Role of TonB1 in Pyoverdine-Mediated Signaling in *Pseudomonas aeruginosa*[▽]

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Pyoverdines are siderophores secreted by *Pseudomonas aeruginosa*. Uptake of ferripyoverdine in *P. aeruginosa* PAO1 occurs via the FpvA receptor protein and requires the energy-transducing protein TonB1. Interaction of (ferri)pyoverdine with FpvA activates pyoverdine gene expression in a signaling process involving the cytoplasmic-membrane-spanning anti-sigma factor FpvR and the sigma factor PvdS. Here, we show that mutation of a region of FpvA that interacts with TonB1 (the TonB box) prevents this signaling process, as well as inhibiting bacterial growth in the presence of the iron-chelating compound ethylenediamine-di(*o*-hydroxyphenylacetic acid). Signaling via wild-type FpvA was also eliminated in strains lacking TonB1 but was unaffected in strains lacking either (or both) of two other TonB proteins in *P. aeruginosa*, TonB2 and TonB3. An absence of pyoverdine-mediated signaling corresponded with proteolysis of PvdS. These data show that interactions between FpvA and TonB1 are required for (ferri)pyoverdine signal transduction, as well as for ferripyoverdine transport, consistent with a mechanistic link between the signaling and transport functions of FpvA.

Pseudomonas aeruginosa is an opportunistic pathogen that is able to cause severe infections in patients with cystic fibrosis and in immunocompromised individuals, such as burn victims. Under conditions of iron limitation, *P. aeruginosa* secretes an iron-scavenging compound (siderophore) called pyoverdine. Ferripyoverdine is transported back into the bacteria by an outer membrane (OM) receptor protein, FpvA. The transport of ferripyoverdine via FpvA requires energy provided by a TonB complex (36, 42, 50). TonB is an energy-transducing protein that couples the energy of the cytoplasmic membrane (CM) to a variety of OM receptors required for the import of ferrisiderophores and other molecules. TonB acts in a complex with two CM-associated proteins, ExbB and ExbD, both of which are required for full TonB function (5, 37). The TonB-ExbB-ExbD complex has been identified in many gram-negative bacterial species and is thought to be a conserved mechanism for energy transduction to OM receptor proteins (31). TonB-dependent receptors contain a conserved protein motif known as the TonB box (5). Direct interaction between TonB and the TonB box has been demonstrated for several TonB-dependent receptors (8, 26, 33, 35, 47). Mutations of the TonB box, particularly mutations that are likely to affect the secondary structure, can result in a TonB-uncoupled phenotype characterized by loss of TonB-dependent functions (ferrisiderophore transport) with no loss of TonB-independent functions, such as internalization of bacteriophage (37).

The *P. aeruginosa* PAO1 genome contains three *tonB* genes, *tonB1* (PA5531) (36), *tonB2* (PA0197) (55), and *tonB3* (PA0406) (20), encoding proteins of 342, 270, and 319 amino acids (aa), respectively. The TonB1 and TonB2 amino acid

sequences display 31% identity over a section of 187 aa, but otherwise, the three PAO1 TonB proteins show similarity (30 to 40% aa identity) to each other only over short (<70-aa) regions. TonB1 is considered to be the primary TonB protein involved in iron transport in *P. aeruginosa*. *tonB1* mutants are impaired for growth in iron-limited medium and are defective for siderophore-mediated iron transport and heme utilization (36, 50, 55). Moreover, direct interaction between TonB1 and the ferripyoverdine receptor FpvA has been demonstrated in vitro (1). The *tonB2* gene is not required for growth in iron-limited medium (55). However, *tonB1 tonB2* double mutants grow even less well under iron limitation than *tonB1* mutants, indicating that TonB2 may be able to partially complement TonB1 in its role in iron acquisition (55). The *tonB3* gene is required for twitching motility and assembly of extracellular pili (20), but it is not known whether TonB3 has a role in iron acquisition. Genes encoding ExbB and ExbD proteins are located directly downstream of *tonB2* (55) but are not found in association with *tonB1* or *tonB3*.

