Dissecting the Machinery That Introduces Disulfide Bonds in Pseudomonas aeruginosa

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ABSTRACT Disulfide bond formation is required for the folding of many bacterial virulence factors. However, whereas the Escherichia coli disulfide bond-forming system is well characterized, not much is known on the pathways that oxidatively fold proteins in pathogenic bacteria. Here, we report the detailed unraveling of the pathway that introduces disulfide bonds in the periplasm of the human pathogen Pseudomonas aeruginosa. The genome of P. aeruginosa uniquely encodes two DsbA proteins (P. aeruginosa DsbA1 [PadsbA1] and PadsbA2) and two DsbB proteins (PadsbB1 and PadsbB2). We found that PadsbA1, the primary donor of disulfide bonds to secreted proteins, is maintained oxidized in vivo by both PadsbB1 and PadsbB2. In vitro reconstitution of the pathway confirms that both PadsbB1 and PadsbB2 shuttle electrons from PadsbA1 to membrane-bound quinones. Accordingly, deletion of both P. aeruginosa dsbB1 (PadsbB1) and PadsbB2 is required to prevent the folding of several P. aeruginosa virulence factors and to lead to a significant decrease in pathogenicity. Using a high-throughput proteomic approach, we also analyzed the impact of PadsbA1 deletion on the global periplasmic proteome of P. aeruginosa, which allowed us to identify more than 20 new potential substrates of this major oxidoreductase. Finally, we report the biochemical and structural characterization of PadsbA2, a highly oxidizing oxidoreductase, which seems to be expressed under specific conditions. By fully dissecting the machinery that introduces disulfide bonds in P. aeruginosa, our work opens the way to the design of novel antibacterial molecules able to disarm this pathogen by preventing the proper assembly of its arsenal of virulence factors.

IMPORTANT The human pathogen Pseudomonas aeruginosa causes life-threatening infections in immunodepressed and cystic fibrosis patients. The emergence of P. aeruginosa strains resistant to all of the available antibacterial agents calls for the urgent development of new antibiotics active against this bacterium. The pathogenic power of P. aeruginosa is mediated by an arsenal of extracellular virulence factors, most of which are stabilized by disulfide bonds. Thus, targeting the machinery that introduces disulfide bonds appears to be a promising strategy to combat P. aeruginosa. Here, we unraveled the oxidative protein folding system of P. aeruginosa in full detail. The system uniquely consists of two membrane proteins that generate disulfide bonds de novo to deliver them to P. aeruginosa DsbA1 (PadsbA1), a soluble oxidoreductase. PadsbA1 in turn donates disulfide bonds to secreted proteins, including virulence factors. Disruption of the disulfide bond formation machinery dramatically decreases P. aeruginosa virulence, confirming that disulfide formation systems are valid targets for the design of antimicrobial drugs.

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none and then to other terminal electron acceptors such as fumarate and nitrate (6–8). More than 30 EcDsbA substrates have been identified so far using various proteomic techniques, but the number of proteins predicted to depend on EcDsbA for folding is much higher (9–12).

EcDsbA preferentially introduces disulfide bonds in a vectorial manner into proteins entering the periplasm (13), i.e., between cysteine residues that are consecutive in the amino acid sequence. Therefore, EcDsbA often incorrectly oxidizes proteins whose folding involves the formation of disulfide bonds between nonconsecutive cysteines. In E. coli, these nonnative disulfide bonds are corrected by an isomerization system, which involves EcDsbC and EcDsbD (14–18).

The E. coli disulfide bond formation pathway is often considered the paradigm of oxidative protein folding machinery in bacteria. However, genome analyses have revealed that the E. coli system cannot serve as a model for all bacteria (19, 20). For instance, many bacterial genomes encode a repertoire of thio-disulfide oxidoreductases that is significantly expanded than the E. coli DsbA-DsbB system (19). As an illustrative example, the pathogenic bacterium Neisseria meningitidis has three DsbA proteins, which differ in terms of localization, redox properties, and surface characteristics (21–23). Furthermore, in certain bacteria, such as Mycobacterium tuberculosis, DsbA is not reoxidized by DsbB, but by a membrane protein homologous to the eukaryotic vitamin K epoxide reductase (VKOR) (20).

In this work, we characterize in detail the machinery that forms disulfide bonds in the periplasm of Pseudomonas aeruginosa, an opportunistic human pathogen that causes life-threatening infections in patients suffering from leukemia, AIDS, cancer, and cystic fibrosis (24). We show that the P. aeruginosa genome encodes multiple Dsb proteins, including two DsbA proteins (P. aeruginosa DsbA1 [PaDsbA1] and PaDsbA2) and two DsbB proteins (PaDsbB1 and PaDsbB2). PaDsbA1, which is the primary donor of disulfide bonds to proteins secreted to the envelope, is recycled by both PaDsbB1 and PaDsbB2. Accordingly, the simultaneous deletion of both P. aeruginosa dsbB (PadsbB) genes is required to impair the folding of several P. aeruginosa virulence factors, leading to a global decrease in pathogenicity. We also reconstituted the P. aeruginosa disulfide bond formation system in vitro and determined the kinetic parameters of the oxidation reaction of PaDsbA1 by both PaDsbB1 and PaDsbB2. Finally, we report the identification of 22 new potential substrates of PaDsbA1 and the biochemical and structural characterization of PaDsbA2, an oxidoreductase which seems to be expressed under specific conditions.

