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DsbA of *Pseudomonas aeruginosa* Is Essential for Multiple Virulence Factors

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DsbA is a periplasmic thiol:disulfide oxidoreductase which contributes to the process of protein folding by catalyzing the formation of disulfide bonds. In this study, we demonstrate that the *dsbA* gene is required for the expression of the type III secretion system under low-calcium inducing conditions, intracellular survival of *P. aeruginosa* upon infection of HeLa cells, and twitching motility. The diverse phenotypes of the *dsbA* mutant are likely due to its defect in the folding of proteins that are involved in various biological processes, such as signal sensing, protein secretion, and defense against host clearing. In light of its effect on various virulence factors, DsbA could be an important target for the control of *P. aeruginosa* infections.

As an opportunistic human pathogen, *Pseudomonas aeruginosa* causes infections ranging from minor skin diseases to life-threatening complications in severe-burn patients and patients with leukemia, AIDS, cystic fibrosis, and cancer (2, 4, 29, 36). *P. aeruginosa* is able to grow in diverse environments by utilizing a wide variety of carbon and nitrogen sources (26). This adaptability and its intrinsic resistance to many common antibiotics as well as the ability to form biofilms make *P. aeruginosa* difficult to eradicate from the hospital environment (1, 7, 28). Moreover, *P. aeruginosa* has many virulence factors, such as proteases, cytotoxins, phospholipases, neuraminidase, capsular polysaccharides, and lipopolysaccharides, contributing to its ability to colonize, penetrate, and survive the host immune defense (9, 26, 36). The *P. aeruginosa* clinical isolate PA103 has been categorized as a noninvasive (cytolytic) strain based on its interaction with nonphagocytic corneal epithelial cells (12). This noninvasive strain carries *exoT* and *exoU*, whose products are translocated into the host cells via type III secretion machinery (11). Expression of these exoenzymes is coordinately regulated by a transcriptional activator, ExsA, in response to various environmental signals, including low calcium and direct contact with tissue culture cells (11, 19, 35).

DsbA is a periplasmic protein in gram-negative bacteria and functions as a soluble thiol:disulfide oxidoreductase. It contains a conserved motif, Cys-X-X-Cys, which is commonly found in other disulfide oxidoreductases (10). In a catalytic cascade pathway, the activity of DsbA is maintained by the function of DsbB (14). DsbA is required for catalyzing the oxidative folding and assembly of many secreted proteins, such as cholera toxin, *Escherichia coli* heat-labile toxin, pertussis toxin, elastase, alkaline phosphatase, and lipase (27, 31, 34, 38). Besides having a role in facilitating protein folding, DsbA is essential in balancing periplasmic redox potential, and a *dsbA* mutant is sensitive to reducing reagents such as dithiothreitol (25).

In this study, we demonstrate that *dsbA* is required for the expression of the type III secretion system as well as the intracellular survival of *P. aeruginosa* during infection of HeLa cells. Furthermore, we show that DsbA affects the expression of *pilA*, which could partially explain the twitching motility defect in the *dsbA* mutant. Overall, DsbA affects multiple virulence factors and thus may be important in the pathogenesis of *P. aeruginosa*.

Secretion of type III effector molecules is defective in a *dsbA* mutant. In a screening of a transposon insertion library for *P. aeruginosa* mutants that are noncytotoxic to HeLa cells, two types of mutants were identified, a type III-defective mutant and a *dsbA* mutant, suggesting that the type III secretion system could be defective in the *dsbA* mutant background. To test the effect of the *dsbA* gene on the secretion of type III effector molecules in *P. aeruginosa*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to examine the secreted proteins under a type III inducing condition. Because the type III secretion system of *P. aeruginosa* is known to respond to a low-calcium signal (37), treatment with EGTA, a strong chelator for divalent cations, was used to activate the type III secretion system. To generate a defined *dsbA* mutant (Table 1), the *dsbA* gene of PA103 was amplified by PCR by using the oligonucleotides 5-dsbA (5′-CGC CTA CTT CGC CAG CCA GAA GAT GAG CGT-3′) and dsbA-3 (5′-GCA GGG GCG AGT TTT CCA GAA GAT CGA CGG-3′). An amplified 1.8-kb DNA fragment was cloned into a PCR cloning vector, pCR2.1-TOPO (Invitrogen), resulting in pHW0206. A 1.6-kb blunt-ended gentamicin resistance cassette was then inserted into the unique *Mlu*I site, located 5′ of *dsbA*, in pHW0206, and the resulting construct was named pHW0212. A 3.4-kb *Hin*dIII-*Xba*I fragment from pHW0212 containing the *dsbA* gene disrupted by the insertion of a gentamicin resistance cassette was cloned into *Hin*dIII-*Xba*I-digested pEX18Tc, yielding pHW0216. pHW0216 was transformed into the wild-type PA103 background to generate a PA103 *dsbA* mutant through homologous recombination into its chromosome by double crossover (18). The genotype of the resulting mutant was confirmed by Southern hybridization (data not shown). Then, pHW0210 containing the intact *dsbA* gene was further transformed into PA103 *dsbA* for a complementation test. Bacterial strains were cultured in L broth containing ap-

