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

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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*.**

Received 4 September 2013 **Accepted** 1 October 2013 **Published** 29 October 2013

Citation Li K, Xu C, Jin Y, Sun Z, Liu C, Shi J, Chen G, Chen R, Jin S, Wu W. 2013. SuhB is a regulator of multiple virulence genes and essential for pathogenesis of *Pseudomonas aeruginosa*. mBio 4(6):e00419-13. doi:10.1128/mBio.00419-13.

Editor Gerald Pier, Harvard Medical School

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Pseudomonas aeruginosa is a versatile Gram-negative bacterium which causes a variety of acute and chronic infections (1). Acute infections, such as nosocomial pneumonia, rely on the expression of specific virulence factors, including flagella, pili, exotoxin, and the type III secretion system (T3SS) (2). During chronic infections, such as pulmonary infections in cystic fibrosis (CF) patients, *P. aeruginosa* exhibits slow growth and mucoid and biofilm-forming characteristics (3). Most virulence factors associated with acute and chronic infections are inversely regulated (4).

The T3SS is a complex protein secretion and delivery machinery encoded by many animal and plant pathogens. The T3SS directly translocates bacterial effector molecules into the cytosols of host cells, causing disruption of intracellular signaling or cell death (5). Four effector proteins have been identified in *P. aeruginosa*, i.e., ExoS, ExoT, ExoY, and ExoU (6). The T3SS is an important virulence factor of *P. aeruginosa* which inhibits host defense by inducing cell death in polymorphonuclear phagocytes, macrophages, and epithelial cells (7–9). Loss of the T3SS attenuates *P. aeruginosa* virulence in mouse acute infection models (10, 11).

The T3SS can be activated by direct contact with host cells or growth in a Ca^{2+}-depleted environment (12, 13). Expression of T3SS-related genes is activated by ExsA (14), an AraC-type DNA binding protein that recognizes a consensus sequence located upstream of the transcriptional start site of type III secretion genes (14). In addition, the T3SS is coordinately regulated with other regulatory pathways. Overexpression of alginate and multidrug efflux systems MexCD-OprJ and MexEF-OprN leads to inhibition of the T3SS (15–17). Upregulation of PtrA or PtrB, which is induced by copper stress or SOS response, represses the T3SS (18–20). Metabolic imbalance, representing nutritional stress, was also shown to cause inhibition of the T3SS (21–23). These discoveries indicate that the T3SS of *P. aeruginosa* is effectively turned off under conditions of various environmental stresses, which might be an important survival strategy for this microorganism.

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 bacterium 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 L-broth (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, *sahH*, *gltD*, and *gabD* 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 InfB 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 (Δ*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 5×10^8 CFU of wild-type PAK or the Δ*suhB* mutant or the Δ*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