Besides its role in ferripyoverdine transport, FpvA is part of a signal transduction pathway and thus belongs to a subset of TonB-dependent receptors known as TonB-dependent transducers (reviewed in references 23 and 51). Mutational analysis has shown that the ferripyoverdine transport and signaling roles of FpvA are separate and discrete functions (21, 46). Besides FpvA, the signal transduction pathway involves a CM-spanning anti-sigma factor protein, FpvR, and (ferri)pyoverdine. (It was previously thought that both ferri- and apopyoverdine could bind FpvA (43). However, it was recently reported that only ferripyoverdine is able to form a high-affinity interaction with FpvA (13). The designation (ferri)pyoverdine will be used here to represent the active signaling molecule. FpvA and (ferri)pyoverdine regulate the activity of FpvR, which in turn regulates the activities of two extracytoplasmic function family sigma factors, PvdS and FpvI (3, 25). Upon binding of (ferri)pyoverdine to FpvA, a signal is transmitted to FpvR,

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TABLE 1. *P. aeruginosa* strains used in this study

<i>P. aeruginosa</i> strain	Relevant characteristics	Reference
PAO1	Wild-type strain	Lab stock
PAO <i>pvdF</i>	Derivative of PAO1; <i>pvdF</i> ::Kan	27
PAO <i>fpvA</i>	Derivative of PAO1; Δ <i>fpvA</i>	This study
PAO <i>pvdF fpvA</i>	Derivative of PAO <i>pvdF</i> ; Δ <i>fpvA</i>	This study
PAO <i>fpvA attP::fpvA_{WT}</i>	Derivative of PAO <i>fpvA</i> ; <i>fpvA</i> (including promoter region), encoding wild-type FpvA, chromosomally integrated at the <i>ctx attP</i> attachment site	This study
PAO <i>fpvA attP::fpvA_{TBM}</i>	Derivative of PAO <i>fpvA</i> ; <i>fpvA_{TBM}</i> (including the promoter region), encoding FpvA with an altered TonB box, chromosomally integrated at the <i>ctx attP</i> attachment site	This study
PAO <i>pvdF fpvA attP::fpvA_{WT}</i>	Derivative of PAO <i>pvdF fpvA</i> ; <i>fpvA</i> (including the promoter region), encoding wild-type FpvA, chromosomally integrated at the <i>ctx attP</i> attachment site	This study
PAO <i>pvdF fpvA attP::fpvA_{TBM}</i>	Derivative of PAO <i>pvdF fpvA</i> ; <i>fpvA_{TBM}</i> (including the promoter region), encoding FpvA with an altered TonB box, chromosomally integrated at the <i>attP</i> attachment site	This study
PAO6609	<i>pvd9 met9011 amiE200 strA</i>	19
K1040 (<i>tonB1</i>)	Derivative of PAO6609; <i>tonB1</i> :: Ω Hg	36, 55
K1407 (<i>tonB2</i>)	Derivative of PAO6609; Δ <i>tonB2</i>	55
MS231 (<i>tonB3</i>)	Derivative of PAO6609; Δ <i>tonB3</i>	This study
K1408 (<i>tonB1 tonB2</i>)	Derivative of K1407; <i>tonB1</i> :: Ω Hg	55
MS232 (<i>tonB1 tonB3</i>)	Derivative of K1040; Δ <i>tonB3</i>	This study
MS233 (<i>tonB2 tonB3</i>)	Derivative of K1407; Δ <i>tonB3</i>	This study
MS234 (<i>tonB1 tonB2 tonB3</i>)	Derivative of K1408; Δ <i>tonB3</i>	This study

resulting in activation of PvdS and FpvI. Activation of PvdS is required for maximal synthesis of pyoverdine itself, as well as two secreted proteins (25). Activation of FpvI leads to increased expression of *fpvA* (3, 39). In the absence of pyoverdine-mediated signaling, caused by the lack of FpvA or pyoverdine or overexpression of FpvR, suppression of PvdS- and FpvI-dependent gene expression occurs (3, 25), and this is associated with proteolysis of PvdS (49).