RESULTS

The P. aeruginosa genome encodes two DsbA proteins and two DsbB proteins. We initiated our study by searching the genome of P. aeruginosa strain PA14 for homologs of EcDsbA and EcDsbB using the psi-BLAST algorithm (25). We found two proteins homologous to EcDsbA (PaDsbA1 [PA14_72450] and PaDsbA2 [PA14_59960] that have 27% and 14% sequence identity with EcDsbA, respectively) and two proteins homologous to EcDsbB (PaDsbB1 [PA14_07000] and PaDsbB2 [PA14_69400] that have 29% and 27% sequence identity with EcDsbB, respectively). The structural and biochemical properties of PaDsbA1 have been reported elsewhere (24, 26). Moreover, PaDsbA1 has been shown to be involved in the oxidative folding of various virulence factors, including elastase (26, 27), and PaDsbA1 mutants have been described as less virulent than wild-type strains in several models, including Drosophila melanogaster (28), Caenorhabditis elegans (29), Arabidopsis thaliana, and mice (29, 30). However, nothing is known about PaDsbA2, PaDsbB1, and PaDsbB2. Interestingly, we noticed that PaDsbA2 belongs to a different subclass of DsbA proteins than EcDsbA and PaDsbA1 (see Fig. S1A in the supplemental material). The proteins from this subclass, mostly present in alp-haproteobacteria, are characterized by the presence of an additional pair of conserved cysteine residues (Fig. S1B), as discussed further below. Thus, the machinery at work in P. aeruginosa differs from the E. coli system by the fact that multiple DsbA and DsbB proteins are encoded by the genome of this microorganism, raising the question of their respective role and contribution in oxidative protein folding.

PaDsbB1 and PaDsbB2 cooperate in the recycling of PaDsbA1. It was important to first confirm the physiological importance and the functional roles of the various Dsb proteins identified by the bioinformatic analysis. First, we decided to determine the in vivo redox state of both PaDsbA1 and PaDsbA2 to confirm their involvement in a pathway that introduces disulfide bonds into substrates. In general, proteins that function in an oxidizing pathway accumulate in the oxidized state in vivo, whereas proteins that function as a reductase or isomerase involved in the correction of nonnative disulfide bonds, such as EcDsbC, accumulate in the reduced state (31). The in vivo redox state of PaDsbA1 and PaDsbA2 was determined using 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), a 490-Da reagent that covalently reacts with free thiol groups, leading to a mobility shift of the modified protein in SDS-polyacrylamide gels. As shown in Fig. 1, we found that PaDsbA1 is predominantly oxidized in vivo (Fig. 1, compare lane 1 to lane 6 showing the migration of reduced PaDsbA1 modified with AMS), which is consistent with PaDsbA1 playing a role as a thiol oxidase. PaDsbA2 was not detected, despite repeated attempts in different culture media, which suggests that this protein is expressed only under specific conditions that remain to be determined. For that reason, we were not able to further investigate the in vivo function of PaDsbA2. However, the results from the biochemical and structural characterization of this protein are presented below.
We then sought to determine the respective roles of PaDsbB1 and PaDsbB2 in the reoxidation of PaDsbA1 by monitoring the redox state of this oxidoreductase in PadsbB1 and PadsbB2 mutants. As shown in Fig. 1, deletion of either PadsbB1 or PadsbB2 does not have a significant impact on the redox state of PaDsbA1 (Fig. 1, lanes 3 and 4). However, when both PaDsbB proteins (PaDsbBs) are absent, PaDsbA1 accumulates mostly in the reduced form (Fig. 1, lane 5). These results indicate that both PaDsbB1 and PaDsbB2 control the redox state of PaDsbA1 and that both proteins are able to compensate for each other. It is noteworthy that expression of either PaDsbB protein in an E. coli dsbB (EcdsbB) mutant complements the motility defect of this mutant, indicating that both PaDsbB1 and PaDsbB2 are able to reoxidize EcDsbA (see Fig. S2 in the supplemental material).

**In vitro reconstitution of the disulfide bond formation machinery of P. aeruginosa.** We decided to reconstitute the disulfide bond formation pathway of *P. aeruginosa* in vitro to determine the kinetic parameters of the reoxidation reaction of PaDsbA1 by PaDsbB1 and PaDsbB2. PaDsbB1 and PaDsbB2 were expressed in an EcdsbB mutant and partially purified. We then tested the ability of these enzymes to directly oxidize PaDsbA1 by using a fluorescence assay developed for DsbB activity (6, 32, 33). This assay is based on the 1.7-fold fluorescence decrease that accompanies PaDsbA1 oxidation (see Fig. S3 in the supplemental material). As shown in Fig. 2A, the fluorescence of reduced PaDsbA1 decreases slowly when the protein is incubated at 30°C, probably as a result of air oxidation. Addition of quinones that function as electron acceptors in the DsbB-catalyzed reaction (6, 34) has no impact. In contrast, addition of PaDsbB1 to the mixture leads to a rapid decrease in PaDsbA1 fluorescence (Fig. 2A). Similar results were obtained for PaDsbB2 (not shown), confirming that both PaDsbB1 and PaDsbB2 are able to reoxidize PaDsbA1. Importantly, we confirmed using AMS trapping experiments that the observed decrease in fluorescence is due to the oxidation of PaDsbA1 cysteines (not shown). We also measured the dependence of the initial velocities of fluorescence decrease on the concentration of PaDsbA1. As shown in Fig. 2, the obtained data could be fit to the Michaelis-Menten equation for both PaDsbB1 (Fig. 2B) and PaDsbB2 (Fig. 2C). We calculated a $K_m$ of 8.1 μM PaDsbA1 for PaDsbB1 and 9.0 μM for PaDsbB2. These values are similar to the value reported for the $K_m$ of EcDsbB for EcDsbA (33). Thus, PaDsbB1 and PaDsbB2 have similar affinities for PaDsbA1.