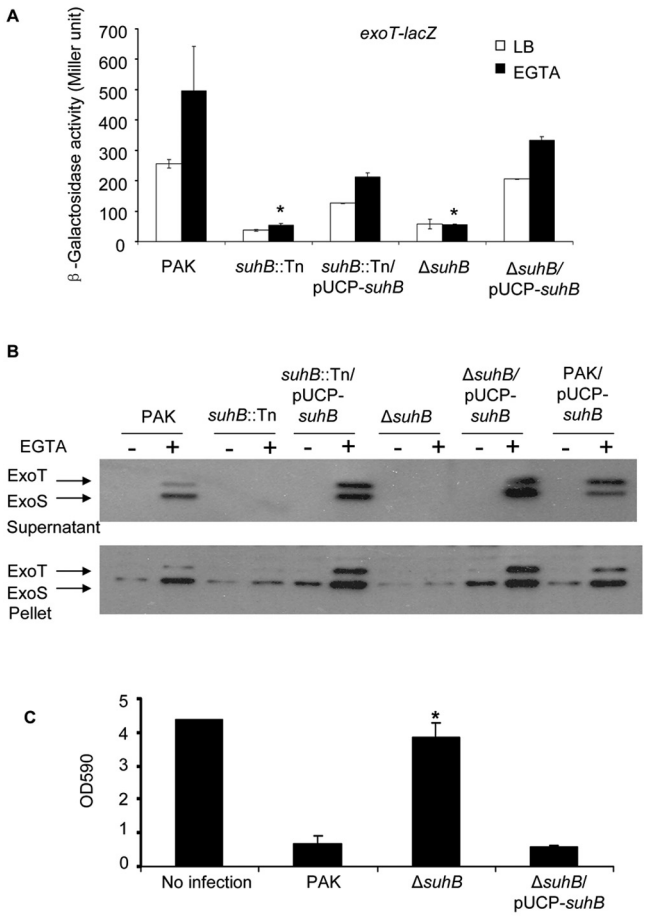

FIG 1 SuhB is required for the expression of the T3SS. (A) The expression of *exoT-lacZ* in the backgrounds of *P. aeruginosa* strains PAK, *suhB*::Tn*5*, *suhB*::Tn*5* containing pUCP-*suhB*, Δ*suhB*, and Δ*suhB* containing pUCP-*suhB*. Bacteria were grown to an OD_{600} of 1 to 2 in LB with or without 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 *suhB* mutant. A549 cells were infected with 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.

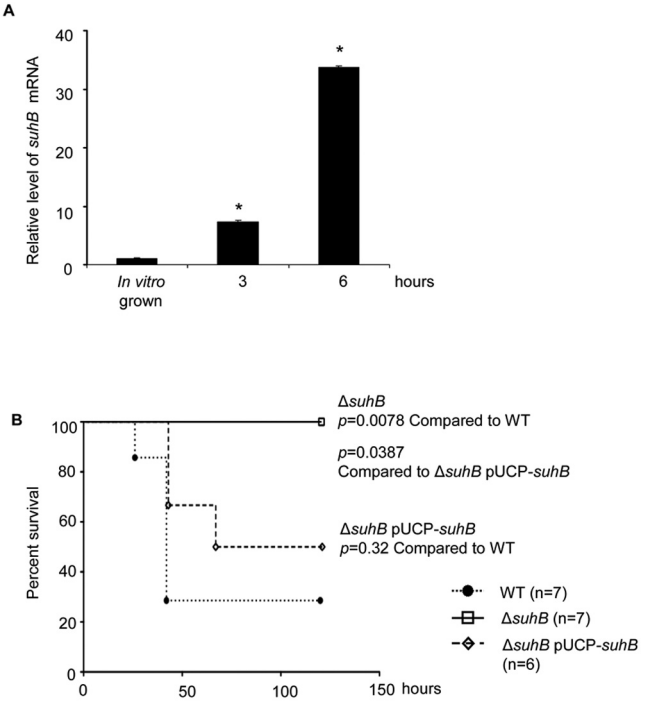

FIG 2 SuhB is induced during infection and plays an essential role in pathogenesis. Mice were challenged with the WT PAK, Δ*suhB*, or Δ*suhB*/pUCP19-*suhB* strains (5×10^8 CFU per mouse) intranasally. (A) BALF from WT strain-infected mice was collected at the indicated time points. Total RNA was isolated 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).

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* mutants might be due to a reduced *exsA* mRNA or protein level. To address this issue, we replaced the *exsC* promoter (P*exsC*) with a *tac* promoter (P*tac*) and inserted a *lacI* gene on the chromosomes 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 P*tac* 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, Δ*suhB* 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 P*tac* 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*::Ω 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.