Analogous siderophore transport and signaling systems involving an OM TonB-dependent transducer, a CM-bound anti-sigma factor, and an extracytoplasmic function family sigma factor have been described in other bacteria, including the ferric citrate (Fec) system in *Escherichia coli* and the pseudobactin (Pup) system in *Pseudomonas putida* (reviewed in reference 6). The TonB protein is required for signaling in both the Fec (14, 33) and Pup (24) systems. Similarly, a TonB system is required for hemophore transport and signaling in *Serratia marcescens* (4). The aim of this study was to investigate whether TonB was required for pyoverdine-mediated signaling in *P. aeruginosa*, and if so, to identify which of the three TonB proteins was involved.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* strains used in this study are listed in Table 1. All strains were maintained on Luria Bertani (LB) agar. For *tonB* mutants, FeCl₃ (100 μ M) was added to the agar to assist growth. The bacteria were grown at 37°C; broth cultures were aerated by shaking (260 rpm). Antibiotics were included in media as appropriate at the following concentrations: for *E. coli*, 100 μ g/ml ampicillin and 5 μ g/ml tetracycline; for *P. aeruginosa*, 7 μ g/ml tetracycline, 50 to 300 μ g/ml chloramphenicol, and 25 μ g/ml gentamicin. DNA manipulations were performed according to standard methods (41). Pyoverdine, purified from *P. aeruginosa* PAO1 (29), and desferrioxamine (Sigma-Aldrich) were added to cultures to a final concentration of 60 μ M where stated. Pyoverdine-deficient *tonB1* (K1040) (36, 55), *tonB2* (K1407) (55), and *tonB1 tonB2* (K1408) (55) mutants were generously provided by K. Poole (Queen's University, Canada).

Construction of Δ *fpvA* mutant strains. *P. aeruginosa* PAO *fpvA* and PAO *pvdF fpvA* strains, in which the entire *fpvA* gene had been deleted, were constructed using pJSS2 as described previously (46); the latter strain also has a mutation in

the *pvdF* gene that is required for pyoverdine synthesis (27). Deletion of *fpvA* was confirmed using PCR with primer A (5'-CAGGGCAAGCAGGCTTCG-3') and *pvdD* for (5'-TGCTGATGCTCAAGGAGCGG-3'), which flank the deletion.

Disruption of the FpvA TonB box. The TonB box of FpvA, represented by residues 129 to 135 of the unprocessed FpvA protein (11, 54), was changed from ATMTSN to ATAAAN using overlap extension PCR (15, 16). PCR was first performed using the primer pairs FpvApromF (5'-AAAGGATCCGGCAAGACCATCATCGTGA-3') with TonBoxmutR (5'-GGCCGCTGCAGCGGTGGC GCCGAGATCGAC-3') and TonBoxmutF (5'-GCTGCAGCGGCCAACCAG TTGGGCACCAT-3') with FpvAmidR (5'-GACGTCCAGTTCGGAGCGGTA A-3'), using *P. aeruginosa* PAO1 genomic DNA as a template. The resulting PCR products were mixed, and a second PCR was performed using primers FpvA promF and FpvAmidR. The product of this reaction was digested with BamHI, which cuts at an introduced site (underlined) in FpvApromF, and EcoRI, which cuts at a naturally occurring site in *fpvA*. The resulting product was ligated with the 1.6-kb EcoRI-HindIII fragment of pUCP22::*fpvA* (21) and cloned into the BamHI-HindIII sites of pUCP22 (52) to give pUCP22::*fpvA_{TBM}*. The wild-type *fpvA* gene, including the promoter region, was cloned in pUCP22 by ligating the 1.6-kb EcoRI-HindIII fragment of pUCP22::*fpvA* to a fragment amplified from PAO1 genomic DNA using PCR primers FpvApromF and FpvAmidR and digested with BamHI and EcoRI. This construct was designated pUCP22::*fpvA_{WT}*. The cloned DNA of pUCP22::*fpvA_{WT}* and pUCP22::*fpvA_{TBM}* was sequenced to confirm the absence of any other mutations and then subcloned into the integration vector mini-CTX2 (18). Mini-CTX2::*fpvA_{WT}* and mini-CTX2 *fpvA_{TBM}* were introduced into *E. coli* S17-1 (48) via transformation (28) and selection on LB agar with tetracycline. The plasmids were then introduced by conjugation (18) into *Pseudomonas* PAO *fpvA* and PAO *pvdF fpvA*. Selection for transconjugants was on LB agar with tetracycline, to select for the presence of mini-CTX plasmids that had integrated at the *attP* attachment site, and kanamycin (50 μ g/ml), to counterselect the S17-1 donor. Vector backbone DNA was removed using the Flp recombinase procedure (17). The resulting strains carried the *fpvA* gene (either wild type or containing the TonB box mutation) on the chromosome in single copies under the control of the native *fpvA* promoter. PCR was performed with primers FpvAmidF (5'-GACCGGGTGGGAAGTACTCAA-3') and FpvAmidR2 (5'-CCCAGATTTCGCCCTCGTA-3') to demonstrate the presence of the *fpvA* gene (Fig. 1B).

