Scal and Smal to delete an internal 500 bp, as shown in Fig. 1. During PCR, one C was deleted resulting in an in-frame deletion. The self-ligated vector was transformed into *E. coli* DH5α and used for a double crossover as previously described (Lee et al., 2014). The specific mutation was confirmed by PCR.

**Construction of the bmsD mutant.** To make an internal deletion of bmsD, a gene fragment containing 65.5% of the internal region of bmsD was created by overlap extension PCR as previously described (Ho et al., 1989). The primers BmsD-F and RR2_dRK were used for amplification of the 5’ fragment and the primers RR2_dFK and BmsD-R for the 3’ fragment (Table 1). The internally deleted gene fragment was cloned at the XbaI/HindIII sites in pK18mobSacB to form pK18mobSacBΔbmsD. The recombinant *E. coli* DH5α (pK18mobSacBΔbmsD) was used to delete the bmsD gene via triple mating. The resulting strain was confirmed by PCR.

**Construction of the bmsR mutant.** The bmsR mutant was constructed by the insertion of an intervening plasmid of pK18mobSacB via a homologous single crossover. The internal fragment of bmsR was PCR amplified with primers BmsR_FE and BmsR_RH (Table 1). The amplified fragment was cloned into the EcoRI/HindIII sites in pK18mobSacB. The remainder of the experimental process is as previously described for algD mutant selection (Lee et al., 2014).

**Construction of the rsmZ promoter reporter vector.** The promoter region of rsmZ (228 bp) was PCR amplified with the primers RsmZp-F and RsmZp-R (Table 1) using *P. alkylphenolica* KL28 chromosomal DNA as the template, and it was cloned into the HindIII and EcoRI sites in a promoterless GFP reporter vector, pPROBE-GT (Miller & Mekalanos, 1988). The resulting plasmid, RsmZp-gfp (an rsmZ-gfp reporter), was grown in *E. coli* DH5α and introduced to *P. alkylphenolica* via conjugation. The expression of GFP was measured as described previously (Veeranagouda et al., 2011a).

**RNA-seq analysis.** Two different derivatives of *P. alkylphenolica*, ΔbmsA(pBBR1MCS-5) and ΔbmsA(pBmsA), were used to monitor the expression levels of genes controlled by the bmsC promoter. Total RNA was isolated from cells cultured for 16 h in LB liquid medium containing gentamicin in a shaking incubator at 160 r.p.m. at 28°C. The experimental details for total RNA purification, subsequent nucleotide sequencing and RNA-seq data analysis have been described previously (Kim et al., 2014).

**Statistical analysis.** All experiments were performed in triplicate. Data are expressed as the means±SD. Statistical significance was
RESULTS

Identification of BmsA as a sensory protein containing an extracellular CHASE3 domain