Both PaDsbB1 and PaDsbB2 play a role in the oxidation of *P. aeruginosa* virulence factors. From the data presented above, we can conclude that PaDsbB1 and PaDsbB2 control the redox state of PaDsbA1 both in *vitro* and *in vivo*. However, the respective importance of the two DsbB proteins for the pathogenicity of *P. aeruginosa* remains unknown, which prompted us to investigate their roles in the assembly of virulence factors.

We first tested the involvement of PaDsbB1 and PaDsbB2 in the assembly of type IV pili and flagellum, two structures involved in bacterial pathogenesis and whose formation requires PaDsbA1 (Fig. 3A) (26, 35). As shown in Fig. 3A, we found that the PadsbB1B2 double mutant is defective in both twitching and swimming motilities, which require functional type IV pili and flagellum, respectively, but that the single PadsbB mutants have a normal phenotype. The PadsbB1B2 mutant also exhibits a significant defect in the early steps of biofilm formation, which most likely results from defective assembly of the pilus and flagellum (see FIG 2). Both PaDsbB1 and PaDsbB2 recycle PaDsbA1 in *vitro*. (A) The reoxidation of PaDsbA1 by PaDsbB1 was monitored by measuring the decrease in fluorescence (excitation wavelength, 295 nm; emission wavelength, 330 nm) that accompanies PaDsbA1 oxidation. The reaction was performed in the presence of quinones (decylobiquinone). The various components were added sequentially to the reaction mixture as indicated. (B and C) We measured the initial velocities (v) of PaDsbA1 reoxidation by PaDsbB1 (B) and PaDsbB2 (C) to determine the kinetic parameters of the reaction. The plots show a fit of the data to the Michaelis-Menten equation. The experimental conditions are described in Materials and Methods.
exoproteins affected by the impairment of the disulfide formation machinery, we analyzed the culture supernatants prepared from the wild type (WT) and the various PadsB and PadsB1A1 described above could be complemented by PaDsbA1 (26, 27). It is important to note that the phenotypes structure (36) whose formation has been reported to depend on an extracellular protease with two disulfide bonds in its native exhibits a reduced elastase activity (Fig. S4B). The elastase LasB is PadsB1 prepared from the wild type, the double mutant, but not the single PadsB1B2 mutant. These four proteins have not yet been reported to oxidatively fold in the periplasm prior to secretion (27). In the Discussion, we explain why we think that the observed decrease in secretion levels results from an impairment of the folding process of these proteins and not from defective assembly of the type II secretion machinery.

Finally, we investigated the global roles of PaDsbB1 and PaDsbB2 in P. aeruginosa virulence by measuring the ability of the various PaDsbB mutant strains to kill C. elegans using a previously established assay (38). Remarkably, we found that only the PaDsbB1B2 mutant is significantly less virulent than the wild-type strain but that the single PaDsbB mutants are not less virulent (see Fig. S4D in the supplemental material). Altogether, these data lead to the conclusion that both PaDsbB1 and PaDsbB2 participate in the folding of several virulence factors of P. aeruginosa and that both PadsB genes need to be deleted to impact the global assembly of virulence factors, confirming that these genes have a redundant function.

New proteins that depend on the disulfide bond formation pathway for folding. A few P. aeruginosa virulence factors have been shown to depend on PaDsbA1 for folding, including the protease LasB (27) and the pilus component PilA (35). However, most PaDsbA1 substrates remain to be identified. Thus, in order to fully grasp the role of the disulfide bond-forming system involving PaDsbA1, PaDsbB1, and PaDsbB2 in the folding of periplasmic proteins in P. aeruginosa and to identify new virulence factors depending on the disulfide bond formation machinery for assembly, we used a differential proteomic approach based on label-free two-dimensional liquid chromatography coupled to tandem mass spectrometry (2D-LC-MS/MS) to compare the periplasmic proteome of PadsB1A1 and wild-type strains. The rationale behind this approach, which has proved to be useful in identifying the substrates of EcDsbA (12) and the E. coli periplasmic chaperone SurA (39, 40), is that proteins whose folding depends on the presence of a functional disulfide bond formation pathway will be less abundant in the PadsB1A1 strain than in the wild type due to misfolding and protease digestion.

We prepared periplasmic extracts containing cell envelope proteins using a protocol adapted from the method of Hiniker and Bardwell (11) and optimized for P. aeruginosa in order to limit contamination by cytoplasmic proteins. Periplasmic proteins were digested with trypsin, and the peptides were analyzed by 2D-LC-MS/MS. The experiments were repeated on three biological replicates for both the PadsB1A1 and wild-type strains. For quantification of protein abundance, we used the number of normalized spectral counts (SC) reported for each protein (see Ta-
We examined the relative abundance of identified proteins in wild-type and PaDsbA1 strains to determine whether the global impact of the PaDsbA1 deletion on the periplasm proteome was significant. Postulating that misfolding of PaDsbA1 substrates leads to degradation, we selected proteins whose abundance was decreased by at least twofold (Table 1). These proteins include periplasmic binding proteins (PA14_03930, PA14_71030, and PA14_07870), putative enzymes involved in peptidoglycan remodeling (PA14_24690 and PA14_25000), and potential extracellular virulence factors (PA14_26020 and PA14_39780). Importantly, the exclusion of PbpG (PA14_53020) and ChiC (PA14_34870), both of which are less abundant proteins containing an even number of cysteine residues, strongly suggests that they are PaDsbA1 substrates. Moreover, CbpD, IMPa, and PaAP, three proteins identified as PaDsbA1 substrates (35), were also found to be less abundant in the PaDsbA1 mutant than in a wild-type strain.

