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BswR controls bacterial motility and biofilm formation in *Pseudomonas aeruginosa* through modulation of the small RNA *rsmZ*

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ABSTRACT

*Pseudomonas aeruginosa* relies on cell motility and ability to form biofilms to establish infections; however, the mechanism of regulation remains obscure. Here we report that BswR, a xenobiotic response element-type transcriptional regulator, plays a critical role in regulation of bacterial motility and biofilm formation in *P. aeruginosa*. Transcriptomic and biochemical analyses showed that BswR counteracts the repressor activity of MvaT, controls the transcription of small RNA *rsmZ* and regulates the biogenesis of bacterial flagella. The crystal structure of BswR was determined at 2.3 Å resolution; the monomer comprises a DNA-binding domain with a helix-turn-helix motif in the N terminus and two helices (α6 and α7) with a V-shaped arrangement in the C-terminus. In addition to the contacts between the parallel helices α5 of two monomers, the two helical extensions (α6 and α7) intertwine together to form a homodimer, which is the biological function unit. Based on the result of DNase I protection assay together with structural analysis of BswR homodimer, we proposed a BswR–DNA model, which suggests a molecular mechanism with which BswR could interact with DNA. Taken together, our results unveiled a novel regulatory mechanism, in which BswR controls the motility and biofilm formation of *P. aeruginosa* by modulating the transcription of small RNA *rsmZ*.

INTRODUCTION

*Pseudomonas aeruginosa* is an important opportunistic human pathogen that causes acute or chronic infections in immunocompromised patients (1). The infectivity of *P. aeruginosa* is associated with its motility and biofilm formation (2), which is mutually exclusively switching between motile and sessile states on environmental conditions. Switching between motility and sessility aids the pathogen to survive harsh environmental conditions by increasing the efficiency of nutrient acquisition, escaping from toxic substances and accessing to favorable colonization sites (3,4). Mutants lacking motility and biofilm formation showed attenuated virulence in a burned mouse model (5), reduced invasion in corneal epithelial cells (6) and decreased adhesion to human respiratory mucin (7).

Swarming is one of the types of bacterial motilities through which bacterial cells move around to aid systemic infection and biofilm formation. It is a complex adaptation process in response to various environmental cues (8). In *P. aeruginosa*, the swarming motility is intimately associated with flagella assembly and type IV pilus (T4P) biogenesis (9). The assembly of flagella involves >50 genes that are coordinated by an array of regulatory proteins including the master regulator FleQ, the sigma factor RpoN, transcription factors FleR and FliA (10). Pilus biogenesis, an equally complicated process, also involves >40 genes and multiple signal transduction pathways (11). These signaling pathways include the two-component regulatory systems PilR/PilS and AlgR/FimS (12), the global regulator Crc (13), the virulence factor regulator Vfr (14) and the chemosensory...
systems encoded by the pilGHIJK-chpABC gene clusters (15,16).

In addition to these regulatory proteins, the small RNA regulatory system RsmA/RsmZ is also known to play critical roles in the regulation of bacterial motility and biofilm formation (17,18). RsmA, an RNA-binding protein, regulates swarming by positively modulating the biosynthesis of flagella and T4P (19,20). RsmZ, a noncoding regulatory small RNA, is an antagonist of the functional RsmA. Overexpression of RsmZ inactivates RsmA and abolishes bacterial swarming motility. Transcription of rsmZ is subject to the direct control of the GacS/GacA two-component system, and influenced by the sensor kinases RetS and LadS (21,22). Among them, it is the response regulator GacA that activates the rsmZ transcription by directly binding to the rsmZ promoter (17,23). Additionally, the H-NS-like protein MvaT acts as an rsmZ transcriptional repressor by binding to its promoter (23,24). Adding further to the complexity of the regulatory mechanisms that control motility and biofilm formation, evidence suggests the presence of some yet to be identified factors associated with the MvaT modulation of rsmZ expression (24).

Given the central role of RsmA/RsmZ system in modulation of P. aeruginosa cell motility and biofilm formation, it is of considerable interest to investigate the detailed regulatory mechanisms that govern its expression. Here we identified a novel transcription factor Bswr, which is involved in the regulation of P. aeruginosa swarming motility and biofilm formation. Overexpression of bswr enhanced the transcription of rsmZ, downregulated the expression of flagellar and T4P genes and attenuated the bacterial swarming motility. We also found that Bswr binds to the promoter of rsmZ and counteracts the repression of MvaT. Furthermore, we determined the crystal structure of Bswr, which reveals that Bswr forms a homodimer as the functional unit and provides clues on how Bswr could interact with target promoter to regulate gene expression. These genetic and structural findings provide a new insight into the complicated and sophisticated regulatory mechanisms that govern the RsmZ/RsmA regulatory system and bacterial motility and biofilm formation.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study were listed in Supplementary Table S1. Both Escherichia coli and P. aeruginosa strains were maintained in Luria–Bertani (LB) broth with shaking at 250 rpm or on LB agar plates at 37°C. When necessary, antibiotics were included in medium as follows: 150 and 300 mg·ml⁻¹ carbenicillin, 5 and 50 mg·ml⁻¹ gentamicin and 5 and 100 mg·ml⁻¹ tetracycline for E. coli and P. aeruginosa, respectively.