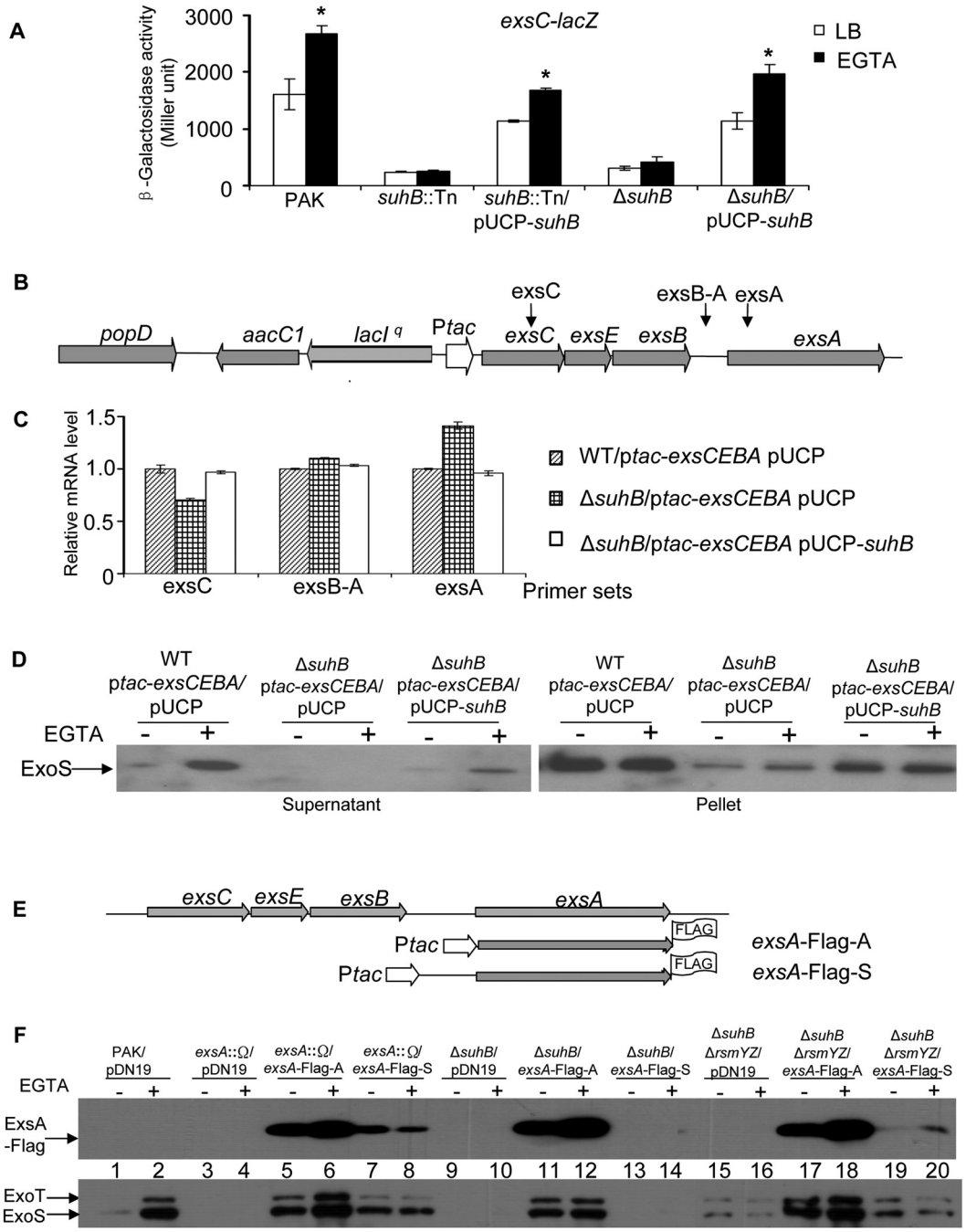

FIG 3 SuhB regulates the expression of *exsA* on the posttranscriptional level. (A) The expression of *exsC-lacZ* in indicated strains. *, $P < 0.05$ compared to WT or complemented strains by Student's *t* test. (B) The construct of the P*tac*-driven *exsCEBA* operon. (C) Relative mRNA levels of *exsC*, *exsA*, and the region between *exsB* and *exsA* under T3SS-inducing conditions. Bacteria were grown to an OD_{600} of 1 in LB containing 1 mM IPTG and 5 mM EGTA before RNA purification. The mRNA levels were tested by real-time PCR. The locations of real-time PCR primers were indicated by arrows in panel B. (D) Expression and secretion of ExoS in indicated strains. (E) Constructs of *exsA*-FLAGs. (F) Expression of ExsA-FLAG and ExoS in indicated strains.

