Role of Cell Surface Signaling in Proteolysis of an Alternative Sigma Factor in *Pseudomonas aeruginosa*[▽]

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Alternative sigma factor proteins enable transcription of specific sets of genes in bacterial cells. Their activities can be controlled by posttranslational mechanisms including inhibition by antisigma proteins and proteolytic degradation. PvdS is an alternative sigma factor that is required for expression of genes involved in synthesis of a siderophore, pyoverdine, by *Pseudomonas aeruginosa*. In the absence of pyoverdine, the activity of PvdS is inhibited by a membrane-spanning antisigma factor, FpvR. Inhibition is relieved by a cell surface signaling pathway. In this pathway, a combination of pyoverdine and a cell surface receptor protein, FpvA, suppresses the antisigma activity of FpvR, enabling transcription of PvdS-dependent genes. In this research, we investigated proteolytic degradation of PvdS in response to the signaling pathway. Proteolysis of PvdS was observed in strains of *P. aeruginosa* in which FpvR had anti-sigma factor activity due to the absence of pyoverdine or the FpvA receptor protein or overproduction of FpvR. Suppression of antisigma activity by addition of pyoverdine or through the absence of FpvR prevented detectable proteolysis of PvdS. The amounts of PvdS were less in bacteria in which proteolysis was observed, and reporter gene assays showed that this reduction was not due to decreased expression of PvdS. In wild-type bacteria, there was an average of 730 molecules of PvdS per cell in late exponential growth phase. Our results show that proteolysis and amounts of PvdS are affected by the antisigma factor FpvR and that this activity of FpvR is controlled by the cell surface signaling pathway.

Pseudomonas aeruginosa can be isolated from a wide range of environmental sources and is also a major pathogen of immunocompromised individuals and of patients with cystic fibrosis. Strains of *P. aeruginosa* secrete pyoverdines, yellow-green fluorescent siderophores that have a high affinity ($\sim 10^{32}$) for Fe^{3+} ions, with different strains secreting different pyoverdines (25). Following iron chelation, ferripyoverdines are taken up by the bacteria via high-affinity cell surface receptors and the iron is released for incorporation into bacterial proteins (30). Pyoverdines are required for infection in animal models of disease (24, 37) and can also be isolated from the sputum of patients with cystic fibrosis (12; I. L. Lamont and L. W. Martin, unpublished data). Pyoverdine-deficient mutants are altered in their ability to form biofilms (3), emphasizing the biological importance of these molecules.

Pyoverdine synthesis and iron acquisition have been best studied with *P. aeruginosa* strain PAO1. Genes required for pyoverdine synthesis in this strain have been characterized, and some of the corresponding enzymes have also been studied (reviewed in reference 39). Pyoverdine synthesis is dependent on an alternative sigma factor, PvdS, that enables RNA polymerase to recognize the promoters of pyoverdine synthesis genes, promoters that are not recognized by other sigma factors (10, 26, 43). Sigma factors have been grouped into families

on the basis of their protein sequences (29), and PvdS is a member of group IV (also known as the extracytoplasmic sigma factors). Sigma factors in this group are typically synthesized in response to specific environmental cues and direct the expression of genes related to extracytoplasmic functions (15).

The activities of many alternative sigma factors are controlled by antisigma factors that can bind the sigma factor to prevent it from interacting with RNA polymerase or target promoters (8, 14, 17, 34). FpvR is an antisigma factor that binds PvdS (32), with the antisigma activity of FpvR being controlled by the concentration of pyoverdine (22). In the absence of pyoverdine, FpvR inhibits the activity of PvdS. The presence of pyoverdine overcomes this inhibition so that PvdS is active and pyoverdine synthesis genes are expressed. This requires the active participation of FpvA, a cell surface (outer membrane) receptor for ferripyoverdine. FpvA is one of a subgroup of ferrisiderophore receptors that enables both uptake of a ferrisiderophore and control of gene expression in response to the presence of the cognate ferrisiderophore (reviewed in reference 21). This process depends on a domain of FpvA (the signaling domain) that is located in the periplasm (44). Genetic evidence (18, 22, 33) indicates that in the presence of ferripyoverdine, the signaling domain of FpvA interacts with FpvR to suppress its antisigma activity; in the absence of ferripyoverdine (or through mutation the FpvA signaling domain), this region does not interact with FpvR, which consequently inhibits the activity of PvdS and suppresses gene expression. Systems of this sort, in which control of gene expression involves a cell surface receptor, a membrane-spanning antisigma factor, and an alternative sigma factor, have been termed cell surface signaling systems (6, 40).

The activities of a number of sigma factors are controlled

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through proteolytic degradation. These include the starvation and general starvation sigma factor RpoS (σ^{38}), which is degraded in logarithmic-phase cells following tagging by RssB (reviewed in reference 16); the heat shock sigma factor RpoH (σ^{32}), which is bound by chaperones including DnaK and GroEL and then rapidly degraded by proteases in the absence of heat shock but is stabilized during heat shock due to titration of chaperones by denaturation of other proteins (11, 35); and FliA (σ^{28}), which directs expression of flagellar genes of *Escherichia coli* (4). A common theme in these systems is binding of a partner protein prior to proteolytic degradation of the sigma factor.

