SuhB Is a Regulator of Multiple Virulence Genes and Essential for Pathogenesis of Pseudomonas aeruginosa

Kewei Li, Chang Xu, Yongxin Jin, Ziyu Sun, Chang Liu, Jing Shi, Guikui Chen, Ronghao Chen, Shouguang Jin, Weihui Wu

Key Laboratory of Molecular Microbiology and Technology of the Ministry of Education, Department of Microbiology, College of Life Sciences, Nankai University, Tianjin, China; Institute of Radiation Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin Key Laboratory of Molecular Nuclear Medicine, Tianjin, China; Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida, USA

ABSTRACT During initial colonization and chronic infection, pathogenic bacteria encounter distinct host environments. Adjusting gene expression accordingly is essential for the pathogenesis. Pseudomonas aeruginosa has evolved complicated regulatory networks to regulate different sets of virulence factors to facilitate colonization and persistence. The type III secretion system (T3SS) and motility are associated with acute infections, while biofilm formation and the type VI secretion system (T6SS) are associated with chronic persistence. To identify novel regulatory genes required for pathogenesis, we screened a P. aeruginosa transposon (Tn) insertion library and found suhB to be an essential gene for the T3SS gene expression. The expression of suhB was upregulated in a mouse acute lung infection model, and loss of suhB resulted in avirulence. Suppression of T3SS gene expression in the suhB mutant is linked to a defective translation of the T3SS master regulator, ExsA. Further studies demonstrated that suhB mutation led to the upregulation of GacA and its downstream small RNAs, RsmY and RsmZ, triggering T6SS expression and biofilm formation while inhibiting the T3SS. Our results demonstrate that an in vivo-inducible gene, suhB, reciprocally regulates genes associated with acute and chronic infections and plays an essential role in the pathogenesis of P. aeruginosa.

IMPORTANCE A variety of bacterial pathogens, such as Pseudomonas aeruginosa, cause acute and chronic infections in humans. During infections, pathogens produce different sets of virulence genes for colonization, tissue damage, and dissemination and for countering host immune responses. Complex regulatory networks control the delicate tuning of gene expression in response to host environments to enable the survival and growth of invading pathogens. Here we identified suhB as a critical gene for the regulation of virulence factors in P. aeruginosa. The expression of suhB was upregulated during acute infection in an animal model, and mutation of suhB rendered P. aeruginosa avirulent. Moreover, we demonstrate that SuhB is required for the activation of virulence factors associated with acute infections while suppressing virulence factors associated with chronic infections. Our report provides new insights into the multilayered regulatory network of virulence genes in P. aeruginosa.
Besides environmental stresses, the host environment under chronic infections might also provide signals that turn off the T3SS while turning on biofilm formation (24). In *P. aeruginosa*, a global posttranscriptional regulatory protein, RsmA, was shown to control a switch between T3SS activation and biofilm formation (25, 26). Mutation in *rsmA* results in the inhibition of the T3SS and upregulation of biofilm formation (27, 28). Two small regulatory RNAs, RsmY and RsmZ (RsmY/Z), bind RsmA to antagonize its function. Upregulation of RsmY and RsmZ leads to T3SS inhibition and a hyperbiofilm phenotype (29, 30). The expression of RsmY and RsmZ is regulated by a variety of regulatory genes (4). It has been reported that the GacS/GacA two-component regulatory system exclusively controls the expression of RsmY/Z (29). GacS is a membrane-bound sensor kinase which phosphorylates GacA (31), and phosphorylated GacA directly activates the transcription of RsmY and RsmZ through binding to their promoters (29). A membrane-bound hybrid sensor kinase, RetS, represses the GacS/GacA signaling, leading to the downregulation of RsmY/Z (31). Mutation in the *retS* results in suppression of the T3SS and hyperbiofilm formation (32). Another hybrid sensor kinase, LadS, positively regulates the GacS/GacA pathway (33). Fine-tuning of virulence factors involved in acute and chronic infections plays an essential role in *P. aeruginosa* pathogenesis.

A *suhB* gene was initially identified in *Escherichia coli* as an extragenic suppressor of a sec Y24 mutation, a component of the type II protein secretion system. A point mutation in the *suhB* gene suppresses the growth defect of the secY24 mutation at 42°C while rendering the bacterial cold sensitive (unable to grow at 30°C) (34). Further studies in *E. coli* demonstrated that mutation in *suhB* suppresses the heat-sensitive phenotype of a dnaB (encoding a protein involved in DNA replication) mutant (35) or an rpoH (the heat shock sigma factor, σ^{32}) mutant (36) while also rendering the mutants cold sensitive. These reports suggest that SuhB might play an important role in stress responses.