Expression plasmid constructs were generated by standard methods and verified by DNA sequencing. The plasmids were transformed into E. coli by heat shock and P. aeruginosa strain by electroporation unless otherwise stated. To generate the bswr-overexpressing construct p19-bswr, the encoding region of bswr was amplified with polymerase chain reaction (PCR) primers 5'-GCGAATTCTGCTAGATTCCGCTAAAG-3' and 5'-GCATTTCTCACGTTCACCTCTTGTGC-3', digested with EcoRI and inserted into the corresponding site of pUCP19. The p19-PA2781 was constructed similarly by using the PCR primers 5'-GGAAATCCAGTACGGGAAAACCTTAG-3' and 5'-GGAAATCCAGTACGGGAAAACCTTAG-3'. The rsmZ reporter construct pME-rsmZ was generated by cloning the EcoRI-digested rsmZ promoter into the enzyme site of pME6016 (25). PCR primers for pME-rsmZ construction were 5'-GTTGATTCTCTGAAACAAA CTTGGGATTGAG-3' and 5'-GCTGACGGGAAAACCTTAG-3'. To delete the 16th–99th amino acids of Bswr, the construct pEX18G-bswr1 was prepared by overlapping PCR to generate the bswr allele bswr1, which lacks an internal fragment corresponding to nucleotides from 48th through 297th base pair (bp). The mutated allele was integrated into the chromosome of P. aeruginosa by homologous recombination, and the plasmid vector fragment was removed as previously described (26). The resultant mutants were verified by PCR and DNA sequencing.

β-Galactosidase assays

β-Galactosidase activity was measured in duplicates and repeated at least twice. Bacterial start cultures were diluted (1:1000) in LB, grown for 8 h at 37°C and then aliquots were taken for measurement of enzyme activity. The β-galactosidase activity was quantified as previously described (27).

Transposon mutagenesis

The mariner transposon carried by plasmid pBT20 was used for mutagenesis of P. aeruginosa PAO1 following the procedures as described (28). Mutants were screened on agar plates, which is a basic minimal nutrient medium supplemented with 0.2% mannitol as sole carbon source, 0.2% ammonia sulphate as sole nitrogen source and gentamicin (50 mg·ml⁻¹) for selection of transposon mutants. The colonies showing smaller size than wild-type control were selected for further analysis. Arbitrary PCR, using primers of 5'-GTCGASGAWGAWGAW-3' with 5'-GTGCAACAGATACGGTAAC-3' and 5'-TGACGATCAGGTGGGCTAC-3', was used to identify the genes disrupted by transposon insertion. The DNA sequences flanking the transposon insertion were analyzed with the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/blast/) and the Pseudomonas genome database (http://www.pseudomonas.com/).

Transcriptional profiling

PAO1, PAO1(p19), ΔbswrR and ΔbswrR(bswR) cultures were grown overnight in LB medium. Bacterial cells were then diluted to OD₆₀₀ of 0.05 and grown at 37°C with shaking at 250 rpm. When OD₆₀₀ reached ~1.0, cells were harvested and total RNAs were extracted with RNeasy Protect Bacteria Mini kit (Qiagen). DNA
contaminants were removed by DNase I. The quality of purified RNAs was analyzed by electrophoresis and quantified by Nanodrop 1000 Spectrophotometer (NanoDrop Technologies). Reverse transcription, fragmentation and complementary DNA labeling were conducted as described by the manufacturer (Affymetrix). The processed samples were hybridized to the Affymetrix GeneChip of *P. aeruginosa*, and chips were washed, scanned and analyzed following the instructions from the manufacturer. Hybridization signals were processed using the statistics software MAS-5.0 from Affymetrix.

**Quantitative real-time reverse transcription polymerase chain reaction**

RNA samples were prepared as described above for transcriptional profiling analysis. An aliquot of 0.1 µg total RNAs was used as template, and real-time reverse transcription-PCR analysis was performed on Lightcycler v1.5 (Roche), using the SYBR® Green RT-PCR kit (Qiagen). The housekeeping gene *rpoC*, which encodes the β’ subunit of RNA polymerase, was included as an internal control. Results were presented as the ratio of target gene expression level versus the control gene (29).

Real-time RT-PCR primers specific to *rsmZ* were 5’-CGT ACAGGGAAACCGCAAC-3’ and 5’-ATTACCCGCCC CACTCTTC-3’, and the primers for *rpoC* were 5’-CTGTT CAAGCC GTTCATTTTC-3’ and 5’-CTTGGATTGTTGG TGGCCATA-3’, respectively.

**Protein expression and preparation**

For biochemical assays, protein expression constructs were generated using the vector pET28a. For preparation of BswR, the encoding region of *bswR* was amplified with PCR primers 5’-CGGGATCCCTAGGTACCCGGCTAA AAGC-3’ and 5’-CGGATGCGTGTCGCGTAG-3’, digested with BamHI and HindIII and cloned into the corresponding sites of pET28a (Novagen). For preparation of SpdH, the full-length *spdH* was amplified from the genomic DNA using the PCR primer pair 5’-CGGGATCCATGAC CATCTCCGCGCCGA-3’ and 5’-CCCAAGCTTCCGC TGGCGTCGGCGTCTC-3’, and cloned into pET28a in the same way as described for *bswR*. After verification by DNA sequencing, pET28a-*bswR* and pET28a-*spdH* were, respectively, transferred into *E. coli* BL21(DE3), and expressed as described above. The BswR protein was first isolated by Ni-NTA column (GE healthcare) under a linear gradient elution of 0.0–0.5 M imidazole in the buffer 50 mM NaH 2PO4, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol (β-ME), and further purified by HiLoad superdex 75 26/60 gel filtration column (GE healthcare), with the elution buffer 200 mM Tris, pH 7.5, 200 mM NaCl and 5 mM β-ME. The purified proteins were concentrated to 5.2 mg·ml⁻¹ and stocked in the buffer (20 mM Tris pH 7.5, 200 mM NaCl, 5 mM β-ME) for crystallization trials.