As shown by the *exsA*-FLAG-A construct results, the ExsA-FLAG protein levels were similar in the *exsA*::Ω and *suhB* mutants (Fig. 3F, lanes 5, 6, 11, and 12), demonstrating that the P*tac* activity is not affected by the *suhB* mutation. Accordingly, expression of the ExoS protein was restored in both *exsA*::Ω 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*::Ω 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*

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				
PA0044	*exoT*	Exoenzyme T	0.083214	0.000311
PA1690	*pscU*	Translocation protein in type III secretion	0.193606	0.024589
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	0.008856
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	0.000649
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	*spcS*	Chaperone	0.231546	0.027402
Flagellum biogenesis genes				
PA1077	*flgB*	Flagellar basal body rod protein FlgB	0.218089	0.007676
PA1080	*flgE*	Flagellar hook protein FlgE	0.219233	0.004098
PA1082	*flgG*	Flagellar basal body rod protein FlgG	0.2187	0.009148
PA1088		Hypothetical protein	0.180182	0.005874
PA1092	*fliC*	Flagellin type B	0.217321	0.007287
PA1104	*fliI*	Flagellum-specific ATP synthase	0.383827	0.009766
Type VI secretion genes				
PA0070	*tagQ1*	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	7.66824	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	0.149172	0.002497
PA0638		Bacteriophage protein	0.154734	0.001363
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

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 β-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,

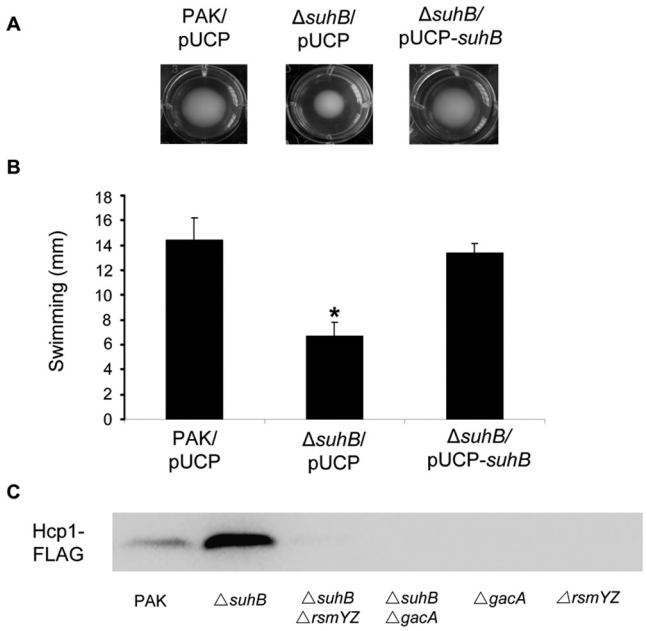

FIG 4 SuhB regulates swimming motility and Hcp1 expression. (A) The swimming ability of the indicated strains was tested in soft agar. (B) Sizes of swimming zones were measured from three experiments. *, $P < 0.05$ compared to WT or complemented strains by Student's *t* test. (C) The indicated strains carrying an *hcp1*-FLAG on their chromosomes were grown for 7 h. Samples from equivalent bacterial cell numbers were loaded onto an SDS-PAGE gel and probed with an anti-FLAG antibody.