In this study, we identified *suhB* as an essential gene for the expression of the T3SS in *P. aeruginosa*. In vivo studies suggest that *suhB* plays a role in signal transduction during infection. Further studies demonstrated that the *suhB* and *retS* mutants share similar phenotypes, including a suppressed T3SS, an elevated T6SS, and hyperbiofilm formation. Both pathways regulate the expression of RsmY/Z through the GacS-GacA two-component regulatory system. Whereas RetS controls GacA activity through inhibiting the kinase activity of GacS, SuhB controls the expression of GacA. Therefore, we have identified a new gene in the regulatory network that controls acute- and chronic-infection-associated genes in *P. aeruginosa*.

**RESULTS**

**Isolation of mutants defective in ExoS expression/secretion.** To identify novel T3SS-related genes, a transposon (Tn) insertion library was generated in a wild-type PAK strain containing a pHW0029 plasmid, encoding ExoS-FLAG fusion protein under the control of the native exoS promoter (37). Individual mutant colonies were picked and cultured in 96-well plates in LB medium with 5 mM EGTA. Secreted ExoS-FLAG in the culture supernatant was quantitated by a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (38). Of 5,000 mutants, a total of 69 mutants showed reduced ExoS-FLAG secretion; 39 of them had Tn insertions at 23 different genes on the chromosome (see Table S1 in the supplemental material), while the remaining 30 had Tn on the plasmid, disrupting the *exoS*-flag coding sequence.

Among those 23 genes, *suhH, gbdD,* and *gbdH* are involved in amino acid metabolism, while *nuoL* and *fadE* encode proteins for energy metabolism (http://www.pseudomonas.com). These results are consistent with previous reports which suggest that metabolic imbalance leads to T3SS inhibition (21, 22). Two heat shock proteins, DnaK and HtpG, were found to be required for the expression or secretion of ExoS as well. Loss of these genes might cause protein misfolding and affect the assembly or function of the T3SS apparatus. SecG is a component of protein export machinery which may indirectly affect membrane integrity and the T3SS apparatus. RNase E, PNP, and DeaD are involved in RNA processing, while InhB and TufAB are translation initiation and elongation factors, respectively (http://www.pseudomonas.com). Mutation in those genes might affect mRNA stability and gene translation, indirectly inhibiting ExoS expression.

**SuhB is required for ExoS/T expression and cytotoxicity.** Among the 23 genes, we further pursued the role of SuhB in the regulation of the T3SS. To confirm the relationship between *suhB* and the T3SS, a chromosomal *suhB* deletion mutation (∆*suhB*) was generated in the wild-type strain PAK. The resulting mutant displayed reduced levels of *exoT* transcription (Fig. 1A) as well as decreased expression of ExoS (Fig. 1B). Complementation with a *suhB*-expressing plasmid partially restored the *exoT* transcription (Fig. 1A) and the expression as well as secretion of ExoS (Fig. 1B). T3SS-mediated cytotoxicity was examined by measuring cells remaining attached after infection. When A549 cells were infected with wild-type PAK at a multiplicity of infection (MOI) of 20, the majority of the cells were rounded and detached 4 h postinfection. Loss of *suhB* rendered the strain noncytotoxic, and complementation with a *suhB* gene restored the cytotoxicity (Fig. 1C). These results suggest that SuhB is required for the T3SS activity in *P. aeruginosa*.

**SuhB is induced in vivo and is required for virulence in a mouse acute pneumonia model.** The T3SS is activated upon infection and plays an essential role in the pathogenesis of acute infections (10, 11, 39). The functional connection between SuhB and the T3SS prompted us to test the expression pattern of *suhB* during infection and its role in pathogenesis. In a mouse acute pneumonia model, mice were challenged intranasally with 5 × 10^6 CFU of wild-type PAK or the ∆*suhB* mutant or the ∆*suhB* mutant with the *suhB* complementation plasmid (pUCP19-*suhB*), using a previously described method (40). At 3 or 6 h postinfection, bronchoalveolar lavage fluid (BALF) was collected from the PAK-infected mice and total RNA was purified from the BALF. Expression levels of *suhB* were determined by real-time PCR. As shown in Fig. 2A, expression of *suhB* increased drastically during the infection, suggesting that *suhB* may play an important role in the pathogenesis. Indeed, no mice died when infected with the *suhB* mutant. In contrast, the wild-type strain and the complementation strain caused 75% and 50% mortality, respectively (Fig. 2B). The results presented above demonstrate that *suhB* is an *in vivo*-inducible gene and that it is essential for the virulence of *P. aeruginosa* in the acute mouse infection model.