**Electrophoretic mobility shift assay**

The promoter region of *rsmZ* was labeled with biotin by PCR using primers 5’-Biotin-TTCAGTGACCGAAAA CTCTAG-3’ and 5’-Biotin-TTCCTGTCAGCAGG TGAATA-3’. The control probe pexC that extended the promoter region of *exsC* from *P. aeruginosa* PAO1 was PCR-amplified using 5’-biotinylated primers 5’-CTCTCT AAGCTAGGCATGAG-3’ and 5’-GTCAGTCTCTTATT CACCCAG-3’. The biotinylated promoter was purified with QIAquick PCR Purification kit (Qiagen) and quantified by Nanodrop spectrophotometer. EMSA was carried out using LightShift™ Chemiluminescent EMSA kit following the manufacturer’s instruction with modifications (Pierce). Each reaction (10 µl) contained the biotin-labeled probe (0.2 ng), 1 µg poly (dI:dC) (2'-deoxyinosinic-2'-deoxyctydilic acid) and various amounts of His6-BswR protein as indicated. In parallel, His6-SpdH was included in the assay as control. The reaction mixtures were incubated at 25°C for 20 min, and then the samples were separated on 6% polyacrylamide gels. The DNA probes were then transferred onto a nylon membrane, cross-linked by ultraviolet light at 254 nm and detected by chemiluminescence as described by the manufacturer (Pierce).

**Motility assay**

The motility of *P. aeruginosa* was examined on LB agar (1.5%) plates at 37°C. Unless stated otherwise, 2 µl of fresh *P. aeruginosa* cultures was spotted in the center of plates and grown at 37°C for 2 days. Swarming, swimming and twitching motilities were respectively determined as described previously (4). Results were recorded after incubation overnight at 30°C.
Biofilm assay

Biofilm formation was determined in 15-ml borosilicate tubes. Briefly, 1 ml of LB medium was inoculated to a final OD_{600} of 0.1 and statically incubated at 37°C for 6 h. Biofilm cell mass was visualized by staining with 0.1% crystal violet and quantified by measuring the absorbance at 595 nm after solubilizing crystal violet in 96% ethanol. Each experiment was independently repeated for at least three times.

DNase I protection assay

The promoter region of rmsZ was amplified by PCR with FAM-labeled primers 5'-(6-FAM)-ATTCTGGAGAAGA ATGGGCCCTGTC-3' and 5'-TTGGTGTTCCTGTACG C-3' from the genomic DNA of P. aeruginosa PAO1. The experiment was done as described previously (30) in four repetitions with some modifications. Briefly, 100 ng of DNA was diluted in 20 μl of gel shift buffer, then 5 μl of purified BswR and 13 μl of water were added to the reaction; binding was allowed to proceed for 20 min at room temperature. Following several trials, 10 U of DNase I (Roche) was added and incubated for 5 min, and then the enzyme was heat-inactivated for 5 min at 95°C; DNA was purified by using Qiagen PCR kit and eluted on 25 μl of water for further analysis. 5’-end labeled DNA was diluted in 20 μl of gel shift buffer, then 5 μl of purified BswR and 13 μl of water were added to the reaction; binding was allowed to proceed for 20 min at room temperature. Following several trials, 10 U of DNase I (Roche) was added and incubated for 5 min, and then the enzyme was heat-inactivated for 5 min at 95°C; DNA was purified by using Qiagen PCR kit and eluted on 25 μl of water for further analysis. 5’-end labeled DNA was diluted in 20 μl of gel shift buffer, then 5 μl of purified BswR and 13 μl of water were added to the reaction; binding was allowed to proceed for 20 min at room temperature. Following several trials, 10 U of DNase I (Roche) was added and incubated for 5 min, and then the enzyme was heat-inactivated for 5 min at 95°C; DNA was purified by using Qiagen PCR kit and eluted on 25 μl of water for further analysis.

Crystallization and structure determination

Crystallization trials were carried out using sitting-drop vapor diffusion method by Robot Phoenix (Art Robbins Instruments) at 20°C. The diffraction quality crystals of BswR were obtained in the drop by mixing protein sample and reservoir solution (0.1 M HEPES, pH 7.5, 20% w/v polyethylene glycol 10 000) with a volume ratio of 100 nl to 200 nl. For data collection, crystals were cryoprotected in reservoir solution supplemented with 20% glycerol, and flash-frozen in liquid nitrogen.

Diffraction data set was collected in Bruker AXS X8 Proteum X-ray system (wavelength = 1.5418 Å) at National Institute of Education Library, Serials Unit on May 21, 2014. The data set was indexed, scaled and integrated with iMosflm (31). Using 3LFP [Protein Data Bank (PDB) code] as a search model, initial phases were obtained by molecular replacement using program Balbes in CCP4 suite (32), followed by model building with ARP/wARP (33). Model visualization and manual model building were carried out with COOT (34), and the refinement was done with CNS (35). The final model was examined using PROCHECK (36), crystallographic data and refinement statistics are listed in Table 1. Protein interface areas were calculated using PISA server (37). Structural illustrations were generated using PyMol (DeLano Scientific).