We then compared the relative abundance of identified proteins in wild-type and PaDsbA1 strains to determine the global impact of the PaDsbA1 deletion on the periplasm proteome. Postulating that misfolding of PaDsbA1 substrates leads to degradation, we selected proteins whose abundance was decreased by at least twofold (Table 1) in the PaDsbA1 mutant. To test the statistical significance of the data, we used the unpaired Student’s t test and defined statistical significance as a P value of <0.05 (two-tailed two-sample equal variance test). We found 23 envelope proteins whose abundance was significantly decreased in the mutant (Table 1). These proteins include periplasmic binding proteins (PA14_03930, PA14_71030, and PA14_07870), putative enzymes involved in peptidoglycan remodeling (PA14_24690 and PA14_25000), and potential extracellular virulence factors (PA14_26020 and PA14_39780). Importantly, the exclusion of PbpG (PA14_53020) and ChiC (PA14_34870), both of which are less abundant proteins containing an even number of cysteine residues, strongly suggests that they are PaDsbA1 substrates. Moreover, CbpD, IMPa, and PaAP, three proteins identified as PaDsbA1 substrates (35), were also found to be less abundant in the periplasm of the PaDsbA1 mutant, which further validates our proteomic data.

**Biochemical and structural characterization of PaDsbA2.** As explained above, the genome of *P. aeruginosa* encodes a second DsbA, PaDsbA2, which belongs to a different subclass of DsbA proteins characterized by the presence of an additional pair of disulfide bonds (DsbA2). Since DsbA2 is homologous to DsbA, we compared the localizations of proteins containing at least one conserved disulfide bond, identified in the proteomes of wild-type and PaDsbA1 strains, with those listed in the UniProtKB database.

**Table 1. Proteins more than twofold less abundant in a PaDsbA1 mutant than in a wild-type *P. aeruginosa* PA14 strain (P < 0.05)**

| UniProtKB/TrEMBL accession no. | Description of protein | No. of Cys<sup>a</sup> | Localization<sup>b</sup> | Ratio (PaDsbA1/WT)<sup>c</sup> |
|-------------------------------|------------------------|----------------------|-----------------|------------------|
| Q02I29                        | d-Alanyl-d-alanine-endopeptidase PbpG (PA14_53020) | 1 | P | 0.31 |
| Q02UB6                        | Polyamine transport protein SpuE (PA14_03930) | 2<sup>d</sup> | P | 0.42 |
| Q02DY4                        | Putative glycine betaine/-proline ABC transporter periplasmic component (PA14_71030) | 2 | P | 0.29 |
| Q02N84                        | Putative periplasmic spermidine/polyamine-binding protein Pof (PA14_30570) | 2<sup>d</sup> | P | 0.21 |
| Q02TF9                        | Putative binding protein component of ABC transporter (PA14_07870) | 2 | P | 0.11 |
| Q02QW4                        | Cyclohexaderyl dehydratase Phc (PA14_19140) | 2<sup>d</sup> | P | 0.33 |
| Q02E10                        | Putative periplasmic monofunctional chorismate mutase (PA14_68480) | 2<sup>d</sup> | P | 0.00 |
| Q02PI4                        | Putative soluble lytic transglycosylase Slh (PA14_25000) | 2 | P | 0.00 |
| Q02PL1                        | Putative d-alanyl-d-alanine-carboxypeptidase DacB (PA14_24690) | 2 | P | 0.00 |
| Q02GT9                        | Putative binding protein component of ABC dipeptide transporter (PA14_58420) | 4<sup>d</sup> | P | 0.26 |
| Q02E45                        | Putative binding protein component of ABC dipeptide transporter (PA14_70200) | 4<sup>d</sup> | P | 0.23 |
| Q02GU4                        | Putative binding protein component of ABC transporter (PA14_58360) | 4<sup>d</sup> | P | 0.40 |
| Q02ER3                        | Putative ABC transporter periplasmic substrate-binding protein (PA14_67400) | 4 | P | 0.08 |
| Q02S18                        | Putative amino acid ABC transporter-binding protein YhdW (PA14_14100) | 4 | P | 0.00 |
| Q02TC1                        | Putative phage-related protein tail component JF1 (PA14_08300) | 4 | OM | 0.38 |
| Q02DR8                        | Putative uncharacterized protein (PA14_71840) | 8 | OM | 0.06 |
| Q02M97                        | Chitinase ChiC (PA14_34870) | 1 | E | 0.47 |
| Q02GR5                        | Type IV pilin structural subunit PilA (PA14_58730) | 2<sup>d</sup> | E | 0.32 |
| Q02L18                        | Staphyloytic protease preproenzyme LasA (PA14_40290) | 4<sup>d</sup> | E | 0.18 |
| Q02PA2                        | Putative aminopeptidase (PA14_26020) | 6 | E | 0.10 |
| Q02I11                        | Chitin-binding protein CbpD (PA14_53250) | 8<sup>d</sup> | E | 0.22 |
| Q02TJ3                        | Putative uncharacterized protein (PA14_07430) | 8 | E | 0.03 |
| Q02L61                        | Putative halovibrin Hvn (PA14_39780) | 10 | E | 0.17 |