**Role of SuhB in the transcription of exsCEBA operon.** ExsA is the master activator for the T3SS genes carried in the *exsCEBA* operon (14). We addressed whether the drastic reduction of ExoS and ExoT expression in the *suhB* mutant is due to the repression of
SuhB is induced during infection and plays an essential role in pathogenesis. Mice were challenged with the WT PAK, ΔsuhB, or ΔsuhB/pUCP19-suhB strains (5 × 10^8 CFU per mouse) intranasally. (A) BALF from WT strain-infected mice was collected at the indicated time points. Total RNA was isolated from bacteria in the BALF. The expression levels of suhB were determined by real-time PCR. The 30S ribosomal protein-coding gene rpsL was used as an internal control. *, P < 0.05 compared to bacteria in vitro by analysis of variance (ANOVA) with Tukey’s multiple-comparison test. (B) Virulence of the suhB mutant in a murine acute pneumonia model. Infected mice were monitored for 5 days. P values were calculated by Kaplan-Meier survival analysis with a log rank test with Prism software (Graphpad Software).

**FIG 2** SuhB is required for the expression of the T3SS. (A) The expression of exoT-lacZ in the backgrounds of *P. aeruginosa* strains PAK, suhB::Tn5, suhB::Tn5 containing pUCP-suhB, ΔsuhB, and ΔsuhB containing pUCP-suhB. Bacteria were grown to an OD$_{600}$ of 1 to 2 in LB with or without EGTA before β-galactosidase assays. *, P < 0.05 compared to wild-type (WT) or complemented strains by Student’s t test. (B) Expression and secretion of ExoS in the indicated strains. (C) Cytotoxicity of the indicated strains at a MOI of 20. At 4 h postinfection, cells attached to the plate were measured with crystal violet staining. *, P < 0.05 compared to WT or complemented strains by Student’s t test.

**FIG 1** SuhB is required for the expression of the T3SS. (A) The expression of exoT-lacZ in the backgrounds of *P. aeruginosa* strains PAK, suhB::Tn5, suhB::Tn5 containing pUCP-suhB, ΔsuhB, and ΔsuhB containing pUCP-suhB. Bacteria were grown to an OD$_{600}$ of 1 to 2 in LB with or without EGTA before β-galactosidase assays. *, P < 0.05 compared to wild-type (WT) or complemented strains by Student’s t test. (B) Expression and secretion of ExoS in the indicated strains. (C) Cytotoxicity of the indicated strains at a MOI of 20. At 4 h postinfection, cells attached to the plate were measured with crystal violet staining. *, P < 0.05 compared to WT or complemented strains by Student’s t test.

**exsA** expression. To test the transcription of *exsCEBA* operon, an *exsC-lacZ* transcriptional fusion reporter plasmid was introduced into the suhB mutant. As shown in Fig. 3A, expression of the *exsCEBA* operon was drastically reduced in the *suhB* mutant whereas complementation with a *suhB* gene partially restored the *exsC* promoter activity (Fig. 3A). These results suggest that SuhB is directly or indirectly involved in the regulation of the *exsCEBA* operon expression.

ExsA is an activator of its own operon (14); thus, the inhibition of the *exsCEBA* transcription in the *suhB* mutant might be due to a reduced *exsA* mRNA or protein level. To address this issue, we replaced the *exsC* promoter (PexsC) with a *tac* promoter (Ptac) and inserted a *lacZ* gene on the chromosones of wild-type PAK as well as in the *suhB* mutant (Fig. 3B); thus, the transcription of *exsCEBA* operon was under the control of IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *Ptac* promoter. The strains were grown under T3SS-inducing conditions in the presence of IPTG, and the mRNA levels of *exsC* and *exsA* and of a region between *exsB* and *exsA* were measured by real-time PCR. The mRNA levels of these three regions were similar among the wild-type, ΔsuhB mutant, and *suhB* complementation strains, with the *exsC* mRNA level slightly lower and the *exsA* mRNA level slightly higher in the *suhB* mutant (Fig. 3C). Despite of similar levels of *exsC* and *exsA* mRNA, the *suhB* mutant was still defective in ExoS expression and secretion (Fig. 3D), suggesting a possible defect of *exsA* expression at the posttranscriptional level.