The formation of a highly branched, aerial mushroom-like structure by P. alkylphenolica in p-cresol vapour is an unprecedented observation in bacteria. The development proceeds with two distinct stages: first, a dome structure formation and then a spike growth from the dome to form highly branched aerial structures (Lee & Veeranagouda, 2009). We have been expanding our investigations to explore the novel adaptation mechanisms at the molecular and cellular levels. In this study, several mutants, named C21, YVS23 and VYS90, formed by transposon mutagenesis using pRL27 were shown to be defective in the ability to form highly branched, mature aerial structures and to be blocked at the dome structure level during growth under p-cresol vapour (Fig. S1a, available in the online Supplementary Material). These mutants were also collectively defective for surface spreading on soft agar LB medium and showed blue fluorescence under UV light at 364 nm (Fig. S1b). The genetic analysis of these mutants revealed that all contained a transposon insertion in a gene tag, PSAKL28_21690, in the P. alkylphenolica chromosome (Lim et al., 2014). The gene encodes a multi-sensor hybrid histidine kinase that consists of 1164 aa, with a predicted molecular mass of 130.3 kDa, and was named bmsA for biofilm formation and motility sensor (Fig. 1). An analysis of the protein domains in BmsA with PSI-BLAST and SMART revealed that it contains a sensory CHASE3 domain (cyclases/histidine kinases associated sensing extracellular) spanning residues 44–179 between two transmembrane domains at the N-terminus (spanning residues 20–42 and 183–205) and, serially, a GAF domain (found in cGMP phosphodiesterases, adenylate cyclases and FhlA) spanning residues 253–397, a histidine kinase and HATPase_c spanning residues 493–720 and three CheY-like response regulator domains spanning residues 772–1154 at the C-terminus of the BmsA (Fig. 1). The conserved phosphorylation site in the His kinase domain is identified at position 503. BmsA orthologues in databases are found in Pseudomonas and other genera, and examples include Pseudomonas plecoglossicida (RK21_01406), Pseudomonas monteilii (X970_15200), Pseudomonas putida KT2440 (PP_3761), P. putida S16 (PPS_3231), Pseudomonas entomophila (PSEEN3205), Pseudomonas mossellii (O165_012200), Pseudomonas parafulva (NZ69_05125), Pseudomonas cremonicola (LK03_18655), Pseudomonas protegens Pf-5 (PFL_3251), Pseudomonas fluorescens LBUM223 (VO64_0369), Pseudomonas chlororaphis subsp. aurantiaca (JM49_14270), Pseudomonas poae (H045_08570), Pseudomonas mandellii (OU5_0320), Pseudomonas syringae pv. tomato DC3000 (PSPTO_2712), Pseudomonas rhizospherae (LT40_09620), Pseudomonas brassicaearum DF41 (CD58_13730), Pseudomonas cichorii (PCH70_22250), Pseudomonas fulva (Pseuf_0573) and Pseudomonas mendo- cina ymp (Pmen_0169), with 81.7–65.3 % amino acid sequence identities. In addition, the same genes are found in some species of the genera Burkholderia, Acidovorax, Herbaspirillum, Ochrobactrum and Stigmatella. However, the homologous protein is not identified in Pseudomonas aeuruginosa. The function of the BmsA homologues has not been characterized previously.

In the genome sequence of P. alkylphenolica, downstream of the bmsA gene are PSAKL28_21700, _21710 and _21720, which encode proteins and show amino acid sequence homologies to the chemotactic CheR-type methyltransferase (87.5 % identity to CheR3, ppu:PP_3760, in P. putida KT2440), the chemotactic CheB-like methylesterase (70.7 % identity to CheB, ppu:PP_3759, in P. putida KT2440) and a response regulator receiver sensor (CheY) with HisKA and HATPase_c domains, respectively. They are named bmsABCD, respectively (Fig. 1). The nucleotide sequences coding these genes overlap, suggesting that the gene cluster forms a single transcriptional unit. Interestingly, the bmsB gene uses TTG as an initiation codon, whereas the other genes in the cluster use the ATG codon. The genes flanking the gene cluster are divergently oriented. The gene

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**Fig. 2.** Aerial and pellicle structure formation by P. alkylphenolica and its derivatives. The photographs of the aerial structures and pellicles were taken after 1 month and 48 h incubations, respectively. The experimental details are described in Methods.
BmsA is required for the formation of multicellular biofilm structures

To substantiate the results obtained from transposon mutagenesis and to determine the role of each gene in the bms gene cluster, mutants in genes bmsA, bmsBC, bmsD and bmsR were constructed as described in Methods. The results of aerial structure formation by the mutants are shown in Fig. 2. All the strains except the bmsA mutant formed aerial structures similar to those of the WT strain. The bmsA mutant formed dome structures by the indicated incubation time similar to those observed for the bmsA transposon mutants; furthermore, extended incubation did not lead to the formation of highly branched aerial structures. The bmsA mutant complemented with the bmsA gene in an expression vector, pBmsA, began to develop the aerial structure after 5 days of incubation compared to 10 days for the WT, and the area occupied by the aerial structures of the complemented mutant was wider (r=14.0±0.2 mm) than the area of the WT structures (r=5.3±0.6 mm, P<0.05) (Fig. 2).