Growth assays. To assay the growth of strains in the presence of the iron-chelating compound ethylenediamine-di(*o*-hydroxy-phenylacetic acid) (EDDHA), cultures grown overnight in King's B medium (22) were subcultured into King's B medium containing EDDHA (200 μ g/ml) (BDG Synthesis) to an optical density at 600 nm (OD₆₀₀) of ~0.01. Growth was monitored by measuring the OD₆₀₀. Growth assays in iron-deficient succinate minimal medium were performed as described by Zhao and Poole (55).

FIG. 1. PAO *fpvA* mutants expressing FpvA with an altered TonB box. (A) Deletion of *fpvA*. PCR was carried out with primer A and *pvdDfor*, which flank *fpvA*. Amplification of a 1.6-kb fragment indicated deletion of a 2.4-kb genomic fragment containing the entire *fpvA* gene. Under the PCR conditions used, the 4-kb region in bacteria containing wild-type *fpvA* does not amplify. Lanes: M, 1-kb Plus DNA ladder (Invitrogen); 1, PAO1; 2, PAO *fpvA*; 3, PAO *fpvA attP::fpvA_{WT}*; 4, PAO *fpvA attP::fpvA_{TBM}*; 5, PAO *pvdF*; 6, PAO *pvdF fpvA*; 7, PAO *pvdF fpvA attP::fpvA_{WT}*; 8, PAO *pvdF fpvA attP::fpvA_{TBM}*. Size markers are shown in kb. (B) Restoration of *fpvA*. PCR with primers FpvA midF and FpvAmidR amplified a 1.3-kb DNA fragment that is internal to *fpvA*. The lanes are as for panel A. (C) Detection of FpvA_{WT} and FpvA_{TBM} proteins. Western analysis of OM preparations was carried out with a polyclonal antibody against FpvA. Lanes: 1, PAO *pvdF fpvA*; 2, PAO *pvdF fpvA attP::fpvA_{WT}*; 3, PAO *pvdF fpvA attP::fpvA_{TBM}*. The mass marker is shown in kDa. The predicted size of FpvA is 86 kDa. The faint band visible in the PAO *pvdF fpvA* OM preparation is due to contaminating antibodies or nonspecific hybridization of the polyclonal antibody to another OM protein. (D) Growth in the presence of 200 μ g/ml EDDHA. Strains: PAO1, $\blacklozenge$; PAO *fpvA* ($\blacktriangle$); PAO *fpvA attP::fpvA_{WT}* ($\blacksquare$); PAO *fpvA attP::fpvA_{TBM}* ($\times$). The data are averages of two independent experiments.

Measurement of pyoverdine production. Strains were grown in King's B medium to stationary phase (24 h). The absorbances of culture supernatants were measured at OD₄₀₃, a wavelength at which pyoverdine has a characteristic peak (19).