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TABLE 1. Strains and plasmids used in this study

Strain, phage, or plasmid	Description[a]	Reference or source
P. aeruginosa		
PA103	Clinical isolate of wild-type cytotoxic strain	22
PA103 *exsA*	PA103 with chromosomal disruption of the *exsA* locus; Sp^r Sm^r	This study
PA103 Δ*exoU exoT*	PA103 with *exoU* deletion and *exoT* disruption; Tc^r	35
PA103 *dsbA*	PA103 with chromosomal disruption of the *dsbA* locus; Sp^r Sm^r	This study
PA103 *exsA dsbA*	PA103 with chromosomal disruption of both *exsA* and *dsbA* loci; Sp^r Sm^r Gm^r	This study
PO4 phage	Pillus-specific lytic phage	David Bradley
Plasmids		
pCR2.1-TOPO	PCR cloning vector, Ap^r Km^r	Invitrogen
pDN19*lac*Ω	Broad-host-range plasmid containing a promoterless *lacZ* gene; Sp^r Sm^r Tc^r	33
pEX18Tc	Suicide vector for recombination by site-specific excision; Suc^s Tc^r	18
pHW0018	*exoT* promoter of PA103 fused to promoterless *lacZ* in pDN19*lac*Ω; Sp^r Sm^r Tc^r	15
pHW0203	*exsA* promoter of PA103 fused to promoterless *lacZ* in pDN19*lac*Ω; Sp^r Sm^r Tc^r	This study
pHW0206	PCR-amplified *dsbA* gene from PA103 in pCR2.1-TOPO; Ap^r Km^r	This study
pHW0210	*dsbA* gene of PA103 cloned in pUCP19; Ap^r	This study
pHW0212	Disruption of *dsbA* gene by Gm^r cassette in pHW0206; Ap^r Km^r Gm^r	This study
pHW0216	*dsbA* gene with Gm^r cassette insertion cloned in pEX18Tc; Tc^r	This study
pMSZ5	PAK *pilA* promoter fused to a promoterless *lacZ* in pDN19*lac*Ω; Sp^r Sm^r Tc^r	20
pUCP19	Broad-host-range shuttle vector, Ap^r	30

[a] Resistance markers: Ap^r, ampicillin; Km^r, kanamycin; Gm^r, gentamicin; Sp^r, spectinomycin; Sm^r, streptomycin; Tc^r, tetracycline. Suc^s, sucrose-sensitive marker encoded by *sacB*.

propriate antibiotics at 37°C overnight. The cultured bacteria were reinoculated into L broth containing 5 mM EGTA to an optical density at 600 nm of 0.1 and then vigorously shaken at 37°C for 12 h. Supernatant of bacterial culture was collected and precipitated with 15% trichloroacetic acid at 4°C for 12 h. The precipitated pellet of the protein sample was completely suspended by sonication in 1× protein sample buffer and subjected to SDS–12% PAGE. As shown in Fig. 1, PA103 *dsbA* did not secrete any detectable amount of ExoU and ExoT proteins, unlike wild-type PA103. However, complementation with *dsbA* fully restored the ability of PA103 *dsbA* to secrete the two effector molecules, to the level of wild-type PA103. These results indicate that the DsbA of *P. aeruginosa* is required for the secretion of type III effector molecules.