<sup>a</sup> Number of cysteines present in the mature protein sequence.  
<sup>b</sup> The localizations of the proteins are shown as follows: P, periplasm; OM, outer membrane; and E, extracellular.  
<sup>c</sup> Ratio of the amount of protein in a PaDsbA1 mutant to the amount of protein in the wild type (WT).  
<sup>d</sup> Homologous proteins from other bacteria that possess at least one conserved disulfide bond in their three-dimensional structure.
cysteine residues. We found that expression of PaDsbA2 from a plasmid does not complement the phenotype of a PadsbA1 mutant (not shown), suggesting that these two proteins have different roles in P. aeruginosa and different substrate specificities. However, as we failed to find the conditions under which PaDsbA2 is expressed, we could not investigate further the in vivo function of this protein.

In order to gain some insights into the role of PaDsbA2, we decided to characterize its biochemical, redox, and structural properties. First, we tested whether PaDsbA2 exhibits an oxidoreductase activity by evaluating its ability to catalyze the reduction of insulin by dithiothreitol (DTT). The reduction of insulin disulfides causes precipitation of the β-chain, which can be monitored by following the absorbance at 650 nm (43). As shown in Fig. 4A, PaDsbA2 is able to catalyze insulin reduction, although less efficiently than EcDsbA and PaDsbA1. Second, we determined whether PaDsbA2 can be reoxidized in vitro by PaDsbB1 and PaDsbB2, using purified components. Because PaDsbA2 does not exhibit a change in fluorescence, we followed its reoxidation using AMS trapping experiments. As shown in Fig. 5, we found that PaDsbA2 can be reoxidized by PaDsbB1 and PaDsbB2, confirming that it is a substrate for both P. aeruginosa DsbBs. We then determined the redox potential (E°) of the CXXC catalytic motif of PaDsbA2 by equilibrating the protein in various glutathione buffers. The redox potential of the CXXC motif was found to have a value of −67.5 ± 2.5 mV, which makes PaDsbA2 one of the most oxidizing oxidoreductases ever characterized (Fig. 4B). Such an oxidizing redox potential is also consistent with a function for PaDsbA2 as a thiol oxidase. Interestingly, mutation of the additional cysteine residues to serines decreases the redox potential of PaDsbA2 as a thiol oxidase. Interestingly, mutation of the additional cysteine residues to serines decreases the redox potential of PaDsbA2 as a thiol oxidase. Interestingly, mutation of the additional cysteine residues to serines decreases the redox potential of PaDsbA2 as a thiol oxidase. Interestingly, mutation of the additional cysteine residues to serines decreases the redox potential of PaDsbA2 as a thiol oxidase.

We also solved the structure of PaDsbA2 using single anomalous dispersion (SAD) (see Text S1 in the supplemental material for more information on the crystallization conditions and data analysis). The structure, which was refined to 1.3 Å with an R/Rfree ratio of 19.32%/21.96% (Text S1), consists of a thioredoxin fold in which a helical domain is inserted (Fig. 6A). This architecture resembles that of other DsbA proteins, confirming the identification of PaDsbA2 as a DsbA. In particular, the structure of PaDsbA2 superimposes onto the structures of PaDsbA1 (PDB accession no. 3H93, 14% sequence identity) and EcDsbA (PDB accession no. 1FVK, 14% sequence identity) with root mean square deviations (RMSDs) of 2.4 Å and 2.9 Å, respectively. The closest structural homolog of PaDsbA2 from a Dali search (44) is a 27-kDa outer membrane protein from Slibacter pomeroyi DSS-3, a bacterioplankton (PDB accession no. 3GYK, 24% sequence identity, RMSD 2.2 Å). Wolbachia pipientis alpha-DsbA1 (α-DsbA1) (PDB accession no. 3F4R), whose sequence presents 26% identity with PaDsbA2, has a lower Z score and an RMSD of 2.7 Å. A major difference in the structure of PaDsbA2 compared to its closest neighbors and other DsbAs is located in the helical domain where residues 131 to 151 form a coil instead of a helix. This coil links helix 4 to helix 5 that is disulfide bonded to helix 3. The first cysteine (Cys66) of the CXXC motif, which is reduced in the structure, is located at the beginning of helix 1. The S-γ of the catalytic cysteines are 3.6 Å apart, which is too long for a stable hydrogen bond, whereas the S-γ of other Dsb proteins are generally within hydrogen bond distance (45–47). On the other hand, the S-γ of Cys66 receives five hydrogen bonds: two from the N-α of Cys69 and Phe68, two from the O-α and O-γ of Thr183, a residue of the conserved cis-Pro loop of proteins with a thioredoxin fold, and one from a water molecule (Fig. 6B). These five hydrogen bonds to the S-γ of Cys66 contribute in stabilizing the thiolate state, which could explain the very oxidizing redox potential of PaDsbA2. As observed in the structure of α-DsbA1 from W. pipientis, a disulfide bond is formed between the additional cysteines of the helical domain, Cys111 and Cys157. This disulfide bond links helices 3 and 5 of the helical domain and is approximately 20 Å away from the CXXC catalytic motif. An important feature that varies be-
between DsbA proteins is their surface electrostatics. Here, we see that the surface of PaDsbA2 is more basic than that of EcDsbA and, to a lesser extent, that of PaDsbA1 (Fig. 6C). Moreover, EcDsbA has a hydrophobic patch and a hydrophobic groove surrounding the CXXC active site. These regions, the hydrophobic patch and hydrophobic groove, which are involved in substrate and DsbB binding, respectively (45), were shown to be truncated in PaDsbA1 compared to EcDsbA (24). In the case of PaDsbA2, many amino acids contributing to the hydrophobic groove of EcDsbA are replaced by polar or charged residues, as in the structure of α-DsbA1 from W. pipientis (48). As the hydrophobic groove is important for the interaction between EcDsbA and EcDsbB (45), this may explain why EcDsbB does not reoxidize PaDsbA2 (not shown).