**SuhB controls the expression of ExsA at the posttranscriptional level.** To test the translation of ExsA in the *suhB* mutant, we cloned *exsA*-FLAG fusions together with a *lacI* gene onto plasmid pDN19, where the transcription of the *exsA*-FLAG is driven by a *Ptac* from the vector. Two *exsA*-FLAG fusions with different *exsA* upstream regions, *exsA*-Flag-A and *exsA*-Flag-S, were constructed (Fig. 3E). In the *exsA*-FLAG-A fusion, only the *exsA* coding region was fused with FLAG tag; the translation start codon as well as the ribosome binding site were from the vector, thus abolishing potential translational control upstream of the *exsA* gene. In the *exsA*-FLAG-S fusion, a 225-bp fragment upstream of *exsA* was included, in addition to endogenous ribosome binding site and start codon. These constructs were transferred into an *exsA*-ΔΩ mutant or the *suhB* mutant background. Expression levels of the *ExsA*-FLAG as well as ExoS proteins were tested under T3SS-inducing and noninducing conditions.
As shown by the exsA-FLAG-A construct results, the ExsA-FLAG protein levels were similar in the exsA::H9024 and suhB mutants (Fig. 3F, lanes 5, 6, 11, and 12), demonstrating that the Ptac activity is not affected by the suhB mutation. Accordingly, expression of the ExoS protein was restored in both exsA::H9024 and suhB mutants (Fig. 3F, lanes 5, 6, 11, and 12). However, when exsA endogenous ribosome binding site and neighboring regions were included (as in the exsA-Flag-S plasmid), the expression levels of ExsA-FLAG and ExoS were diminished in the suhB mutant compared to those in the exsA::H9024 mutant (Fig. 3F, lanes 7, 8, 13, and 14). These results indicated that SuhB might regulate the expression of ExsA posttranscriptionally through the exsA upstream region.

Identification of genes regulated by SuhB. To further understand the role of SuhB, we performed transcriptome sequencing (RNA-seq) to examine the global gene expression of the suhB
mutant and wild-type PAK under T3SS-inducing conditions. Compared to wild-type PAK results, expression of 547 genes was altered in the suhB mutant (see Table S2 in the supplemental material). Consistent with our /H9252-galactosidase assay and Western blotting results, the mRNA levels of exoS, exoT, and other T3SS genes were lower in the suhB mutant (Table 1). In addition, flagellum biosynthesis genes were downregulated whereas type VI secretion system (T6SS) genes were upregulated in the suhB mutant (Table 1). Real-time PCR results also showed downregulation of the fliC flagellin gene and upregulation of the tssK1, tssJ1, and tssF1 T6SS structural genes and of effector gene hcp1 in the suhB mutant (see Fig. S1 in the supplemental material). To further confirm the expression patterns of flagellum and T6SS genes, we examined their related phenotypes. As expected, the suhB mutant displayed reduced swimming motility (Fig. 4A and B) and increased expression of the T6SS effector Hcp1 (Fig. 4C).

In P. aeruginosa, the RetS-RsmA regulatory pathway reciprocally controls the T3SS and the T6SS (4). Mutation in either retS or rsmA results in similar phenotypes, in terms of the T3SS and T6SS expression as well as attenuated virulence in acute infection (26, 32). These results led us to compare the the suhB mutant transcriptome results with the microarray data from retS (performed under T3SS-inducing conditions) and rsmA (performed under normal growth conditions) mutants in the PAK background (26, 32).