DsbA is required for the expression of the type III secretion system. The defect in the secretion of type III effector molecules by the *dsbA* mutant could be due to either low expression of the type III effector molecules or defects in the type III secretion apparatus. To determine whether DsbA is essential for the expression of the type III secretion system, the promoter activity of *exoT*, one of the major type III effector molecules expressed by PA103, was monitored. Both the pDN19*lac*Ω vector and pHW0018, an *exoT*::*lacZ* fusion construct, were first introduced into wild-type PA103 and PA103 *dsbA*. Then, pUCP19 vector or pHW0210, containing the intact *dsbA* gene, was further transformed into the resulting strains for the purpose of complementation. The resulting transformants were cultured in L broth containing appropriate antibiotics with or without 5 mM EGTA at 37°C overnight. As shown in Fig. 2A, the expression of *exoT* in PA103 *dsbA* with pUCP19 vector did not respond to EGTA treatment. However, complementation of PA103 *dsbA* with a *dsbA* gene fully restored the expression level of *exoT* in response to EGTA treatment, comparable to that of wild-type PA103. Since the expression of *exoT* is regulated by the transcriptional activator ExsA, the expression of *exsA* promoter was also monitored. Similar to earlier experiments, both pDN19*lac*Ω vector and pHW0203 containing an *exsA*::*lacZ* fusion were transformed into the wild-type PA103 and PA103 *dsbA*. pUCP19 vector or pHW0210 was further transformed into the resulting transfor-

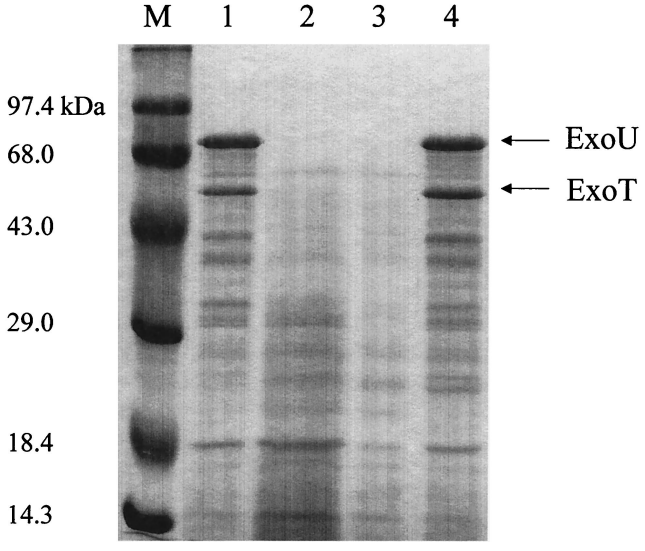

FIG. 1. The *dsbA* mutant is defective in the secretion of type III effector molecules. Bacterial strains were cultured in L broth containing appropriate antibiotics at 37°C overnight. The cultured bacterial cells were reinoculated into fresh L broth containing 5 mM EGTA and vigorously shaken at 37°C for 12 h. The bacterial culture supernatant was subjected to trichloroacetic acid precipitation. Standard SDS-PAGE was used to observe the secreted protein profiles. M, high-molecular-weight protein marker; lane 1, wild-type PA103; lane 2, PA103 Δ*exoU exoT*; lane 3, PA103 *dsbA*; lane 4, PA103 *dsbA* complemented by pHW0210 harboring an intact *dsbA* gene.