Previously, we have shown that the biofilm formation capacity of P. alkylphenolica is highly related to colony phenotype, the mutants forming wrinkling colonies exhibiting strong biofilm ability and vice versa (Choi et al., 2007; Lee et al., 2014; Lee & Veeranagouda, 2009). When streaked on LB medium containing 0.3 % agar, the colonies of the bmsA mutant became smaller (r=0.11±0.03 mm), thinner and more irregular compared to those of the WT and the other mutants (r=0.19±0.01 mm, P<0.05) (Fig. 2). When the bmsA mutant was complemented with pBmsA, the strain exhibited pellicles covering the majority of the surface of the medium with an OD_{260}/OD_{660} ratio of approximately 5, together with isolated pellicles that had reverted to the original pellicle type. This suggested that the bmsA gene product is responsible for biofilm formation by P. alkylphenolica.

BmsA enhances surface spreading but suppresses swimming of cells

Previously, we have shown that the surface spreading mobility of P. alkylphenolica on soft LB agar has a positive correlation with lifestyles related to aerial structure and pellicle biofilm formation (Lee & Veeranagouda, 2009). Because the deduced amino acids of BmsB and BmsC showed CheR and CheB domains, respectively, the bms gene cluster may have a functional role in cell motility. To test the role of the bms genes in motility, each mutant was stab inoculated on LB medium containing 0.3 % agar. Depending on the incubation temperature, P. alkylphenolica showed different levels of motility. As shown in Fig. 4(a), at 25 °C, the WT and the bmsBC, bmsD and bmsR mutants showed wrinkled surface spreading with almost the same motility zone sizes (r=1.25±0.1 cm). In sharp contrast, the bmsA mutant showed defective surface spreading with a smoother surface. The bmsA mutant complemented with the pBmsA plasmid reverted to the WT phenotype (r=1.30±0.2 cm), although the mutant with a control vector was still defective (Fig. 4b). Under these conditions, only bmsA mutants with or without pBBR1MCS-5 produced non-diffusible fluorescent pigments as detected in the transposon mutants (Fig. S1b). The other mutants did not produce the pigments (data not shown).

At 30 °C, the WT and the bmsR mutant did not show surface spreading, but rather motility inside the agar matrix (swimming motility) with a radius of 1.95±0.1 cm (Fig. 4c). These radii were quantified from the inoculation site to the boundaries not facing the other boundaries formed by adjacent cell growth. However, in contrast to incubation at 25 °C, at 30 °C the bmsA mutant showed a swimming circle of greater diameter (r=2.3±0.1 cm, P<0.05) than the WT. Under the same conditions, the swimming circle sizes formed by bmsBC and bmsD mutants were smaller (r=0.8±0.1 cm, P<0.05) than those formed by the WT. Further incubation resulted in swimming circle sizes similar to those of WT. This indicated that those genes are also involved in the modulation of swimming motility by P. alkylphenolica. The bmsA mutant complemented with pBmsA showed strong surface spreading with very wrinkled surfaces (r=2.11±0.2 cm, P<0.05), and the spreading size was larger than that of the WT (r=1.23±0.1 cm) (Fig. 4d). Under the same incubation conditions, the bmsA mutant with a control vector showed strong swimming activity (r=1.91±0.1 cm) as shown in Fig. 4(c). These results suggested that BmsA is required for surface spreading motility and that it represses the swimming motility of P. alkylphenolica. A transposon mutant at flaC gene encoding a subunit of proximal rod for flagella functioning showed no swimming motility, but rather surface spreading motility (data not shown).