### TABLE 1  Genes of altered expression in the suhB mutant compared to wild-type PAK

| Gene category and designation | Gene | Gene function | Fold change (suhB/WT) | P value |
|-----------------------------|------|---------------|-----------------------|--------|
| Type III secretion genes    | exoT | Exoenzyme T    | 0.083214              | 0.00331 |
| PA1690 | pscU | Translocation protein in type III secretion | 0.193606 | 0.023589 |
| PA1703 | pcrD | Type III secretory apparatus protein PcrD | 0.356385 | 0.010725 |
| PA1705 | pcrG | Regulator in type III secretion | 0.434703 | 0.008231 |
| PA1706 | pcrV | Type III secretion protein PcrV | 0.254249 | 0.008586 |
| PA1708 | popB | Translocator protein PopB | 0.107812 | 0.005814 |
| PA1709 | popD | Translocator outer membrane protein PopD precursor | 0.101252 | 0.00077 |
| PA1712 | exsB | Exoenzyme S synthesis protein B | 0.644707 | 0.00472 |
| PA1719 | pscF | Type III export protein PscF | 0.134511 | 0.006694 |
| PA1725 | pscL | Type III secretion system protein | 0.122295 | 0.001139 |
| PA2191 | exoY | Adenylate cyclase | 0.361816 | 0.009486 |
| PA3841 | exoS | Exoenzyme S | 0.028808 | 4.12E-07 |
| PA3842 | sceS | Chaperone | 0.231546 | 0.027402 |
| Flagellum biogenesis genes  | flgB | Flagellar basal body rod protein FlgB | 0.218089 | 0.007676 |
| PA1077 | flgE | Flagellar hook protein FlgE | 0.219233 | 0.004098 |
| PA1080 | flgG | Flagellar basal body rod protein FlgG | 0.2187 | 0.009148 |
| PA1082 | Hypothetical protein | 0.180182 | 0.005874 |
| PA1088 | fliC | Flagellin type B | 0.217321 | 0.007287 |
| PA1104 | fliI | Flagellum-specific ATP synthase | 0.383827 | 0.009766 |
| Type VI secretion genes     | tssQ1 | Membrane proteins; protein secretion/export apparatus | 0.31983 | 0.043719 |
| PA0079 | tssK1 | Protein secretion/export apparatus | 10.6766 | 0.000804 |
| PA0080 | tssJ1 | Protein secretion/export apparatus | 7.68624 | 0.004379 |
| PA0088 | tssF1 | Protein secretion/export apparatus | 11.89032 | 0.005502 |
| PA0090 | clpV1 | ClpV1 protein | 2.022019 | 0.009987 |
| PA0091 | vgrG1 | VgrG1 | 45.37001 | 0.009213 |
| Pyocin synthesis genes      | PA0613 | Hypothetical protein | 0.209161 | 0.031819 |
| PA0614 | Hypothetical protein | 0.138015 | 0.021263 |
| PA0622 | Bacteriophage protein | 0.079474 | 3.87E-05 |
| PA0623 | Bacteriophage protein | 0.109028 | 0.000165 |
| PA0624 | Hypothetical protein | 0.100469 | 8.56E-05 |
| PA0625 | Hypothetical protein | 0.142139 | 0.00168 |
| PA0626 | Hypothetical protein | 0.186657 | 0.005785 |
| PA0633 | Hypothetical protein | 0.065348 | 1.20E-05 |
| PA0634 | Hypothetical protein | 0.22264 | 0.008216 |
| PA0635 | Hypothetical protein | 0.162455 | 0.009963 |
| PA0636 | Hypothetical protein | 0.148172 | 0.002497 |
| PA0638 | Bacteriophage protein | 0.154734 | 0.003163 |
| PA0639 | Hypothetical protein | 0.12724 | 0.000413 |
| PA0641 | Bacteriophage protein | 0.297345 | 0.049771 |
| PA0643 | Probable bacteriophage protein | 0.248638 | 0.015289 |
| PA0984 | Colicin immunity protein | 0.062835 | 3.68E-06 |
| PA0985 | pyoS5 | Pyocin S5 | 0.228836 | 0.016219 |
Similar to the retS and rsmA mutant results, the expression of type IV pilus biogenesis genes (pilA and pilY1) and a type II secretion gene (xcpQ) was reduced in the suhB mutant (see Table S2 in the supplemental material). In the PAK rsmA mutant, genes involved in iron homeostasis are downregulated (26); similarly, pyoverdine synthesis genes pvdP (PA2392) and pvdG (PA2425) and genes PA4359, PA4514, hitA (PA4687), hitB (PA4688), and PA5217 involved in iron transportation were downregulated in the suhB mutant (Table S2). These results suggest that SuhB-mediated regulatory pathways might partially overlap those under the control of RetS and RsmA.

RsmY/Z and GacA are involved in SuhB-mediated regulation. The RetS-RsmA pathway mainly regulates T3SS and T6SS genes through modulation of RsmY and RsmZ levels (29, 31). Interestingly, levels of RsmY/Z were also upregulated in the suhB mutant (see Table S2 in the supplemental material). In the PAK rsmA mutant, genes involved in iron homeostasis were downregulated (26); similarly, pyoverdine synthesis genes pvdP (PA2392) and pvdG (PA2425) and genes PA4359, PA4514, hitA (PA4687), hitB (PA4688), and PA5217 involved in iron transportation were downregulated in the suhB mutant (Table S2). These results suggest that SuhB-mediated regulatory pathways might partially overlap those under the control of RetS and RsmA.

Next, we examined the expression levels of GacS, GacA, and LadS, which positively regulate the expression of RsmY/Z. The mRNA level of gacA, but not gacS or ladS, was increased in the suhB mutant (Fig. S2A and B in the supplemental material). Overexpression of retS did not restore the expression of ExoS in the suhB mutant. Similarly, overexpression of suhB in the retS mutant had no effect on the expression of ExoS (Fig. S2C). These results suggest that RetS and SuhB might independently control RsmY/Z.