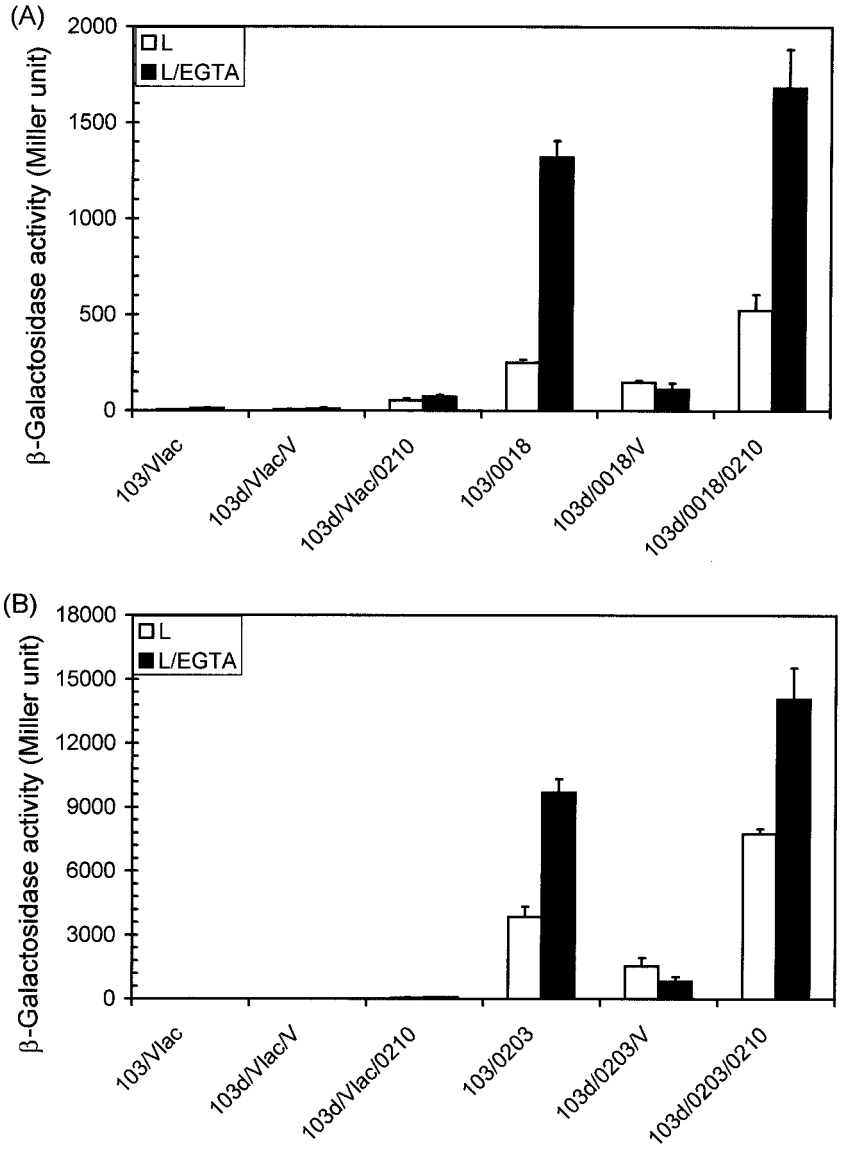

FIG. 2. The *dsbA* mutant is defective in the expression of both the type III effector molecule ExoT (A) and the type III transcriptional activator ExsA (B). Bacterial strains were cultured in L broth with (L/EGTA) or without (L) 5 mM EGTA at 37°C overnight. The cultured bacterial cells were subjected to a standard β-galactosidase assay. 103, wild-type strain PA103; 103d, *dsbA* mutant of PA103; Vlac, pDN19*lac*Ω vector; V, pUCP19 vector; 0210, pHW0210 harboring an intact *dsbA* gene in pUCP19 vector; 0018, pHW0018 harboring *lacZ* fusion to the *exoT* promoter in pDN19*lac*Ω; 0203, pHW0203 harboring *lacZ* fusion to the *exsA* promoter in pDN19*lac*Ω. Average values from four separate tests are shown.

mants. As shown in Fig. 2B, there was no expression of *exsA* in the *dsbA* mutant background under a type III inducing condition. However, complementation of PA103 *dsbA* with the *dsbA* gene fully restored the expression of *exsA* in response to EGTA treatment. These results indicate that the function of DsbA is required for type III gene expression in response to the type III inducing signal; thus, the signal sensor molecule might need a disulfide bond to be functional.

DsbA is required for intracellular survival during infection of HeLa cells. In several bacteria, including *P. aeruginosa* PAO1, mutations in the *dsbA* gene conferred sensitivity to strong reducing reagents, such as dithiothreitol (23, 25). Since the host intracellular compartment is known to be a reduced environment due to the high ratio of reduced to oxidized glutathione, estimated to be between 30:1 and 100:1 (13), the *dsbA* mutant is likely less able to survive within the host cells. To test this, a *dsbA* mutant was generated in a PA103 *exsA* background; PA103 *exsA* is a type III secretion null mutant which has no cytotoxic activity and is capable of maintaining viability within HeLa cells. The viability of PA103 *exsA dsbA* was compared to that of its parental strain, PA103 *exsA*, during HeLa cell infection. Bacterial strains were cultured in L broth containing appropriate antibiotics at 37°C overnight. HeLa S3 epithelial cells (3.0×10^5) in 3 ml of Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS) were seeded into six-well plates and incubated at 37°C in