RESULTS

BswR regulates P. aeruginosa motility and biofilm formation

To identify unknown regulatory factors associated with the motility of P. aeruginosa, a transposon mutant library was screened for alteration in colony size on LB agar plates. Of ~2000 mutants screened, three mutants with colony size smaller than the wild-type control were selected and designated as Mot1, Mot2 and Mot3 (Supplementary Figure S1A). Sequence analysis of the three mutants showed that transposon was inserted in PA2285 (Mot1), PA3966 (Mot2) and the intergenic region of PA2779 and PA2780 (Mot3), respectively. Among them, PA2285 encodes the transcriptional factor AmrZ associated with the regulation of P. aeruginosa motility (38), and PA3966 encodes a hypothetical protein with unknown function, whereas PA2779 and PA2780 encode a hypothetical protein and a putative DNA-binding protein, respectively. The mutant Mot3, in which the transcriptional expression of PA2780 was significantly upregulated by transposon insertion (Supplementary Figure S2), was selected for further analysis in this study.

As shown in Figure 1A, the ORFs PA2779 and PA2780 were divergently orientated, sharing an intergenic region of 407 bp as putative promoters. The mariner transposon was inserted at the 12th bp upstream of PA2780, and the transposon-borne tac promoter was orientated in the same direction as that of PA2780, which is known to increase the transcriptional expression of downstream genes (28). Following PA2780 is PA2781, a 339-bp ORF, which overlaps the last 1 bp of PA2780 and possibly forms an operon with PA2780. Similar to PA2780, PA2781 also encodes a hypothetical protein with unknown function. No discernible motifs were found in both proteins, but bioinformatics analysis suggested that PA2780, comprising 114 amino acid residues, possesses a potential xenobiotic response element (XRE)-type DNA-binding domain at its N-terminus (Figure 1A). According to its role in bacterial swarming regulation as described below, PA2780 was designated hereafter as bswR (Bacterial SWarming Regulator).

In other bacterial species, the proteins containing the XRE-type DNA-binding domain have been implicated in regulating plasmid copy number and transcriptional expression of bacteriophage genes (39). However, the biological function of BswR in P. aeruginosa has not yet been characterized. To elucidate its role in bacterial motility, we generated the mutant ΔbswR by in-frame deletion of bswR in wild-type strain PAO1. We first checked the colony size on LB agar plates and found the deletion mutant formed much larger colonies than the wild-type and the bswR-overexpressing derivative (Supplementary Figure S1B), which was not due to altered bacterial growth rate (Supplementary Figure S3). We then examined the bacterial motility on specific agar plates for analysis of swarming, swimming and twitching motility (4). As shown in Figure 1B, we found deletion of bswR dramatically increased the bacterial swarming motility and the changed phenotype was rescued by
Table 1. Summary of crystallographic data and refinement statistics

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*\( R_{\text{sym}} = \sum |I_i - <I>|/|I_i| \) where \( I_i \) is the intensity of the \( i \)th measurement, and \( <I> \) is the mean intensity for that reflection.

*Reflections with \( I_o > 0 \) were used in the refinement.

*\( R_{\text{work}} = [F_{\text{calc}} - |F_{\text{obs}}|] \) where \( F_{\text{calc}} \) and \( F_{\text{obs}} \) are the calculated and observed structure factor amplitudes, respectively.

*\( R_{\text{free}} \) as for \( R_{\text{work}} \) but for 9.4% of the total reflections chosen at random and omitted from refinement.

*Individual B-factor refinements were calculated.

*In trans* expression of the wild-type *bswR* but not *PA2781* in the deletion mutant Δ*bswR* (Figure 1B). We also tested the effect of *bswR* overexpression on bacterial swimming and twitching motility, but no significant change was observed (data not shown). These data suggest that BswR is specifically associated with the regulation of *P. aeruginosa* swimming motility.

Given that bacterial motility is critical for biofilm formation in *P. aeruginosa* (40), we tested the impact of *bswR* on biofilm formation. As depicted in Figure 1C, biofilm formation was almost completely abolished in the deletion mutant Δ*bswR*. In contrast, overexpression of BswR in the deletion mutant Δ*bswR* dramatically increased the biofilm formation. Surprisingly, overexpression of *PA2781* in Δ*bswR* had no effect on bacterial swimming motility (Figure 1B), whereas this resultant strain produced more biofilm than that produced by the wild-type PAO1 (Figure 1C), suggesting that the hypothetical protein PA2781 may play a specific role in biofilm development, which is currently under investigation.