DISCUSSION

The number of infections with multidrug-resistant Gram-negative bacteria, including *P. aeruginosa*, has dramatically increased in recent years, calling for the urgent development of new classes of antimicrobial drugs. Molecules targeting bacterial virulence rather than bacterial growth appear to be a particularly promising therapeutic approach to combat bacterial infections. Indeed, as virulence factors are usually not required for bacterial survival, antivirulence molecules provide the advantage of decreasing pathogenicity without applying a strong selective pressure for the development of bacterial resistance. By highlighting the crucial role played by PaDsbA1, PaDsbB1, and PaDsbB2 in the assembly of the arsenal of virulence factors of *P. aeruginosa*, our study provides the fundamental knowledge required to develop antivirulence molecules able to disrupt disulfide bond formation in this microorganism. In particular, the central role played by PaDsbA1 in the disulfide bond formation machinery makes it a target of choice for antivirulence strategy. However, it will be important to identify drugs targeting PaDsbA1 without interfering with the cellular proteins from the host that also present a thioredoxin fold. In that respect, the available structure of PaDsbA1 (24) should facilitate the design of molecules interacting with the specific surface characteristics of this protein. An alternative approach is the development of drugs inhibiting recycling of PaDsbA1 by PaDsbB1 and PaDsbB2, which do not have eukaryotic homologs. However, our study shows that only molecules capable of inhibiting both PaDsbBs with high affinity will be useful due to the functional redundancy between these two proteins.

Our work also raises the question of why two seemingly redundant DsbB proteins are encoded by the *P. aeruginosa* genome. Indeed, in the absence of a selective advantage for keeping two redundant genes, one of them should have progressively become nonfunctional. However, this is not the case, as we show that PaDsbB1 and PaDsbB2 are both able to reoxidize PaDsbA1 and PaDsbA2. It has been proposed that redundant genes may provide a cumulative advantage (49), each contributing to the global quantity of a protein involved in a specific cellular process. Thus, a first hypothesis is that under certain growth conditions, the presence of a single *dsbB* gene is not sufficient to drive disulfide bond formation and that efficient oxidative folding requires the presence of both *PadsbB1* and *PadsbB2*. Alternatively, possession of two redundant *dsbB* genes may allow a broader spectrum of regulation than a single one: the expression of *PadsbB1* and *PadsbB2* may be differentially modulated according to the environmental conditions, which would *de facto* give rise to more individualized functions for these two genes. For instance, it has recently been shown that the respective expression levels of two redundant operons involved in phenazine production in *P. aeruginosa* are differentially modulated in liquid cultures and biofilms (50). Thus, the presence of two redundant *dsbB* genes may allow *P. aeruginosa* to adapt to different culture and environmental conditions.

We also report the identification of several new potential PaDsbA1 substrates. First, we found several secreted proteins, including virulence factors that are not secreted by the *PadsbA1* and *PadsbB1B2* mutants. The affected proteins all possess an even number of cysteine residues that are conserved in the sequence, which strongly suggests that they contain disulfide bonds in the native state (20). This is further reinforced by the fact that for one of these proteins, the chitin-binding protein CpbD, disulfide bonds have been observed in the structure of a homolog from *Serratia marcescens* (51, 52). It is striking that the affected exoproteins depend on the Xcp type II machinery for secretion to the extracellular environment (53). This pathway, unlike the type I and III systems that facilitate a one-step secretion across both the inner and outer membrane, generally translocates proteins that are first transported to the periplasm and then fold in this compartment prior to secretion (54). The absence of the exoproteins in the supernatants prepared from the *PadsbB1B2* and *PadsbA1* mutants is therefore likely to result from the inability of these proteins to oxidatively fold in the periplasm of these strains. Alternatively, the decrease in secretion could result from a defective assembly of the type II secretion machinery in the *PadsbA1* and *PadsbB1B2* mutants. However, we think that this is unlikely for at least three reasons. First, the type II secretion apparatus should not be affected by impairment of the disulfide bond machinery, as none of the proteins involved in its assembly contains disulfide bonds (54). Second, the hemolytic phospholipase PlcH, a type II secreted substrate that folds to the active form in the cytoplasm, is efficiently secreted by the *PadsbB1B2* and *PadsbA1* mutants (not shown). Third, three of the exoproteins whose abundance was decreased in the supernatants prepared from the *PadsbB1B2* and *PadsbA1* mutants (CpbD, IMPa, and PaAP) were found to be less abundant in the periplasm of the *PadsbA1* mutant (see the results of the 2D-LC-MS/MS analysis), which is more consistent with a defect in folding than in secretion.