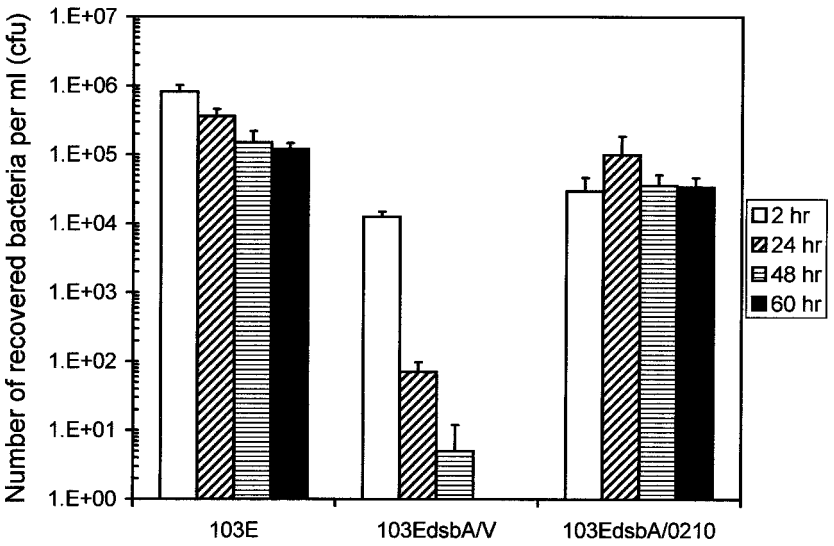

FIG. 3. Effect of DsbA on the intracellular survival of *P. aeruginosa* during infection of HeLa cells. Stationary-phase bacteria were used to infect HeLa S3 cells and incubated at 37°C in 5% CO_2 for 2 h. After three washes with warmed PBS, the HeLa cells were incubated at 37°C in 5% CO_2 for 2, 24, 48, and 60 h in the presence of 400 μg of amikacin per ml to kill extracellular bacteria. The number of intracellular bacteria was calculated by colony counting. Average values for three repeated tests are shown. 103E, PA103 *exsA*; 103EdsbA, PA103 *exsA dsbA*; V, pUCP19 vector; 0210, pHW0210 harboring an intact *dsbA* gene in the pUCP19 vector.

5% CO_2 for 24 h. After two washes with warmed phosphate-buffered saline (PBS), 1 ml of DMEM containing 5% FBS was added to the HeLa cells, followed by the addition of 0.1 ml of bacterial suspension in DMEM containing 5% FBS, giving a multiplicity of infection of 100. The infected HeLa cells were incubated at 37°C in 5% CO_2 for 2 h, followed by three washes with warmed PBS and the addition of 1.5 ml of DMEM containing 5% FBS and 400 μg of amikacin per ml to kill extracellular bacteria. At each incubation time, the HeLa cells on the plate were lysed with 0.25% Triton X-100 and plated by serial dilution on L-agar plates containing appropriate antibiotics to count the number of internalized bacteria. As shown in Fig. 3, the viability of PA103 *exsA dsbA* complemented with vector control (103EdsbA/V) was dramatically reduced, over 100-fold, after 24 h of infection, and no bacteria were recovered after 60 h postinfection. In contrast, complementation of PA103 *exsA dsbA* with the *dsbA* gene (103EdsbA/0210) fully restored its viability compared to the parental strain, PA103 *exsA* (103E) (Fig. 3). The *dsbA* mutant, however, was not defective in growth in either rich or minimal medium, compared to its parental strain PA103 *exsA* (data not shown); thus, the defect of the *dsbA* mutant in intracellular survival is not due to a defect in its growth rate.