(PSAKL28_21680) upstream of bmsA is designated as bmsR and encodes a response regulator receiver protein with a CheY domain. A KEGG SSDB Gene Cluster Search showed that the gene cluster bmsABCD is widely distributed in Pseudomonas and the other G(−) strains listed above for bmsA.
To determine whether the increase in swimming motility caused by the \( bmsA \) mutation is due to the increased expression of genes related to swimming, the transcriptional levels of the expressed genes were compared between the \( bmsA \) mutant and its complementary strain containing pBmsA. In the \( P. \ alkylphenolica \) genome, 66 genes required for flagellar biosynthesis and swimming are encoded in a gene locus PSALK28_3780-37210. The differential RNA sequencing showed that 60 of these genes exhibited increased expression (two- to fourfold) in the \( bmsA \) mutant compared to the complementary strain. The results showing the differential expression of genes are depicted in Fig. 3.

**Fig. 3.** Quantitative measurement of the pellicle formed by \( P. \ alkylphenolica \) and its derivative strains grown in LB medium in a Petri dish. Biofilm (pellicle) growth was measured by the nucleic acid content in cells as described in Methods. One star indicates \( P<0.05 \) by Student's \( t \)-test compared to the WT, and two stars indicate \( P<0.05 \) by Student's \( t \)-test compared to \( \Delta bmsA(pBBR1MCS-5) \). Error bars represent the SD values for three experiments.

**Fig. 4.** Motility of \( P. \ alkylphenolica \) and its derivatives on LB containing 0.3 % agar. Cells were incubated for 48 h at the respective incubation temperatures following stab inoculation. The diameter of the Petri dish was 8.5 cm.
genes encoding flagella constituents are presented in Fig. S2.

**Independent expression of the BmsA and Gac regulons**

Our previous study showed that gacS or gacA mutation led to smoother colonies and defects in the formation of aerial structures and pellicles (Lee & Veeranagouda, 2009). Phenotypic changes also occurred in the bmsA mutant, as shown in this study. Thus, the possibility of an epistatic relationship between the membrane-bound BmsA and the Gac regulons in *P. alkylphenolica* was examined. First, the expression of the *rsmZ* gene in the bmsA mutant was evaluated because the *rsmZ* promoter is one of the targets of the GacS and GacA cascade. Abnormal signalling in GacS and GacA would result in changes in the expression of *rsmZ*. As shown in Fig. S2, neither the gacS nor gacA mutant showed GFP expression, which was under the control of the *rsmZ* promoter (pRsmZ*-gfp) as described in Methods. This result confirmed that *rsmZ* expression is a monitor of the Gac regulon. The expression of GFP from the reporter vector pRsmZ*-gfp in the isogenic bmsA mutant was almost the same as in the WT at the 24 h incubation time but was 71.1±5.2% of that in the WT at 48 h (Fig. 5b). In addition, the levels of the *gacS*, *gacA* and *rsmZ* transcripts were almost the same in the bmsA mutant and its complementary strain containing *pBmsA* (Fig. S2). Next, we examined changes in the phenotypes of the mutants complemented with *pGacA* [Δ*bmsA*(pGacA)] or *pBmsA* [Δ*gacA*(pBmsA)]. The colony morphologies of Δ*bmsA*(pGacA) and Δ*gacA*(pBmsA) were both as smooth as those of the bmsA and gacA mutants (r=0.8±0.1 mm, P<0.05) when they were grown on LB agar containing 0.5% glucose. Under the same conditions, the WT (r=1.6±0.2 mm) and Δ*bmsA*(pBmsA) (r=3.5±0.2 mm) exhibited larger and more wrinkled colonies (Fig. S3). When the Δ*bmsA*(pGacA) and Δ*gacA*(pBmsA) strains were tested for the formation of pellicles on LB liquid medium, they showed more suspension growth than pellicle biofilm formation as did the parental Δ*bmsA* mutant strain (Fig. 3). These results indicate that both the BmsA and Gac sensory cascades are required for the formation of specialized multicellular structures by *P. alkylphenolica*.