**BswR modulates the biosynthesis of flagella and type IV pili**

To understand how BswR could regulate the swarming motility, we conducted microarray analysis to compare the global gene expression profiles of the deletion mutant Δ*bswR* and wild-type strain PAO1. The results showed that deletion of *bswR* significantly changed the expression patterns of 454 genes (≥2-fold), with 258 being upregulated and 196 downregulated (Supplementary Table S2). Considering the critical roles of flagella and T4P in bacterial motility and biofilm formation, we specifically compared the expression patterns of the genes associated with flagella and T4P in PAO1 and Δ*bswR*, and found that >40 flagella and T4P genes, including the gene clusters *flgB-flil*, *cheV-flgN*, *fimT-pilE* and pilQ-pilN, were upregulated (≥2-fold) in the deletion mutant (Figure 2A and B). These upregulation patterns were verified by real-time RT-PCR analysis (Supplementary Figure S4). Consistently, overexpression of *bswR* in the strain Δ*bswR* resulted in decreased (≥2-fold) transcriptional expression of 14 flagella and 15 T4P genes compared with the wild-type PAO1 (Supplementary Figures S2 and S5). These results suggest that BswR regulates swimming motility likely through modulation of flagella and T4P biogenesis.

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![BswR modulates the motility and biofilm formation of P. aeruginosa.](image-url)
In addition to flagella and T4P, extracellular polysaccharide (EPS) is another factor associated with biofilm formation in *P. aeruginosa* (41); we then analyzed the genes responsible for EPS production based on microarray data. Among the two gene clusters, i.e. the *pel* operon and the *psl* operon, known to be associated with EPS production, we found that only the expression of *pel* operon was significantly decreased in the ΔbswR mutant compared with the wild-type strain (Figure 2C). These data are in line with the previous study that mutation of the *pel* genes results in decreased biofilm formation (42) and establish a link between BswR and the *pel* genes in biofilm formation. Taken together, these results suggest that the influence of BswR on bacterial biofilm formation is not only related to its negative role in regulation of flagella and T4P biogenesis, but may also be associated with its positive effect on modulation of EPS production.

**BswR regulates the transcription of small RNA rsmZ**

Besides the genes related to flagella, T4P and EPS biosynthesis, genes involved in protein secretion were also regulated by *bswR* (Supplementary Table S2). As shown in Figure 3A, expression of the genes for type III secretion system (T3SS) (including *exoT, exsE, pscRQPO, pcr1234, popB and pscEF*) was dramatically increased by deletion of *bswR*. Intriguingly, we noted that these expression patterns were mutually reciprocal with those caused by the deletion of the RNA-binding protein encoded by gene *rsmA* (20), and reminiscent to those caused by double deletion of small RNAs *rsmZ/Y* (Figure 3A) (23). It has been reported previously that deletion of *rsmZ/Y* results in increasing expression of T3SS genes and decreasing expression of T6SS genes, whereas deletion of *rsmA* increases the expression of T6SS genes but decreases the expression of T3SS and T4P genes (20,23).
Given that the RsmZ/Y-RsmA system is known to be associated with the regulation of P. aeruginosa motility (18), the above results motivated us to postulate that BswR might regulate the expression of rsmZ/Y. A specific probes matching rsmZ/Y were designed in the GeneChips from Affimatrix, we tested this hypothesis by generating two reporter constructs by fusing the promoters of rsmZ (PrsmZ) and rsmY (PrsmY) separately to the coding region of lacZ and introduced these constructs into PAO1 and its derivatives. Analysis of the PrsmZ-directed β-galactosidase activity showed that deletion of bswR decreased the rsmZ expression, and overexpression of bswR increased the rsmZ expression level (Figure 3B). However, such differences were not observed when the PrsmY-lacZ fusion gene was assayed (data not shown). These results suggest that BswR regulates the transcriptional expression of rsmZ but not rsmY. Consistent with these results, real-time RT-PCR assay also showed that the transcript level of rsmZ was decreased by ~60% in the bswR-deleted mutant and increased by ~3-fold in the bswR-overexpression strain (Figure 3C).

BswR requires GacA and MvaT for upregulation of rsmZ

GacA, a response regulator of the two-component system GasA/GacS in P. aeruginosa, has been well known to regulate rsmZ by directly binding to its promoter. It has been shown that disruption of GacA by transposon significantly reduced the rsmZ expression (23). To characterize the BswR-mediated regulation of rsmZ, we compared the transcription level of rsmZ in the wild-type strain and the relevant mutants. Consistent with previous report (23), disruption of gacA substantially reduced the transcriptional expression of rsmZ compared with the wild-type strain (Figure 4A). Notably, while in trans expression of bswR in wild-type strain increased the transcriptional expression of rsmZ, expression of bswR in the gacA mutant strain had no effect on rsmZ transcription (Figure 4A). The findings were further consolidated by examination of phenotype changes. As depicted in Figure 4B, overexpression of bswR inhibited the swarming motility of the wild-type PAO1. In contrast, the influence of overexpressed BswR was diminished when gacA was mutated, suggesting that BswR requires a functional GacA for the upregulation of rsmZ transcription.