Promoter activity assays. Pyoverdine-mediated signaling was assessed by measuring PvdS-dependent gene expression using the reporter plasmid pMP190::PpvdE (40, 53). In this plasmid, expression of a *lacZ* reporter gene is under the

FIG. 2. Effect of TonB box mutation on pyoverdine-mediated signaling. PAO *pvdF fpvA attP::fpvA_{WT}* (pMP190::PpvdE) and PAO *pvdF fpvA attP::fpvA_{TBM}* (pMP190::PpvdE) were grown in King's B medium, and β -galactosidase activity was assayed. Black bars, pyoverdine was added. The results are averages of data from three independent experiments. The error bars represent the standard deviations.

control of the PvdS-dependent *pvdE* promoter. pMP190::PpvdE was introduced into appropriate strains via transformation (10). For the assays, overnight cultures grown in King's B medium containing chloramphenicol (50 μ g/ml) were diluted in King's B medium to an OD₆₀₀ of $\sim$ 0.1. Duplicate cultures were grown to an OD₆₀₀ of $\sim$ 0.8, and then pyoverdine (60 μ M) was added to one of the duplicates. Incubation was continued for a further 2 h, and β -galactosidase assays were performed according to the method of Miller (30).

OM preparations. Cultures were grown as described for the promoter activity assays with pyoverdine (except chloramphenicol was omitted from the overnight cultures), and OMs were prepared using the sarcosyl solubilization method of Filip et al. (12) as described previously (3). Protein samples ($\sim$ 2 μ g) were electrophoresed on a 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and submitted to Western analysis with a polyclonal anti-FpvA antibody as described previously (3).

Construction of Δ tonB3 mutant strains. Mutants containing an in-frame deletion of *tonB3* were engineered using the gene replacement vector pEX18Gm (17). A 0.8-kb region of DNA upstream of *tonB3* and a 0.9-kb region of DNA downstream of *tonB3* were amplified from *P. aeruginosa* PAO1 genomic DNA using the PCR primer pairs TB3UF (5'-AAAGGATCCGACCAACCGCTGCACGAAC-3') with TB3UR (5'-AAACTGCAGATGACGCACTCCGCGCCTTG-3') and TB3DF (5'-CCCCTGCAGCGGCTGTCCAGCAAGTAGCG-3') with TB3DR (5'-AAAAAGCTTCTTGGGCTGCCGTCCATGTT-3'), and the products were cloned in pUC19 following digestion at introduced restriction sites (underlined). DNA sequencing confirmed that the cloned DNA was free of mutations. The cloned *tonB3* flanking regions were ligated together at the common PstI site and cloned into the BamHI-HindIII sites of pEX18Gm to give pEX18Gm:: Δ tonB3 containing the *tonB3* flanking DNA with a 933-bp internal deletion in *tonB3*. pEX18Gm:: Δ tonB3 was introduced into *P. aeruginosa* strains PAO6609 (Pvd⁻) (19), K1040, K1407, and K1408 via conjugation (as described

TABLE 2. Pyoverdine production by *P. aeruginosa* strains

<i>P. aeruginosa</i> strain	Pyoverdine production ^a
PAO1	100
PAO <i>pvdF</i>	3.3
PAO <i>fpvA</i>	22.7
PAO <i>pvdF fpvA</i>	3.2
PAO <i>fpvA attP::fpvA_{WT}</i>	54.1
PAO <i>fpvA attP::fpvA_{TBM}</i>	23.3
PAO <i>pvdF fpvA attP::fpvA_{WT}</i>	3.2
PAO <i>pvdF fpvA attP::fpvA_{TBM}</i>	3.2

^a Absorbance of culture supernatant at OD₄₀₃ as a percentage of that of PAO1. The data are averages from two independent experiments.

FIG. 3. Pyoverdine-mediated signaling in TonB mutants. Strains were grown in King's B medium, and β -galactosidase activity was assayed. Black bars, pyoverdine was added; hashed bar, desferrioxamine was added. The results are averages of data from at least three independent experiments. The error bars represent the standard deviations. PAO6609 is the parent strain (Pvd⁻ TonB1⁺ TonB2⁺ TonB3⁺) from which the *tonB* mutant strains are derived. All of the strains carry pMP190::PpvdE.

above) with selection on LB agar with FeCl₃ (100 μ M), gentamicin, and kanamycin (50 μ g/ml). Secondary recombination causing deletion of the *tonB3* gene and removal of vector DNA was selected by sucrose counterselection as described previously (17, 50). Deletion of *tonB3* was confirmed using PCR with primers TB3UF and TB3DR (data not shown).