Consistent with the conclusion presented above, mutation of gagA in the suhB mutant background partially restored the expression of ExoS (Fig. 6). Furthermore, simultaneous deletion of both rsmY and rsmZ partially restored the expression of ExoS (Fig. 6) whereas deletion of rsmY or rsmZ individually did not restore the ExoS expression in the suhB mutant. Since the major role of RsmY and RsmZ is to antagonize RsmA, we investigated whether overexpression of rsmA could restore the T3SS. As expected, overex-
preservation of rsmA partially restored the expression of ExoS in the suhB mutant (see Fig. S4A in the supplemental material).

Next, we examined the involvement of RsmY/Z in the posttranscriptional regulation of exsA with the ExsA-FALG constructs (Fig. 3E). In the suhB mutant, deletion of rsmY/Z had no effect on the expression of ExsA-FLAG with an exogenous ribosome binding site (Fig. 3F, lanes 17 and 18), suggesting that RsmY and RsmZ do not affect the P\textit{tac} promoter activity. However, in the exsA-Flag-S construct where the exsA endogenous ribosome binding site and upstream region were included, deletion of rsmY/Z in the suhB mutant partially restored the translation of ExsA-FLAG as well as the expression of ExoS (Fig. 3F, lanes 19 and 20). Overall, these results suggest that GacA and RsmY/Z do play a role in the SuhB-mediated regulation on T3SS.

GacA and RsmY/Z contribute to the upregulation of T6SS and hyperbiofilm phenotype in the suhB mutant. To investigate the roles of GacA and RsmY/Z in the regulation of T6SS in the suhB mutant, we examined the protein levels of Hcp1 in the Δ\textit{gacA} ΔsuhB Δ\textit{rsmY} Δ\textit{rsmZ} mutant strains. Deletion of \textit{gacA} or rsmY/Z rsmZ reduced the level of Hcp1 in the suhB mutant background (Fig. 4C). In addition, overexpression of rsmA suppressed the expression of Hcp1 in the suhB mutant (see Fig. S4B in the supplemental material). These results suggest that SuhB regulates the expression of Hcp1 through GacA and RsmY/Z, similar to their effect on the T3SS as described above.

The RetS-GacS-GacA and RsmY/Z-RsmA regulatory pathway reciprocally regulates virulence factors associated with acute and chronic infections, including the T3SS, the T6SS, and biofilm formation. A featured phenotype of retS and rsmA mutants as well as wild-type PAK overexpressing RsmY/Z is hyperbiofilm formation (29). The observed upregulation of GacA and RsmY/Z in the suhB mutant hinted at a hyperbiofilm phenotype. Tests of biofilm formation in 96-well plates (29, 33) showed that the suhB mutant does indeed form biofilm at increased levels compared to the wild-type strain (Fig. 7). Deletion of \textit{gacA} or rsmY/Z rsmZ in the suhB mutant abolished the hyperbiofilm phenotype (Fig. 7). Complementation with a suhB gene or overexpression of rsmA reduced the biofilm formation (see Fig. S4C and D in the supplemental material). These results suggest that SuhB-mediated regulation of biofilm formation also goes through the GacA and RsmY/Z pathway.


discussion

In this study, we performed a small-scale Tn mutagenesis screening and identified 23 novel T3SS-related genes. However, no Tn insertion was found in the T3SS gene cluster. One likely explanation is the nonrandom nature of the Tn5 insertion (41). In a previous Tn\textit{5} mutagenesis study (42), it was found that the lowest insertion density of Tn5 in the chromosome was between 1.5 Mbp and 3 Mbp where the T3SS gene cluster is located, suggesting that the T3SS locus might be a cold spot for Tn5. It is also possible that the 5,000 Tn insertion mutants that we had screened were not enough to cover the whole genome (5,570 genes in strain PAO1; http://www.pseudomonas.com).

The T3SS is under the control of a variety of environmental signals. Metabolic stresses inhibit T3SS expression (24). In this study, we found that SahH, GltD, GabD, NuoL, and FadE are required for the T3SS expression. These proteins are involved in the metabolism of amino acids, energy, and nucleotides, which fits into the metabolic-imbalance hypothesis. Proteins involved in RNA processing, such as ribonuclease E (gene \textit{rne}), polynucleotide phosphorylase (gene \textit{pnp}), and a probable ATP-dependent RNA helicase (gene \textit{dealD}), were also found to be essential for T3SS expression. As ribonuclease E, polynucleotide phosphorylase, and another RNA helicase (RhlB) are components of the RNA degradosome (43), these proteins might control T3SS expression through degradation of the mRNAs of T3SS-related genes.