A *dsbA* mutant strain of PA103 is completely defective in twitching motility. During the intracellular survival assay described above, it was observed that binding of the *dsbA* mutant to HeLa cells decreased about 100-fold compared to that of the parental strain (data not shown), indicating that the *dsbA* mutant is also defective in host cell attachment. Since pili are known to be the major adhesin of *P. aeruginosa*, the effect of *dsbA* on pili was further examined. To test the pilus function, bacterial twitching motility was tested with PA103 *exsA dsbA*, which was used in the intracellular survival assay. As shown in Fig. 4A, PA103 *exsA dsbA* completely lost the ability to form a twitching zone, compared to wild-type PA103 and the parental strain, PA103 *exsA* (103E), which formed twitching zones with average diameters of 4.1 and 4.25 mm, respectively. Complementation of PA103 *exsA dsbA* with a *dsbA* gene fully restored the twitching motility, with an average diameter of 4.25 mm, whereas the mutant complemented with vector did not form any twitching zone (Fig. 4A). To understand the defect in twitching motility, we examined the expression level of *pilA* in the *dsbA* mutant background. As shown in Fig. 4B, *pilA* expression in the *dsbA* mutant background was half the level seen in wild-type PA103. *pilA* expression was restored by the introduction of *dsbA*. Indeed, despite its nontwitching phenotype, the mutant was sensitive to the pilus-specific lytic phage PO4 (data not shown), indicating that the *dsbA* mutant still has pili that act as phage receptors. Therefore, it may be that the *dsbA* mutant has a reduced number of pili on the cell surface but is unable to retract them, thus conferring nontwitching motility.

Conclusions. Overall, this study describes the essential role of DsbA for several important virulence factors that affect *P. aeruginosa* pathogenesis. The molecular mechanisms by which DsbA affects type III secretion systems, intracellular survival, and twitching motility are not clear yet. It is reasonable to assume that the direct requirement of DsbA could be due to its catalytic activity of forming a disulfide bond for the functional folding of various proteins required for its virulence. However, it was previously reported that a *dsbA* mutant of *Yersinia pestis* also secretes a dramatically reduced amount of Yop proteins by the type III secretion system due to reduced amounts of full-sized YscC protein, which is required for the formation of a ring-shaped structure in a type III secretion apparatus (21). Like *Yersinia*, the DsbA protein of *P. aeruginosa* might also be required for the formation of the type III secretion apparatus, in addition to its effect on the expression of type III genes. The defect of the type III secretion system in the *dsbA* mutant was also supported by a recent study in which several genetic loci, including a *dsbA* gene of *P. aeruginosa*, were identified by