Additional PaDsbA1 substrates were also identified using a

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**FIG 5** PaDsbA2 is reoxidized by both PaDsbB1 and PaDsbB2 *in vitro*. The reoxidation of PaDsbA2 by PaDsbB1 and PaDsbB2 was monitored over time by AMS trapping. The reaction was performed in the presence of quinones (decylubiquinone) when indicated. PaDsbA2 alone and PaDsbA2 with quinones (+) (top right panel) were used as negative controls. The experimental conditions are described in Materials and Methods. Abbreviations: red, reduced; ox, oxidized.
A high-throughput proteomic approach. Here also, almost all the proteins whose abundance is decreased in the PadsbA1 mutant contain an even number of conserved cysteine residues, strongly suggesting that the decreased abundance results from impaired oxidative folding. Furthermore, for several of these proteins (Table 1), disulfide bonds are observed in the structures of homologs from other bacteria. We cannot rule out the possibility that for some proteins, the decrease in abundance results from decreased synthesis. However, our previous work on the E. coli disulfide bond system showed that for almost all proteins whose abundance is decreased in the periplasm of an EcdsbA mutant, the lower protein abundance does not result from reduced transcription (12, 39).

We also report that P. aeruginosa encodes a second DsbA-like protein, which we named PaDsbA2. Although the in vivo function of PaDsbA2 remains unclear, we provide biochemical evidence that this protein is an oxidoreductase with a highly oxidizing redox potential. The PaDsbA2 protein belongs to the class of α-DsbA proteins, whose most representative members are found in alphaproteobacteria (48). Proteins from this class are charac-

**FIG 6** PaDsbA2 adopts a typical DsbA-like structure, with a thioredoxin (Trx) domain and an α-helical domain. (A) The structure of PaDsbA2 was solved using single anomalous dispersion and refined to 1.3 Å with an R/Rfree ratio of 19.3%/21.96% and consists of a thioredoxin fold (in gray) in which a helical domain is inserted (in blue). The catalytic cysteines (C66 and C69) and the additional cysteines (C111 and C157) are shown in red. (B) The first cysteine (Cys66) of the CXXC motif is located at the beginning of helix 1 and is 3.6 Å away from the second catalytic cysteine (Cys69). The S– of Cys66 receives five hydrogen bonds: two from the N– of Cys69 and Phe68, two from the O– and O– of Thr183, and one from a water molecule. These five hydrogen bonds to the S– of Cys66 help stabilize the thiolate state, which could explain the very oxidizing redox potential of PaDsbA2. (C) The relative electrostatic surface potentials of PaDsbA1, PaDsbA2, and EcDsbA are shown. The electrostatic potential is given from −3 to 3 kT/e with k the Boltzmann constant, T the temperature, and e the elementary charge (charge of a proton). The respective active site cysteines and cis-proline are annotated.
teredized by the presence of four conserved cysteine residues, relatively longer sequences, and the presence of an invariant threonine residue preceding the cis-proline found in proteins with a thioredoxin (Trx) fold (48). The structure and biochemical properties of α-DsbA1 from Wolbachia pipiens, another α-DsbA, have recently been reported (48). This protein, which exhibits 26% sequence identity with PaDsbA2, is significantly more reducing (−163 mV) than PaDsbA2 (48). Importantly, α-DsbA1 also contains a second disulfide bond for which a regulatory role has been proposed (48). Although a single periplasmic DsbA is sufficient for oxidative folding in many bacterial species, the presence of additional Dsbs has been reported in some Gram-negative bacteria where they usually catalyze the oxidation of specific target proteins. In Legionella pneumophila for instance, a newly identified DsbA protein specifically catalyzes disulfide bond formation in proteins involved in the type IV secretion system (55). In Salmonella enterica serovar Typhimurium, DsbL, which is homologous to DsbA, specifically oxidizes a periplasmic aroylsulfate sulfotransferase, an enzyme that detoxifies phenolic substances and antibiotics (56). Further research to explore the conditions under which PaDsbA2 is expressed and to identify the function of this protein remains to be done.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and protein purification. The bacterial strains, plasmids, and growth conditions used in this study and the protein purification methods are described in Text S2 in the supplemental material.

Insulin reduction assay. Insulin reduction assays were performed by using methods described previously (43). Briefly, insulin (150 μM) and the various DsbA proteins (10 μM) was used as a control were mixed in 100 mM KPi (potassium phosphate) (pH 7.0) and 1 mM EDTA. The reaction was started by adding 0.8 mM dithiothreitol (DTT) (final concentration), and the reduction of insulin by DTT was monitored spectrophotometrically at 650 nm.

Redox potential determination. The fractions of reduced and oxidized PaDsbA2 were determined using 4-acetamido-4-acetoxy-2,2-dissulfonic acid (AMS) trapping by the method of Denoncin et al. (57). Briefly, PaDsbA2 and PaDsbA2CSS (1 μM) were incubated overnight at room temperature in 50 mM KPi (pH 7.0), 0.1 mM EDTA and various glutathione (GSH)/glutathione disulfide (GSSG) ratios. After incubation, proteins were precipitated with trichloroacetic acid (TCA) (10% final concentration). After 20-min incubation on ice, the samples were centrifuged (Eppendorf 5418; 15,000 × g, 5 min, 4°C), and the pellets were washed with cold acetone. After a second centrifugation, pellets were dissolved in a buffer containing 20 mM AM, 0.1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 7.5). After 45-min incubation at 37°C, with 1,400 rpm shaking under light protection, solutions were loaded onto 12% SDS-polyacrylamide gels under denaturing conditions. Fractions of reduced and oxidized protein were determined using ImageJ (http://rsweb.nih.gov/). The redox potential was then calculated as described previously (58).