It has been reported that \textit{exsA} is the last gene in the \textit{exsCEBA} operon and that the expression of this operon is activated by ExsA itself (44). Between \textit{exsB} and \textit{exsA}, there is a 298-bp noncoding region (http://www.pseudomonas.com). However, the function of this region was not clear. In this study, we constructed two \textit{exsA}-FLAG tag fusions in which the transcription of \textit{exsA} was driven by an exogenous \textit{tac} promoter and thus should be constitutive in the absence of \textit{laci}. In the \textit{exsA} mutant background, both constructs were able to complement the T3SS expression defect. In the suhB mutant, however, the translation of a \textit{Ptac}-driven \textit{exsA} was defective in the presence of the \textit{exsA} upstream region (225 bp in our construct), and when that fragment was excluded, the translation of \textit{Ptac}-driven \textit{exsA} increased dramatically. These results suggest that the \textit{exsA} upstream region is involved in the posttranscriptional regulation of ExsA and that SuhB seems play a key role in this regulation.

Studies of SuhB in \textit{E. coli} suggest a role in the posttranscriptional regulation. The cold-sensitive phenotype of an \textit{E. coli} suhB mutant was suppressed by an additional mutation in the endoribo nuclease RNase III (45), which is involved in RNA processing.

**FIG 6** Roles of GacA and RsmY/Z in the SuhB-mediated T3SS gene expression. Indicated strains were grown to an OD_{600} of 1 to 2 in LB with or without EGTA. Intracellular ExoS and secreted ExoS were separated by centrifugation. Samples from equivalent bacterial cell numbers were loaded onto SDS-PAGE gels and probed with an anti-ExoS antibody.

**FIG 7** SuhB regulates biofilm formation through GacA and RsmY/Z. Biofilm formation by indicated strains was displayed with crystal violet staining (A) and quantified with optical density measurement (B). *, P < 0.05 compared to WT PAK by Student’s \textit{t} test.
and controls the stability and translation of numerous mRNAs (46). Whether RNA processing is related to the SuhB-mediated posttranscriptional control of ExsA expression is currently under investigation in our laboratory.

In this study, we demonstrated that GacA is upregulated in the suhB mutant, which leads to increased levels of RsmY and RsmZ. Deletion of gacA or rsmYZ diminished the expression of Hcp1 and biofilm formation and partially restored the T3SS activity in the suhB mutant. These results suggest that GacA and RsmYZ are under the control of SuhB and play key roles in SuhB-mediated virulence gene regulation. However, the following observations support the idea of the existence of additional routes for the SuhB-mediated regulation of virulence genes. First, in the suhB gacA double mutant or suhB rsmY rsmZ triple mutant, the expression of ExsS was lower than that in the wild-type strain under T3SS-inducing conditions. Second, deletion of gacA or rsmYZ could not restore the swimming defect of the suhB mutant (data not shown). More in-depth studies are warranted to clarify the detailed regulatory mechanisms.

Besides P. aeruginosa, Rosales-Reyes et al. have recently demonstrated in Burkholderia cenocepacia that SuhB is required for protein secretion, motility, and biofilm formation (47). These results suggest that SuhB might be involved in the pathogenesis of multiple bacterial pathogens. Interestingly, the roles of SuhB in biofilm formation in B. cenocepacia and P. aeruginosa seem to be opposite, indicating functional differences of SuhB in the regulatory networks in different bacteria. Obviously, the mechanisms of SuhB-mediated regulation are complicated and require further studies.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study were listed in Table S3 in the supplemental material. Bacteria were grown in L-broth (LB) medium at 37°C. Antibiotics were used at the following concentrations: for E. coli, ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, gentamicin at 10 μg/ml, spectinomycin at 50 μg/ml, streptomycin at 25 μg/ml, and tetracycline at 10 μg/ml; for P. aeruginosa, carbenicillin at 150 μg/ml, neomycin at 400 μg/ml, gentamicin at 150 μg/ml, spectinomycin at 200 μg/ml, streptomycin at 200 μg/ml, and tetracycline at 50 μg/ml.

Construction of a transposon (Tn5) insertion mutant library, plasmid rescue, and sequence analysis were conducted as previously described (15, 41). Plasmid construction information is provided in the supplemental material. Chromosomal gene mutations were generated as previously described (48).

Western blot analysis. Bacterial overnight cultures were subcultured with 100-fold dilution in fresh LB or 30-fold dilution in LB containing 5 mM EGTA for 3.5 h. The supernatant and pellet were separated by centrifugation. Samples from equivalent numbers of bacterial cells were loaded and separated by 12% SDS-PAGE. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and hybridized with a rabbit polyclonal ExsS antibody (generated in Shouguang Jin’s laboratory) or a mouse monoclonal FLAG antibody (Sigma). The signal was detected by the use of an ECL Plus kit (Amersham Biosciences).