Another of our previous studies showed that mutations in the *epm* genes that encode enzymes for the biosynthesis of a polymannuronate-derived exopolymer led to smoother colonies and defective formation of aerial structures and pellicles (Lee et al., 2014). Thus, under the conditions in which the BmsA regulon acts positively on the expression of the *epm* operon, the bmsA mutant should show the same phenotype as the *epm* mutants. To test this assumption, the expression of *epmD*, the first gene in the *epm* gene cluster, was examined with a *gfp* reporter, pEpmDp-*gfp* in the WT and the bmsA mutant (Fig. 5b). After 48 h incubation, the expression of GFP from pEpmDp-*gfp* in the bmsA mutant was 76.3±7.9% (P<0.05) of that in a parent strain, SG1-WC1-10*, which is a *P. alkylphenolica* spontaneous mutant that overproduces the Epm polymer. However, this slight reduction in *epm* gene expression may not cause the dramatic changes in phenotype observed in the bmsA mutant, which may indicate that the induction of genes for the biosynthesis of the scaffold polymer for the biofilm is not the major target of the BmsA regulon. Furthermore, the RNA-seq analysis showed no apparent difference in the levels of *epm* gene expression between the bmsA mutant and its complementary strain. In addition, the supply of pBmsA to the *epm* transposon mutant (C22) (Lee et al., 2014) resulted in a defect in pellicle biofilm formation (Fig. 3), indicating that the Epm polymer is absolutely required for biofilm formation by *P. alkylphenolica*.

**DISCUSSION**

BmsA, a membrane-anchored His kinase, is regarded as a sensory protein with extracellular CHASE3 and intracellular GAF domains, and which may play a role in sensing signals in a signal transduction system. CHASE3 domain comprises a 130–150 aa domain with four to six putative α-helices and is found in a wide variety of bacterial genomes (Zhu&lin et al., 2003). The ligands for the CHASE3 domain in bacteria have not been rigorously identified, but one report showed that the domain is a salt sensor in KipF, which contains a cytoplasmic kinase catalytic core in *Sphingomonas melonis* Fr1 (Kaczmarczyk et al., 2015). The ligands for the GAF domain were identified as c-GMA, c-AMP and phytochrome, and the proteins with a GAF domain are known to be responsive to oxygen, redox potential and light (Yang et al., 2008). Although *bmsA* and its gene clusters are found in many different species of *Pseudomonas*, including plant and insect pathogens and other bacterial genera, the function of this hybrid sensor kinase has not been reported. In this study, we first demonstrated that BmsA stimulates community-dependent development, including wrinkled colonies, aerial structure formation, pellicle biofilm formation and surface spreading on wet surfaces, but it inhibits fluorochrome biosynthesis and swimming (Fig. 6). Identification of the signals that activate BmsA will provide a clue to understanding a signal transduction system related to multicellular development in bacteria occupying special niches in the environment.