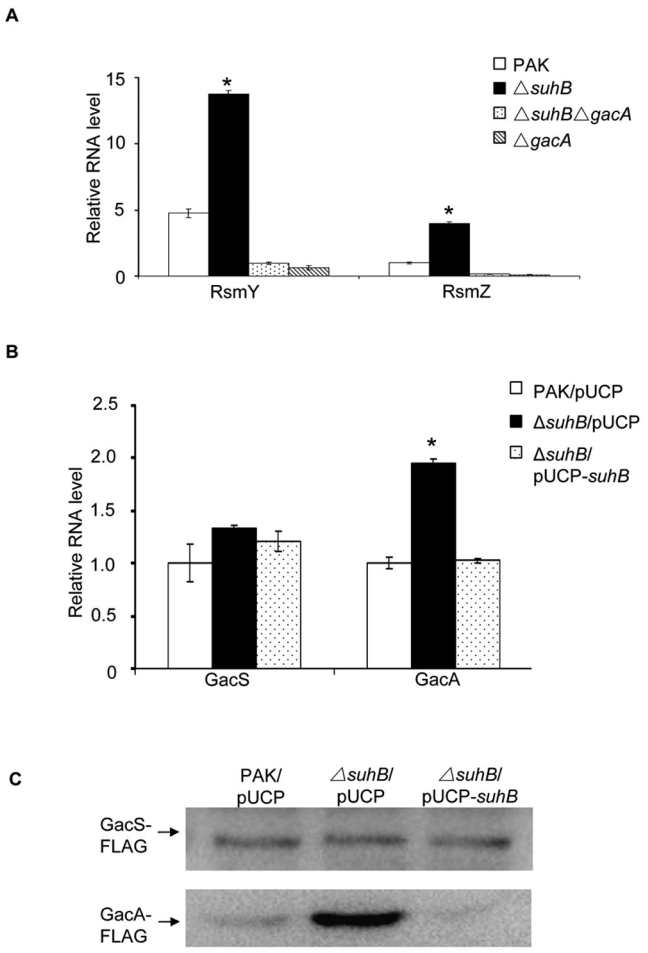

FIG 5 GacA and RsmY/RsmZ are involved in the SuhB-mediated regulation. (A) Relative RNA levels of RsmY and RsmZ in indicated strains. Bacteria were grown to an OD_{600} of 1, and RNA samples were collected and analyzed with real-time PCR. (B) mRNA levels of *gacS* and gacA in indicated strains. *, $P < 0.05$ compared to WT or complemented strains by Student's *t* test. (C) Protein levels of GacS and GacA FLAG fusions. Indicated strains carrying a *gacS*-FLAG or gacA-FLAG on their chromosomes were grown to an OD_{600} of 1. Samples from equivalent bacterial cell numbers were loaded onto SDS-PAGE gels and probed with an anti-FLAG antibody.

32). 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 (Fig. 5A). To understand the SuhB-mediated RsmY/Z regulation, we further tested whether SuhB interacts with regulatory genes upstream of RsmY/Z, including *retS*, *ladS*, *gacS*, and *gacA*. Mutation of *suhB* did not alter the mRNA level of *retS* and vice versa (see 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.

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. 5B; see also Fig. S3 in the supplemental material). To confirm the expression levels of GacS and GacA, we constructed GacS and GacA FLAG fusions driven by their own promoters in the vector pUC18T-mini-Tn7T-Gm and integrated them into the chromosomes of wild-type PAK and the *suhB* mutant. Consistent with the mRNA levels, the protein level of GacA but not GacS was increased in the *suhB* mutant (Fig. 5C). Complementation of *suhB* reduced the mRNA and protein levels of GacA to the wild-type level (Fig. 5B and C). Furthermore, mutation of *gacA* in the *suhB* mutant reduced the levels of RsmY/Z (Fig. 5A). These results suggest that SuhB regulates the expression of RsmY/Z through GacA.

Consistent with the conclusion presented above, mutation of *gacA* 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-

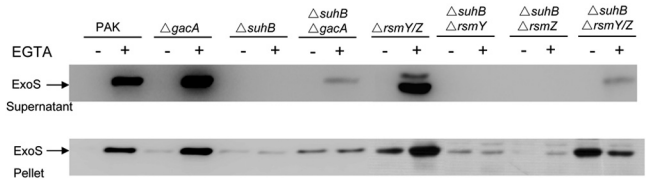

FIG 6 Roles of GacA and RsmY/Z in the SuhB-mediated T3SS gene expression. Indicated strains were grown to an OD_{600} of 1 to 2 in LB with or without EGTA. Intracellular ExoS and secreted ExoS were separated by centrifugation. Samples from equivalent bacterial cell numbers were loaded onto SDS-PAGE gels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