In addition to GacA, the H-NS-like DNA-binding protein MvaT also binds to the rsmZ promoter and negatively regulates the transcription of rsmZ (24). To test whether BswR requires MvaT for the upregulation of rsmZ, we compared the transcription level of rsmZ in the wild-type and the relevant mvaT mutant strains.
Regulation of \textit{bswR} on bacterial motility is dependent on the RsmZ regulators GacA and MvaT. (A) The $\beta$-galactosidase activities of PAO1(\textit{PrsmZ-lacZ}) and derivatives as specified. Bacterial cells were grown in LB medium, and the enzymatic activity was determined at stationary phase. (B) Representative images of swarming motilities of PAO1 and derivatives. Bacterial cells were inoculated at the center of the swarming plates and incubated at 30°C for 20 h before taking photographs. Consistent with the previous study (23), deletion of \textit{mvaT} resulted in a >2-fold increase in \textit{rsmZ} transcription compared with the wild-type strain PAO1 (Figure 4A). In contrast, overexpression or deletion of the \textit{bswR} gene in the background of \textit{mvaT}-deletion mutant did not cause any change at the transcript level of \textit{rsmZ} (Figure 4A). As a control, overexpression of \textit{bswR} in PAO1 inhibited the swarming motility of the wild-type strain, and this inhibition was almost completely relieved in the \textit{mvaT} mutant (Figure 4B). Given that overexpression of \textit{bswR} increases the \textit{rsmZ} transcription in the wild-type strain, these results suggest that \textit{bswR} may act by counteracting the repressor MvaT in upregulation of \textit{rsmZ}. Consistent with this notion, we found that the double deletion mutant \textit{ΔbswRΔmvaT} had a comparable transcription level of \textit{rsmZ} as that of the single deletion mutant \textit{ΔmvaT} (Figure 4A). In line with these findings of genetic analysis, the swarming assay showed deletion of \textit{bswR} in strain PAO1 increased the bacterial motility, but such an effect was vanished when \textit{mvaT} was also deleted (Figure 4B).

\textbf{BswR binds to the promoter region of \textit{rsmZ}}

To characterize the function of BswR, we examined whether BswR could directly interact with the promoter of \textit{rsmZ} \textit{in vitro}. First, we expressed and purified the recombinant BswR as a His$_{6}$-tagged protein and conducted the EMSA. As shown in Figure 5A, the mobility shift of the \textit{rsmZ} promoter was affected by BswR in a dosage-dependent manner, demonstrating BswR binds to the \textit{rsmZ} promoter. In contrast, the control protein SpdH, which is a spermidine hydrogenase in \textit{P. aeruginosa} (43), did not interact with the \textit{rsmZ} promoter. Furthermore, the purified BswR did not bind to the control DNA fragment PexsC, which is the promoter of gene \textit{exsC} in \textit{P. aeruginosa} (Figure 5A). Cumulatively, these results indicate that BswR positively regulates the transcriptional expression of the small RNA \textit{rsmZ} by specifically binding to the promoter region of \textit{rsmZ}.

To identify the binding sites of BswR in the \textit{rsmZ} promoter region, we conducted the DNase I protection analysis, the swarming assay showed deletion of \textit{bswR} in PAO1 inhibited swarming motility of the wild-type strain, and this inhibition was almost completely relieved in the \textit{mvaT} mutant (Figure 4B). Given that overexpression of \textit{bswR} or deletion of the \textit{mvaT} gene increases the swarming motility of the wild-type strain, and this inhibition was almost completely relieved in the \textit{mvaT} mutant (Figure 4B). Given that overexpression of \textit{bswR} increases the \textit{rsmZ} transcription in the wild-type strain, these results suggest that \textit{bswR} may act by counteracting the repressor MvaT in upregulation of \textit{rsmZ}. Consistent with this notion, we found that the double deletion mutant \textit{ΔbswRΔmvaT} had a comparable transcription level of \textit{rsmZ} as that of the single deletion mutant \textit{ΔmvaT} (Figure 4A). In line with these findings of genetic analysis, the swarming assay showed deletion of \textit{bswR} in strain PAO1 increased the bacterial motility, but such an effect was vanished when \textit{mvaT} was also deleted (Figure 4B).
assay by using the PCR-amplified promoter region of rsmZ, which is labeled with the fluorescent dye FAM (fluorescein amidite). The results revealed that the DNA stretch ‘ACCCGGCTTCGCCGTT’ was clearly protected from the DNase I digestion on incubation together with BswR (Figure 5B). This protected region appeared as a palindrome, typical of the transcriptional factor binding sites in bacteria. In the promoter region of rsmZ, it extended from positions −226 to −208 relative to the transcriptional start site of rsmZ, upstream of the binding regions of GacA and MvaT (Figure 5C). Moreover, sequence analysis showed this protected region is absent in the promoter region of rsmY. This result was consistent with our findings that BswR has little effect on the transcription of rsmY, further supporting that BswR specifically regulates rsmZ by directly binding to its promoter region.

Overall structure of monomeric BswR

To further understand the molecular mechanisms with which BswR interacts with the promoter of rsmZ, we proceeded with structural analysis by X-ray crystallography. The crystal structure of BswR was determined and refined to 2.3 Å resolution. While 101 residues were visible in the final model, the last 13 residues and the 6xHis-tag in C-terminus were not well ordered in the electron density map and thus excluded. Then, the BswR protein model was refined and water molecules were located, finally giving R-work/R-free factor to 22.9/28.6%, respectively. The final model was examined using PROCHECK: 94.5% of the residues lie in the most favored regions, with the remaining 4.4 and 1.1% assigned to the additionally and generously allowed regions, respectively. The final refinement statistics for the structure are listed in Table 1.

The overall topology of BswR monomer contains seven helices, of which five form a compact helix bundle and the other two assemble as extended C-terminal helices (Figure 6A). Consistent with the prediction, our structure reveals that the N-terminus of BswR comprises α2-turn-α3, a typical helix-turn-helix (HTH) motif for DNA-binding (in which α2 is for stabilization and α3 for recognition). The two helices (α6 and α7) with a V-shaped arrangement in the C-terminus vary largely across different organisms, which meets the requirement for diverse ligand binding.