Western analysis of PvdS. Western analysis was carried out as described previously (49). Briefly, cultures were grown to late exponential phase in King's B medium with or without pyoverdine (60 μ M). Cells (1.1×10^9 CFU) were lysed by boiling them (5 min), and samples (~15 μ g of protein) were electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Western analysis was performed with an anti-PvdS monoclonal antibody (mAbI308).

RESULTS AND DISCUSSION

The TonB box is required for interactions between TonB proteins and TonB-dependent receptors (35, 38, 47), and the position of the TonB box in the structure of FpvA is consistent with this function (7, 11, 44). *P. aeruginosa* strains were constructed in which *fpvA* was deleted and replaced with wild-type *fpvA* or *fpvA*_{TBM}, in which the TonB box of FpvA, represented by residues 129 to 135 of the unprocessed FpvA protein (11, 54), is changed from ATMTSN to ATAAAN (Fig. 1A and B). Western analysis confirmed that the FpvA proteins were expressed and present in the OM of cells with a mass of 85 kDa (Fig. 1C), as found previously (21).

Alteration of the TonB box region was expected to abolish interactions between FpvA and TonB (and hence TonB-dependent functions of FpvA) without affecting other TonB-dependent functions of the cell. PAO *fpvA attP::fpvA*_{WT} and PAO *fpvA attP::fpvA*_{TBM} were tested for growth in King's B broth medium (22) containing the iron-chelating compound EDDHA, which inhibits the growth of strains defective for pyoverdine-dependent iron uptake (2). PAO *fpvA attP::fpvA*_{WT} had no significant growth deficiency compared to the wild-type strain PAO1, whereas PAO *fpvA attP::fpvA*_{TBM} showed no significant growth after 10 h, similar to the PAO *fpvA* mutant (Fig. 1D). These data show that mutation of the TonB box prevented the ferripyoverdine transport function of the FpvA receptor, consistent with an inability of FpvA_{TBM} to interact with TonB.

Pyoverdine signaling is required for maximal expression of pyoverdine synthesis genes (25). Pyoverdine-mediated signaling was assessed by measuring PvdS-dependent gene expression using the reporter plasmid pMP190::PpvdE (40, 53). Signaling, represented by an increase in PvdS-dependent gene expression in response to added pyoverdine, was observed with bacteria carrying wild-type FpvA, but not with bacteria carrying FpvA with the TonB box mutation (Fig. 2). Furthermore, pyoverdine production was measured in *P. aeruginosa* strains. PAO *fpvA attP::fpvA*_{TBM} (expressing FpvA with the altered TonB box) produced an amount of pyoverdine similar to that produced by the PAO *fpvA* mutant and significantly less than PAO *fpvA attP::fpvA*_{WT} or wild-type bacteria produced (Table 2). Together, these data are consistent with the hypothesis that FpvA-TonB interaction is required for pyoverdine-mediated signaling to occur.

We next investigated which of the *P. aeruginosa* TonB proteins was required for pyoverdine-mediated signaling. Signaling was assessed in *P. aeruginosa tonB* mutants using the pMP190::PpvdE reporter construct (Fig. 3). Mutation of *tonB1*

FIG. 4. Proteolysis of PvdS in the absence of pyoverdine-mediated signaling. Western analysis of whole-cell lysates from cultures grown to late exponential phase was carried out with an anti-PvdS monoclonal antibody (mAbI308). Lanes 1 and 2, PAO *pvdF fpvA attP::fpvA*_{WT}; lanes 3 and 4, PAO *pvdF fpvA attP::fpvA*_{TBM}; lanes 5 and 6, PAO6609 (Pvd⁻ TonB1⁺ TonB2⁺ TonB3⁺); lanes 7 and 8, K1040 (Pvd⁻ TonB1⁻); lanes 9 and 10, MS233 (Pvd⁻ TonB1⁺ TonB2⁻ TonB3⁻); lanes 11 and 12, MS234 (Pvd⁻ TonB1⁻ TonB2⁻ TonB3⁻). In the even-numbered lanes, pyoverdine was added to cultures to 60 μ M. For clarity, the brightness and contrast have been adjusted in the top panel using Multi Gauge (Fujifilm).