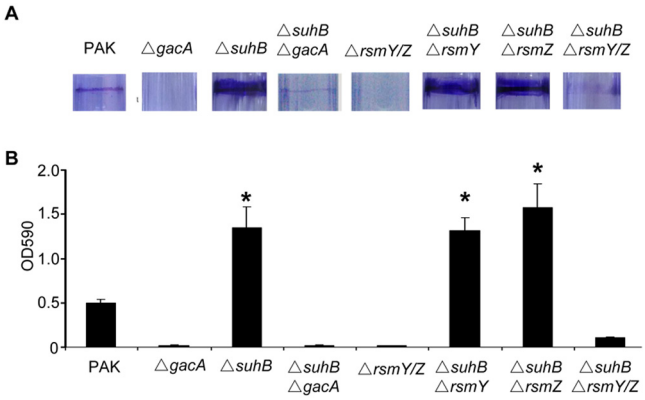

FIG 7 SuhB regulates biofilm formation through GacA and RsmY/Z. Biofilm formation by indicated strains was displayed with crystal violet staining (A) and quantified with optical density measurement (B). *, $P < 0.05$ compared to WT PAK by Student's *t* test.

pression 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*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*suhB* Δ*gacA* and Δ*suhB* Δ*rsmY* Δ*rsmZ* mutant strains. Deletion of *gacA* or *rsmY*/*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-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 *gacA* or *rsmY*/*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 Tn*5* insertion (41). In a previous Tn*5* mutagenesis study (42), it was found that the lowest insertion density of Tn*5* 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 Tn*5*. 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 *rne*), polynucleotide phosphorylase (gene *pnp*), and a probable ATP-dependent RNA helicase (gene *deaD*), 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 *exsA* is the last gene in the *exsCEBA* operon and that the expression of this operon is activated by ExsA itself (44). Between *exsB* and *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 *exsA*-FLAG tag fusions in which the transcription of *exsA* was driven by an exogenous *tac* promoter and thus should be constitutive in the absence of *lacI*. In the *exsA* mutant background, both constructs were able to complement the T3SS expression defect. In the *suhB* mutant, however, the translation of a P*tac*-driven *exsA* was defective in the presence of the *exsA* upstream region (225 bp in our construct), and when that fragment was excluded, the translation of P*tac*-driven *exsA* increased dramatically. These results suggest that the *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 *E. coli* suggest a role in the posttranscriptional regulation. The cold-sensitive phenotype of an *E. coli suhB* mutant was suppressed by an additional mutation in the endoribonuclease RNase III (45), which is involved in RNA processing

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 *rsmY/Z* diminished the expression of Hcp1 and biofilm formation and partially restored the T3SS activity in the *suhB* mutant. These results suggest that GacA and RsmY/Z 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 ExoS was lower than that in the wild-type strain under T3SS-inducing conditions. Second, deletion of *gacA* or *rsmY/Z* 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 (Tn*5*) 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 ExoS 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 (7.5×10^4) 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% CO_2 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 resuspended 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 at 600 nm (OD_{600}) of 1. The bacterial cells were spun down and resuspended in PBS at a concentration of 2.5×10^{10} CFU/ml. Bacterial cells (10 μl) were placed in each nostril of the anesthetized mouse, resulting in inhalation of approximately 5×10^8 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 Trizol extraction method (Trizol; Invitrogen) according to the manufacturer's protocol. The mRNA was isolated from total bacterial RNA by using MICROBExpress Oligo MagBeads (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 $OD_{600} = 0.025$ 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 an RNeasy 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

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00419-13/-/DCSupplemental.

Figure S1, TIF file, 1.8 MB.
Figure S2, TIF file, 4.1 MB.
Figure S3, TIF file, 1.5 MB.
Figure S4, TIF file, 5.1 MB.
Table S1, DOC file, 0.1 MB.
Table S2, XLS file, 0.1 MB.
Table S3, RTF file, 0.1 MB.
Table S4, DOC file, 0.1 MB.

ACKNOWLEDGMENTS

This work was supported in part by National Science Foundation of China (31170128), National Basic Research Program of China (973 Program, 2012CB518700), Ph.D. Programs Foundation of Ministry of Education of China (20120031120019), and a startup fund from Nankai University. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

W.W., S.J., K.L., and C.X. conceived and designed the experiments. K.L., C.X., Y.J., Z.S., C.L., J.S., G.C., R.C., and W.W. performed the experiments. K.L., C.X., Y.J., C.L., S.J., and W.W. analyzed the data. K.L., C.X., Y.J., Z.S., and C.L. contributed reagents/materials/analysis tools. K.L., C.X., S.J., and W.W. wrote the paper.

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