Dimerization of BswR

Based on the gel filtration profile during protein purification, we found that BswR exists as a dimer in solution. This was further confirmed from the crystal structure: the two monomers with the V-shaped arrangement of the C-terminal helices intertwine together to form a homodimer by crystallographic symmetry. Using PISA and Contact (CCP4 suite) programs (33,39), we investigated the dimerization interface of BswR. In either monomer, 94 of 101 amino acids were exposed on the surface. On dimerization, an average interface area of 1438.7 Å² was buried, accounting for 20.8% of the solvent-exposed surface area (6923.4 Å²) of each monomer. In total, 45 contacts were identified between two monomers with an intermolecular distance of <3.5 Å, the majority of which were located at the C-terminus. Furthermore, dimerization was mainly mediated through hydrophobic interaction, which involves the residues Leu71, Leu74, Leu81, Phe85, Leu89, Thr90, Ile93, Leu97 and Ala99 (Figure 6D and E). In addition, the intermolecular hydrogen bonds between the main chain N atom of Ala62 and the main chain O atom of Tyr65 (Ala62-Tyr65', Tyr65-Ala62', where the primes refer to the second monomer, same as below), the salt bridges (Lys77-Glu94, Glu94-Lys77'), as well as the aromatic π-π stacking interactions (Tyr65-Tyr65' and Phe92-Phe92') (Figure 6C and D), were found to contribute to the BswR dimerization. In the dimeric structure, BswR exhibits an uncommon large area of dimer interface, which is formed by the extension of C-terminal helical region (helices 5–7, residues 61–101). Clearly, the dimerization of BswR was mainly achieved by the interleaveing of the two extended helices in its C-terminus, although the parallel helix 5 also contributes to the dimer formation.

XRE comprises the second-most frequently occurring regulator family in bacteria that control diverse biological functions, and these regulators share a characteristic N-terminal DNA-binding domain with a HTH motif, whereas the C-terminal region is extremely variable. Structural comparison of BswR with other XRE family proteins, restriction–modification (R–M) system controller proteins (C protein) Csp2311 from Citrobacter sp. RFL231 (44), SinR from Bacillus subtilis (45), C protein Esp1396I from Enterobacter sp. RFL1396 (46) and EspR from Mycobacterium tuberculosis (47), shows that they share a similar fold for DNA-binding domain (Supplementary Figure S6), despite the variations mainly in the turn of HTH (α2-turn-α3) which could be of importance for orientating the recognition helix α3 for an optimal affinity for DNA (47). Except for C protein Esp1396I with one C-terminal helix, these proteins contain two C-terminal extended helices forming a V-shaped arrangement, which is involved in dimer assembling. The orientation of this V-shaped arrangement relative to the similar N-domain is varied, which consequently impacts on its DNA-binding (47). In contrast to the structural similarity, the sequence identity is relatively low (Supplementary Figure S6).

Structural basis of BswR for DNA binding

BswR possesses a calculated isoelectric point (pI) of 9.2, indicating that it is positively charged under physiological condition. EMSA assay showed that BswR can bind to the promoter region of rsmZ, and DNase I protection assay further revealed that ‘ACCCGGCTTCGCCGTT’, a palindromic sequence (underlined) in the rsmZ promoter, is the binding site of BswR. To elucidate how BswR binds to DNA, a structural alignment of BswR onto Esp1396I-DNA (PDB ID: 3CLC) resulted in a DNA-binding model (Figure 7A), in which no steric clash between BswR and the double-stranded DNA (dsDNA) was observed. In this model, the HTH motif in each monomer of BswR dimer is the major component to interact with the dsDNA, and the
recognition helix α3 was inserted into the consecutive major groove of the dsDNA, in a canonical way to establish contact.

We also mapped the electrostatic surface of BswR dimer, as shown in Figure 7B. The strong positive charge was mainly located at the N-terminal DNA binding domain, and the remaining surface charges were evenly distributed. The dsDNA molecules were laid across the positive-neutral-positive patches in BswR dimer, and most positively charged region colored blue was covered, thereby demonstrating that the model is nicely consistent with the surface charge distribution. Compared with other XRE family transcriptional factors, several conserved residues were mapped out for BswR to bind DNA (Figure 7C). These residues, including Arg34, Gln37, Arg42, His43, Asn46 and Arg48 in the N-terminal domain, face toward the dsDNA and probably contribute to the direct contact with DNA.

**DISCUSSION**

Bacterial motility and biofilm formation has been extensively investigated (3,8–11). In *P. aeruginosa*, swarming and biofilm formation are mutually exclusive, could be regulated by nitrogen limitation, controlled by quorum sensing and governed by a large number of transcriptional regulators (8,9). In this study, we discover a new layer of
regulatory cascade that tailors swarming and biofilm formation in *P. aeruginosa*, by identification of a previously uncharacterized protein BswR (Figure 1). Genome-wide transcriptomic analyses show that BswR specifically controls the activity of flagella and T4P (Figure 2). Alteration of the *bswR* expression affected the swarming of *P. aeruginosa* and also affected the expression of genes involving the assembly of flagella and the biogenesis of T4P, indicating that BswR regulates bacterial swarming likely through compromising the function of flagella and T4P. However, mutation of *bswR* did not significantly influence the flagella-dependent swimming and the T4P-dependent twitching, suggesting that BswR have additional roles in regulation of bacterial swarming under these experimental conditions.