FIG. 5. Growth of *tonB* mutants in succinate minimal medium. Strains: PAO6609 (◆); K1040 (*tonB1*) (■); K1407 (*tonB2*) (▲); MS231 (*tonB3*) (×). The data are averages of three independent experiments.

eliminated pyoverdine-mediated signaling. In contrast, mutations in *tonB2*, *tonB3*, or *tonB2* and *tonB3* together did not affect signaling. Assays in which the unrelated siderophore desferrioxamine was added in place of pyoverdine were also performed. No increase in β -galactosidase production was observed in response to desferrioxamine, indicating that the signaling response is specific for pyoverdine (Fig. 3). Strain PAO6609 and its derivatives yielded approximately threefold more enzyme units than derivatives of PAO *pvdF* (Fig. 2), but the reason for this is not known.

Proteolysis of PvdS occurs in the absence of pyoverdine-mediated signaling (49). We therefore tested whether proteolysis of PvdS would occur in strains with the altered TonB box or in the *tonB1* mutants. Proteolysis of PvdS did indeed occur in strains in which pyoverdine-mediated signaling did not take place (the TonB box mutant and *tonB1* derivatives), even in the presence of pyoverdine, resulting in the occurrence of a subfragment of PvdS (Fig. 4). PvdS proteolysis was suppressed by the presence of pyoverdine in *TonB1*⁺ strains, including the *tonB2 tonB3* double mutant.

Finally, to assess the possible involvement of *tonB3* in iron uptake, growth assays were performed as described by Zhao and Poole (55). Unlike the *tonB1* mutant, the pyoverdine-deficient PAO6609 *tonB3* mutant showed no growth deficiency in iron-deficient succinate minimal medium compared to PAO6609 *TonB*⁺ (Fig. 5), indicating that TonB3 is not required for growth under iron limitation.

Collectively these data show that TonB function is required for pyoverdine-mediated signaling, and of the three TonB proteins in *P. aeruginosa*, only TonB1 is able to perform this function. TonB1 is also the only TonB protein that enables uptake of ferripyoverdine (36). It has recently been proposed (44) that, following binding of ferripyoverdine by FpvA, interaction of TonB with the TonB box is involved in displacement of the signaling domain of FpvA, which then interacts with FpvR to trigger the signaling pathway. The requirement of TonB1 for both transport and signaling is consistent with this proposal, as is the effect of the FpvA TonB box mutation on both functions of FpvA. Expression of *tonB2* is iron regulated, but TonB2 is not required for growth under iron limitation

(55) or for signaling in the pyoverdine pathway, and no role has yet been assigned to TonB2. TonB3 is required for twitching motility and assembly of type IV pili (20), but TonB3, like TonB2, is not required for growth under iron limitation.

Bioinformatics analysis of completed genome sequences suggests that up to 30% of gram-negative bacteria possess more than one *tonB* gene (9). Among other bacterial species with multiple TonB proteins, there are several examples in which different TonB proteins have distinct functions in iron acquisition. For example, both redundant and specific functions have been reported for separate TonB proteins in *S. marcescens* (34). In this species, either TonB or the TonB-like protein HasB can mediate heme utilization. However TonB, but not HasB, is required for ferrisiderophore (enterochelin, ferrichrome, or ferric citrate) utilization. Similarly, in *Vibrio cholerae*, either of two TonB proteins can support growth with hemin, vibriobactin, or ferrichrome as the sole source of iron (32), but only TonB1 supports schizokinen utilization and only TonB2 supports enterobactin utilization (45).

Besides their roles in iron import, it is apparent that TonB proteins have important roles in signaling pathways. There are 35 predicted TonB-dependent receptors in *P. aeruginosa* PAO1, and an unusually high proportion of these (40%) are believed to encode TonB-dependent transducers (23). The occurrence of multiple TonB proteins in PAO1 may reflect an important regulatory role for these proteins, including involvement in siderophore-mediated signaling networks. The data presented here show that TonB1 is an important component of the pyoverdine-mediated signaling pathway in *P. aeruginosa*. It remains to be determined whether TonB2 and TonB3 play roles in iron acquisition or gene expression mediated by other siderophores.

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