In vivo reoxidation state determination. The in vivo reoxidation state of PaDsbA1 has been assessed using AMS trapping experiments as described by Denoncin et al. (57). Briefly, bacteria were cultured at 37°C with 130 rpm shaking in LB medium until they reached an optical density at 600 nm (OD600) of 0.5. The proteins were then TCA precipitated (10% cold TCA) and resuspended in 30 μl of 20 mM AM, 0.1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 7.5). Samples were incubated for 45 min at 37°C, with 1,400 rpm shaking, protected from light. For a positive control, protein pellets were treated with 50 mM DTT in the presence of 200 mM Tris (pH 8) and 1% SDS. Samples were loaded onto 12% SDS-polyacrylamide gels under denaturing conditions. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and probed with an anti-PaDsbA1 antibody (1/3,000) produced from a rabbit immunized with the purified protein (CER, Marloie, Belgium). Anti-rabbit IgG (Sigma) was used as the secondary antibody at a concentration of 1/5,000. Thermo Scientific Pierce enhanced chemiluminescence (ECL) Western blotting substrate and Fuji film (Fujifilm) were used to visualize the protein bands.

Reoxidation of PaDsbA1 and -A2 by PaDsbB1 and -B2. The reoxidation of PaDsbA1 and PaDsbA2 by PaDsbB1 and PaDsbB2 was performed in the presence of 30 μM decylubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone) in a buffer containing 50 mM NaPi, (pH 6.0), 300 mM NaCl, and 0.1% N-dodecyl-β-D-maltoside (DDM). The reoxidation of PaDsbA1 was followed in a spectrophotometer using 295 nm as the excitation wavelength and 330 nm as the emission wavelength. The plot of substrate concentrations versus initial velocity was fitted, using Prism, to the Michaelis-Menten equation, and the K_m values were determined. The reoxidation of PaDsbA2 was followed by AMS trapping as described by Denoncin et al. (57).

E. coli complementation assays. Swarming motility was assayed as described previously (59). Strains IA32 and IA33 were inoculated on M63 minimal medium soft agar plates containing 15 mM (NH_4)_2SO_4, 100 mM KH_2PO_4 (pH 7.0), 1 mM MgSO_4, 0.4% glucose, 0.2% arabinose, 10 μg/ml thiamine, 5 μg/ml mix NAD-riboflavin, 0.4 μg/ml biotin, 174 μg/ml of a mix of amino acids (except cysteine), and 0.3% agar. After 16 h of incubation at 37°C, motility was analyzed. Strains IA34 and IFC234 were used as negative controls.

Analysis of culture supernatants of wild-type and mutant P. aeruginosa strains. P. aeruginosa strains were grown at 37°C in TSB medium (Difco Laboratories). Cells and supernatants were separated by centrifugation (Beckman Coulter JA-10 rotor, 2,375 × g, 10 min, 4°C); proteins contained in the supernatant were TCA precipitated (10% final concentration) for 1 h at 4°C. Samples were subsequently centrifuged (Beckman Coulter JA-10 rotor, 16,000 × g, 30 min, 4°C). The pellets were then washed with 90% acetone and resuspended in SDS-PAGE sample buffer. The samples were normalized according to the OD_600 of the initial bacterial culture (0.1 OD_600 unit of cell culture per μl of sample buffer) and loaded onto a 10.5% SDS-polyacrylamide gel. CbpD and LasB were identified by Western blotting using specific antibodies (not shown), while the three other proteins were identified by mass spectrometry.

Plate assays. The proteolytic activity of P. aeruginosa PA14 wild-type and mutant strains was tested by growing colonies on tryptic soy agar (TSA) plates containing 1.5% skim milk. Elastase activity was tested on TSA plates containing 1% elastin. Flagellar motility assay was performed by spotting bacteria with a toothpick at the surface of LB agar plates (0.3% agar). Bacteria were grown at 30°C, and halos corresponding to the spreading of bacteria from the point of inoculation were observed. Twitching motility assay was performed on LB agar plates by inoculating bacteria through the agar to the bottom of the plate. The plates were incubated overnight at 37°C. The halo at the bottom of the plate was visualized with crystal violet coloration.

Biofilm formation assay and quantification. The biofilm formation assay was performed in 24-well polystyrene microtiter dishes as described by Vallet et al. (60). Phenotypes are visualized after 2, 4, 6, and 8 h of incubation at 30°C. Bacterial cells bound to the walls of the wells were stained with 1% crystal violet. For quantification, these cells were suspended in 400 μl of 95% ethanol and 600 μl of water, and the OD_600 was measured.

Caenorhabditis elegans slow killing assay. The slow killing assay was performed as previously described (38). Each assay consisted of four replicates. Adult stage C. elegans worms were selected from plates containing overnight growth of each bacterial strain, and on a daily basis worms were evaluated for viability. Worm survival was plotted using the Prism 5.00 computer program. Survival curves were considered significantly different from the control when P values were <0.05. Prism calculates survival fractions using the product limit (Kaplan-Meier) method and compares
survival curves by two methods: the log rank test and the Gehan-Breslow-Wilcoxon test.

**Preparation of periplasmic extracts and proteolytic digestion.** Periplasmic extracts of *P. aeruginosa* PA14 were prepared following a protocol adapted from the method of Hiniker and Bardwell (11). *P. aerugi-

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