Our results provide new insights into how the small RNA *rsmZ* is regulated in response to cellular physiology and environmental stimuli (Supplementary Figure S7). RsmZ is a multifunctional regulator in *P. aeruginosa*. By counteracting the activity of RsmA, RsmZ acts as a negative regulator for bacterial swarming and a positive regulator for biofilm formation (18–20). In present study, we found overexpression of *bswR* inhibits bacterial swarming but favors biofilm formation, compatible with the previous results that overexpression of *rsmZ* resulted in loss of the swarming ability and increase of biofilm.

![Figure 7](image-url) A proposed model of BswR interacting with DNA. (A) Model depicting BswR–DNA complex. Structural alignment of one protein dimer of Esp1396I–DNA complex (PDB ID: 3CLC) onto BswR dimer by fitting the two N-terminal domains was carried out with coot, subsequently the excess nucleotides far apart from BswR were removed. The dsDNA was shown as cartoon with sticks, same as (B). (B) A representative portion of the BswR–DNA complex structure with exposure of the HTH regions. Electrostatic potentials were rendered on the protein surface with a color scale between $-40.0 \, kT \, e^{-1}$ (Red) and $+40.0 \, kT \, e^{-1}$ (blue). The DNA was shown as cartoon with sticks. (C) The putative residues of BswR involved in DNA binding. The residues are predicted to interact with DNA are labeled and shown as sticks. For clarity, the DNA was shown as cartoon without sticks.
production (23). Identification of rsmZ as the target of BswR provides a molecular mechanism through which BswR regulates the bacterial swarming and biofilm formation.

Transcription of rsmZ is activated by GacA but repressed by MvaT, both physically binding to the promoter region of rsmZ (17,18,22,23,48). While the activity of GacA is titrated by a few two-component sensors, the repression of MvaT could be attenuated by some yet unidentified transcription factors (23). Our results provide a few lines of evidence suggesting that BswR is one of such factors. First, BswR activates the transcription of rsmZ (Figure 3). Second, BswR requires GacA for its activation (Figure 4). Third, this activation depends on the presence of MvaT (Figure 4). Fourth, BswR directly binds to the rsmZ promoter (Figure 5). Even so, the detailed mechanism through which BswR relieves the repression of MvaT requires further investigation. In the rsmZ promoter, binding site of GacA (TGT AAGCATTAACTTACA) is upstream of that of MvaT (AAATTTAAGTACCACTTTATGAAATATTTAAGGATAAT TTGCAAAAT), separated by a DNA stretch with a length of 56 bp (Figure 5C). DNA footprint unravels that BswR binds to a separate site from that of MvaT, excluding the possibility that BswR and MvaT directly compete the binding sites for expression of RsmZ. In the crystal structure, BswR contains a positively charged DNA-recognition extension that dramatically deforms the promoter region during DNA binding (Figure 7), indicating that BswR may activate rsmZ by influencing the binding affinity of MvaT to the promoter region. However, previous results demonstrate that MvaT could associate with two small regulatory peptides for its repression activity (49), and moreover, MvaT functioning as a member of H-NS family protein usually causes DNA bending and thus facilitates distant protein–protein interactions (50). It is possible that BswR counteracts MvaT by directly binding to each other. In addition, the MvaT of P. aeruginosa has been reported to form both dimers and higher-order oligomers and the binding of MvaT to DNA targets depends on the oligomerization of MvaT dimers (50). Therefore, it is also possible that BswR counteracts MvaT by interfering with the protein–protein interactions between MvaT dimers.

Bacterial motility and biofilm are most important virulence determinants and survival strategies for microbial pathogens to cope with harsh environments and infecting hosts. Expression of these traits is under stringent regulation, and it responds to largely unidentified environmental signals. Although the regulatory cascade has been extensively studied, the environmental signals remain largely unknown. Structural comparison of BswR with other XRE family proteins reveals a similar fold for the N-terminal domain (Supplementary Figure S6). Further structural analyses suggest BswR functions as a homodimer, which uses positively charged helix to fit the major groove of the dsDNA for binding (Figure 7). In particular, BswR shares the most similar structure to C protein Csp231I, with a root-mean-square deviation (rmsd) of 1.169 Å for the main chain (Supplementary Figure S6). In Citrobacter sp. RFL231, Csp231I plays a key role in the temporal regulation of gene expression in bacterial R–M systems and are important mediators of horizontal gene transfer (44). The biological significance of this structural similarity is not known yet. Different from the N-terminal DNA-binding domain, the C-terminal region is structurally diversified with no typical homologs identified in the database. In the crystal structure, it comprises two alpha-helices with a V-shaped arrangement for dimerization. It is unknown whether this arrangement also involves the potential ligand binding for BswR, given that no ligand has been identified for these proteins, and little information is available on how the ligand-binding site could be formed. Analyses of previously published genome-wide microarray results show that the expression of BswR is not significantly influenced under the conditions of nutrient depletion, altered quorum sensing and increased temperatures, all of which have been implicated in bacterial motility and biofilm formation (51–54). Identifying the environmental signals that modulate the BswR expression and functionality will answer whether BswR represents a previously unappreciated signal pathway regulating the virulence and survival capability of P. aeruginosa.

ACESSION NUMBERS

Atomic coordinate and structure factor of BswR have been deposited in PDB with accession code 4O8B.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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