Cytotoxicity assay. Bacterial cytotoxicity was determined by measuring detachment of cells after P. aeruginosa infection as described before (20) with minor modifications. A549 cells (7.5 × 10⁴) were seeded into each well of a 48-well plate. The cells were cultured in RPMI medium with 10% fetal calf serum at 37°C with 5% CO₂ for 24 h. Overnight bacterial culture was subcultured in fresh LB to the log phase before infection. Bacteria were washed once with phosphate-buffered saline (PBS) and re-suspended in PBS. A549 cells were infected with the bacteria at a multiplicity of infection (MOI) of 20. At 4 h after infection, the culture medium in each well was aspirated. Cells were washed twice with PBS and stained with 200 μl 0.1% crystal violet–10% ethanol for 15 min at 37°C. The staining solution was discarded, and the plates were washed twice with 1 ml of water. A 250-μl volume of 95% ethanol was then added into each well, and the reaction mixture was incubated at room temperature for 30 min with gentle shaking. The ethanol solution with dissolved crystal violet dye was subjected to measurement of absorbance at a wavelength of 590 nm.

Murine acute pneumonia model. All animal experiments complied with Nankai University and Chinese national guidelines regarding the use of animals in research. The induction of pneumonia by intranasal inoculation was performed as previously described (40). Briefly, 6-to-8-week-old female Balb/c mice were anesthetized with 0.15 ml of chloral hydrate (7.5%) by intraperitoneal injection. Overnight bacterial cultures were diluted in fresh LB and grown to an optical density of 0.5 (OD₆₀₀) of 1. The bacterial cells were spun down and resuspended in PBS at a concentration of 2.5 × 10⁸ CFU/ml. Bacterial cells (10 μl) were placed in each nostril of the anesthetized mouse, resulting in inhalation of approximately 5 × 10⁶ bacteria per mouse. Survival of the mice was monitored for 5 days after the infection.

Purification of RNA from bronchoalveolar lavage fluid (BALF). At 3 or 6 h postinfection, mice were sacrificed using carbon dioxide. BALF was obtained by cannulation of the trachea followed by two instillations of 1 ml of PBS with 0.5 mM EDTA. Bacteria from BALFs were collected by centrifugation. Total RNA was isolated with Trizol (Invitrogen) and further purified with an RNA cleanup kit (Tiangen Biotech).

Transcriptome sequencing and analysis. The transcriptome sequencing analysis was performed by the Tianjin Biochip Corporation Research Center for Functional Genomics and Biochip. Briefly, total RNA was extracted using the Triol extraction method (Triol; Ambion, TX) and then sheared. The isolated mRNA samples were used for first-strand and second-strand cDNA synthesis performed with random hexamers and Superscript II reverse transcriptase. After end repair and addition of a 3’ dA overhang, the cDNA was ligated to an Illumina paired-end-adapter oligonucleotide mix. Fragments of around 200 bp were purified after electrophoresis. After 16 PCR cycles, the libraries were sequenced using an Illumina GAIIx sequencing platform with the paired-end sequencing module. The RNA expression analysis was based on the predicted genes of strain PAO1 (http://www.pseudomonas.com). First, bow tie was used to map mRNA reads to the genome, and cufflinks was then used to calculate the expected numbers of fragments per kilobase of transcript per million mapped reads (FPKM) as expression values for each transcript.

Biofilm formation analysis. Biofilm formation was measured as previously described (29). Briefly, overnight bacterial cultures were diluted to OD₆₀₀ = 0.025 in LB and incubated in each well of a 96-well plate at 37°C for 24 h. For the quantification of biofilm formation, each well was washed twice with water and stained with 0.1% crystal violet, followed by two washes with water. Then, 200 μl ethanol was added to each well. After a 10-min incubation at room temperature, the OD of each sample was measured at a wavelength of 590 nm.

Real-time PCR. Bacteria were grown under the indicated conditions to log phase, and RNAs were stabilized with RNAprotect Bacteria Reagent (Qiagen). RNAs were purified using a RNasey minikit with in-column DNA digestion (Qiagen). cDNA from each RNA sample was synthesized with reverse transcriptase and random primers (Takara). Real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad). The 30s ribosomal protein coding gene rpsL was used as an internal control. Primers used in real-time PCR are listed in Table S4 in the supplemental material.

Swimming motility assay. The swimming motility was examined as previously described (49). Briefly, the indicated strains were inoculated on 0.3% LB agar by stabbing with a sterile toothpick and incubated at 37°C for 18 h.
Other methods. β-Galactosidase activity assays and Tn mutagenesis were performed as described previously (15, 50).

SUPPLEMENTAL MATERIAL

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