Among the well-developed signal transduction systems in bacteria is a chemotaxis signalling system in which the transmembrane receptors are methyl-accepting chemotaxis proteins (MCPS), as sensors of chemical gradients. The signals from MCPS are transmitted across the plasma membrane into the cytosol, where the Che proteins are activated. Glutamate residues of the cytoplasmic side of MCPS are methylated by CheR (a methyltransferase) and are demethylated by phosphorylated CheB (a methyltransferase). The relative activities of these two enzymes on MCPS depend on the level of signals bound to the MCPS, and they allow bacteria to sense temporal gradients of stimuli leading to the modulation of bacterial swimming patterns (Sampe-dro et al., 2015). In the bms gene cluster, genes encoding
CheR and CheB homologues (bmsBC) were identified. When these genes were mutated, their phenotypes did not affect aerial structure, surface spreading or pellicle biofilm formation but showed delayed onset in swimming motility in LB. In addition, BmsA lacks the amino acid sequence homology of canonical MCP modules, which suggests that canonical MCP modules are unrelated to the function of BmsA signal transduction. These results indicate that BmsA activity is independent of BmsB and BmsC, showing that BmsB and BmsC have their own cognate MCP protein other than BmsA. In the P. alkylphenolica genome, two further CheR methyltransferases (PSAKL28_37870 and PSAKL28_02740) and two further CheB chemotaxis-related methylesterases (PSAKL28_37300 and PSAKL28_41490) were identified. The combinations of these MCP methyltransferases and methylesterases may help control the fine-tuning of chemotaxis to specific attractants and repellants. It is intriguing that the bms gene cluster encodes genes necessary for control of two superficially opposing bacterial behaviours, biofilm and swimming modes of life by BmsA and BmsBC, respectively. It has also been shown that CheB and CheR chemotactic proteins from non-canonical che gene clusters are involved in chemosensory regulation of alternative cellular functions other than motility (Wuichet & Zhulin, 2010). In fact, all three CheR genes have been mutated in P. putida KT2440, which showed the different roles of each gene (Garcia-Fontana et al., 2013). In this study, it has been shown that CheR2 is essential for chemotaxis and CheR1 is involved in biofilm formation but not in chemotaxis. The mutation in CheR3 (PP_3760), equivalent to bmsB, did not affect chemotaxis in soft LB agar medium as we observed with the bmsBC mutant. Further studies are necessary to determine the functional link of BmsB and BmsC to BmsA. The results, showing no dramatic reduction of rsmZ expression in the bmsA mutant and no reversion to the WT phenotype by the bmsA mutant supplemented with the gacA gene or by the gacA mutant supplemented with bmsA, suggested that BmsA does not significantly affect the Gac regulon at least down to rsmZ expression. In P. aeruginosa PAO1, the ladS, retS and PA1611 genes encoding transmembrane sensory domains have been reported to influence the Gac regulon. For example, a mutation of ladS

**Fig. 5.** Specific GFP expression by P. alkylphenolica and its derivatives. Cells were grown on LB medium with gentamicin. GFP levels were measured in cells grown in liquid medium for the indicated incubation time (a) and on agar medium for 48 h (b). In (b), the photograph was taken under UV light at 364 nm. Error bars represent the SD values for three experiments. *P<0.05, Student’s t-test.
resulted in the same level of rsmZ expression as that of gacA or gacS, resulting in a biofilm-deficient phenotype. This finding indicated that LadS is absolutely required for functioning of the Gac regulon (Ventre et al., 2006). LadS is thought to contain an N-terminal 7TMR-DISMED2 (a putative signal-binding domain) followed by a 7TMR-DISM_7TM transmembrane domain, a cytoplasmic C-terminal histidine kinase and a response regulator receiver domain. In contrast, RetS with the same domains as LadS and an additional response regulator domain at its C-terminus, was shown to play opposite roles in biofilm formation through interactions with GacS. The retS mutant showed a hyperadhesive phenotype and overproduction of biofilms. A heterodimer of GacS and RetS has been identified (Goodman et al., 2009). Another hybrid sensor kinase PA1611 in P. aeruginosa is known to regulate transitions between acute and chronic infection through direct interaction with RetS (Kong et al., 2013). In the P. alkylphenolica genome, the LadS orthologue (PSAKL28_06690, 65.3% identity to LadS of P. aeruginosa) without the C-terminal receiver domain such as Psyr_4339 of P. syringae (Records & Gross, 2010), the RetS orthologue (PSAKL28_47240, 59.9% identity to RetS of P. aeruginosa) and the PA1611 orthologue (PSAKL28_35920, 64.7% identity to PA1611 of P. aeruginosa) were identified, but in our transposon mutant screen study, mutants of genes encoding these proteins that influence surface-related phenotypes have never been isolated, including aerial structure formation and colony morphology. These results, together with the absence of the bmsA orthologue in the P. aeruginosa genome, indicated that the Gac regulon in P. alkylphenolica may be controlled differently from that in P. aeruginosa through different signalling networks for biofilm development. In the literature, the intracellular level of c-di-GMP was shown to play a crucial role in the determination of sessile and biofilm lifestyles in bacteria (Hickman & Harwood, 2008). Our future studies will focus on a possible relationship between BmsA signal transduction and biosynthesis of the cellular biofilm messenger c-di-GMP in P. alkylphenolica.

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REFERENCES


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