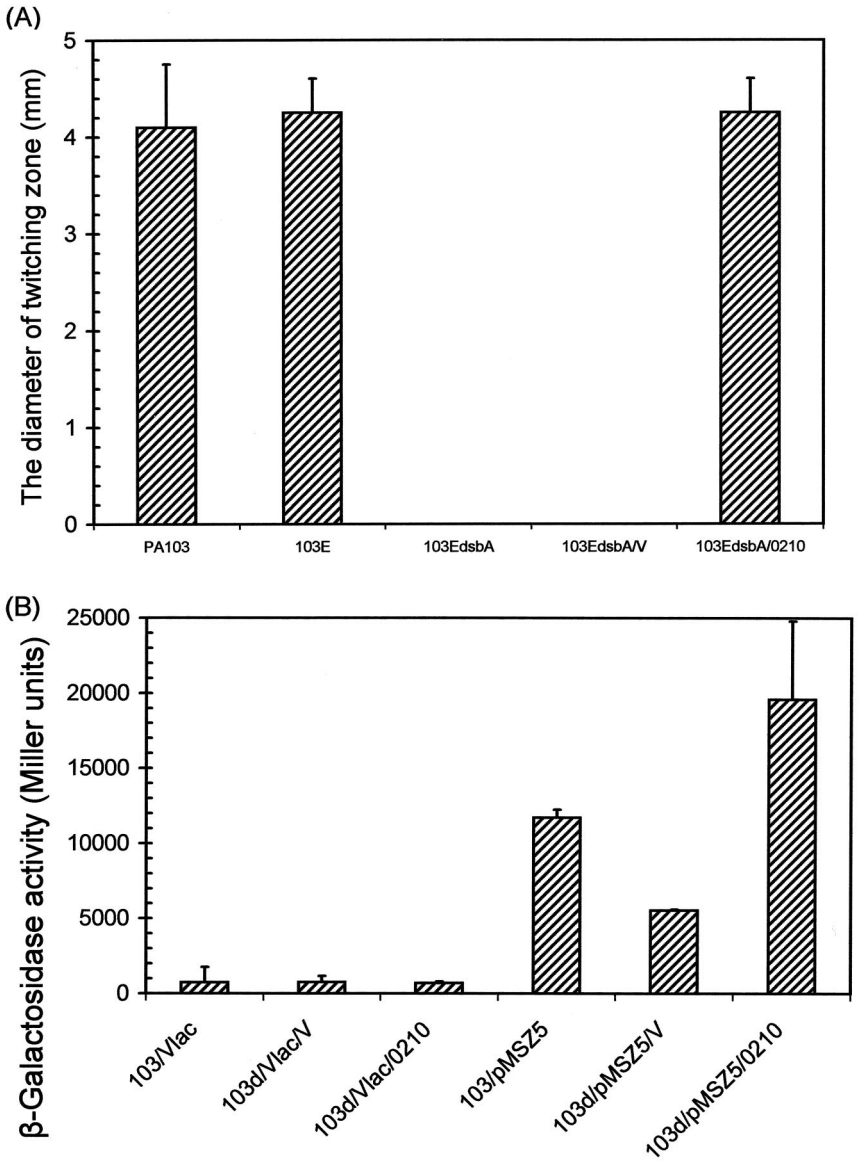

FIG. 4. The *dsbA* mutant is completely defective in twitching motility and has decreased expression of *pilA*. (A) Freshly grown bacterial strains on L-agar plates containing appropriate antibiotics were transferred with a sharp needle to the bottoms of thin-layer L-agar plates without antibiotics. The plates were incubated at 37°C for 12 h and then at 25°C for 48 h. (B) Bacterial strains were cultured in L broth containing appropriate antibiotics at 37°C overnight. The cultured bacterial cells were subjected to a standard β-galactosidase assay. 103, wild-type strain PA103; 103d, PA103 *dsbA*; 103E, PA103 *exsA*; 103EdsbA, PA103 *exsA dsbA*; Vlac, pDN19*lac*Ω vector; V, pUCP19 vector; 0210, pHW0210 harboring an intact *dsbA* gene in the pUCP19 vector; pMSZ5, *lacZ* fusion to the *pilA* promoter in pDN19*lac*Ω.

screening of a transposon insertion library for type III secretion system-deficient noncytotoxic mutants (8). Recently, three proteins in the plant pathogen *Ralstonia solanacearum* were reported to be required for transmitting a type III inducing signal to initiate the transcription of type III secretion system across the bacterial membrane (6). *P. aeruginosa* could also encode similar signal sensing and transducing factors, and the DsbA protein might be required for the formation of functional signal sensor molecules, presumably through catalyzing disulfide bond formation. In *Shigella flexneri*, the *dsbA* gene was also reported to be required for intracellular survival (39), although the exact mechanism is not known. Besides possibly having sensitivity to the reduced environments found in host intracellular compartments (13), the *dsbA* mutant was reported to be defective in the synthesis of *c*-type cytochromes during anaerobic growth (24). This implies that DsbA could be critically involved in the assembly of electron transfer chains induced under anaerobic conditions. Therefore, DsbA could have a vital role in bacterial survival by enabling proper folding of membrane or secreted proteins involved in adapting to stressful conditions or physiological changes. The decrease in the expression of *pilA* could partially explain the defect of *dsbA* mutants in twitching motility. It is also possible that the loss of the disulfide loop at the C terminus of the type IV pilin subunit, important for adhesion to epithelial cells (16), contributes to the reduced twitching motility, since twitching motility re-

quires adhesion and traction on solid surfaces (23). Although the *dsbA* mutation in the PA103 background caused a complete loss of twitching motility, mutation of *dsbA* in a PAO1 background was reported to result in about 60% reduction in the twitching zone compared to that of wild-type PAO1 (23), indicating that the *dsbA* effect on the twitching motility in *P. aeruginosa* seems to be a strain-dependent phenomenon. The defect in twitching motility was also supported by previous reports presenting the requirement of DsbA in the biogenesis of type 4 bundle-forming pili in enteropathogenic *E. coli* and type 4 pili in *Burkholderia cepacia* (3, 17). DsbA of *P. aeruginosa* has been shown to be essential for the production of important virulence factors such as elastase, alkaline phosphatase, and lipase (5, 34), and infection of *Caenorhabditis elegans* with the *dsbA* mutant causes slower killing (32). These previously observed effects of DsbA combined with the roles of DsbA in intracellular survival, the expression of a type III secretion system, and twitching motility shown in this study suggest that DsbA could be an important target for the control of *P. aeruginosa* infections.

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