The aim of the research described here was to determine if binding of PvdS by FpvR can lead to proteolysis of PvdS and affect cellular amounts of the sigma factor. In addition, we quantified the amount of PvdS in wild-type bacteria.

MATERIALS AND METHODS

Strains, growth conditions, and enzyme assays. *Escherichia coli* K12 strain BL21 (36) containing plasmid pPROEx:pvdS was grown in L broth containing ampicillin (25 μ g/ml) as described previously (41). *P. aeruginosa* PAO1 and strains carrying mutations in *fpvA*, *fpvR*, *pvdS*, *pvdD*, *pvdF*, or *pvdD* and *fpvR* have been described previously (1, 22, 23, 27, 31), as has plasmid pUCP::fpvR, which causes overexpression of *fpvR* in *P. aeruginosa* (22). *P. aeruginosa* was grown in King's B broth (20) at 37°C with aeration at 200 rpm, and growth was monitored by measuring the optical density at 600 nm. Pyoverdine was prepared from *P. aeruginosa* PAO1 as described previously (25). β -Galactosidase assays were carried out with bacteria containing pMP190::PpvdS, in which the *pvdS* promoter is transcriptionally fused to a *lacZ* reporter gene, as described previously (22).

Purification of recombinant PvdS. Recombinant PvdS fused to a hexahistidine tag (hPvdS) was purified from *E. coli* BL21 (pPROEx:pvdS) as described previously (41, 42). Purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (5); no contaminating proteins were detected. Amounts of hPvdS were determined using the DC protein assay (Bio-Rad) in conjunction with a standard curve obtained with solutions of known concentrations of bovine serum albumin.

Preparation of cell extracts and immunodetection of PvdS. Samples (1.1×10^9 CFU) of bacterial culture were centrifuged in a bench-top microcentrifuge (13,000 rpm, 10 min), the supernatant discarded, and the cells frozen at -20°C . The cells were thawed and resuspended in 300 μ l of slot blot buffer (NaCl, 300 mM; Tris-HCl, pH 7.5, 50 mM; SDS, 0.1% [wt/vol]) and lysed by heating (100°C, 20 min). Samples were centrifuged (bench-top microcentrifuge, 13,000 rpm, 10 min) to remove any insoluble debris. Samples were then applied to nitrocellulose membrane (Schleicher and Schuell) supported by three sheets of 3MM paper (Whatmann) in a Minifold II slot blotter (Schleicher and Schuell) in accordance with the manufacturers instructions. Each well was first washed by loading slot blot buffer (150 μ l) into the well and applying a vacuum (Red-Evac PV100; Hoefer Scientific Instruments) until most of the liquid had been drawn through the wells. The vacuum was then turned off, and 75 μ l of cell extract or 150 μ l of purified hPvdS in slot blot buffer was applied to different wells, with each sample applied in triplicate, and the vacuum reapplied to draw liquid through the wells. Wells were washed by loading slot blot buffer (150 μ l) and drawing it through the membrane using a vacuum. The nitrocellulose was removed and any debris removed by wiping gently with a damp piece of 3 MM paper (Whatmann). Proteins in cell extracts were also analyzed by SDS-PAGE followed by Western blotting. For Western blotting, proteins were transferred onto a nitrocellulose membrane using a Mini Transblot cell system (Bio-Rad) according to the manufacturer's instructions. Transfer was carried out at 100 V for 1 h.

For immunodetection of PvdS, blocking was carried out by incubating the membranes in ITBS buffer (Tris-HCl, pH 7.5, 50 mM; NaCl, 250 mM; Tween 20, 0.2% [wt/vol]) containing dried milk powder (5%) with rocking for at least 1 h at room temperature. Membranes were washed three times by immersion in ITBS buffer with rocking for 10 min each time. Membranes were then incubated in ITBS buffer containing anti-PvdS monoclonal antibody (MAb) mAb1308 in ascites fluid (1:5,000 dilution) or MAb 209, MAb 683, or MAb 1195 in cell culture supernatant (1:50 dilution) (45) and incubated for at least 2 h. Following three washes in ITBS, the membrane was incubated in 20 ml of ITBS containing secondary antibody (antimouse antibody-horseradish peroxidase conjugate; Bio-

FIG. 1. Proteolysis of PvdS. Cellular extracts were prepared from the strains shown using bacteria that had been grown to stationary phase (lanes 1 to 9) or late exponential phase (lanes 10 to 13). Proteins were separated by SDS-PAGE, and immunodetection was carried out using anti-PvdS monoclonal antibody mAb1308 except where an alternative antibody is indicated. The position of PvdS and that of a sub-fragment are indicated. FpvR⁺⁺, *P. aeruginosa* PAO1 containing pUCP::fpvR.

Rad) (1:10,000 dilution) for 1 to 2 h. Washing in ITBS was then carried out as before. Detection was carried out using chemiluminescence as described previously (13).

Measurement of PvdS. Following slot blotting and immunodetection, the relative signal intensities of different cell extracts were determined using a Powerlook 1120UTI scanner (Amersham Biosciences Ltd.). Films were scanned at a resolution of 1,200 dots per inch using a blank filter. The ImageQuant TL v2003.01 software program (Amersham Biosciences Ltd.) was used to quantify individual slots, using the "array analysis" function. Negative control values (obtained for cell extracts prepared from PAO1 *pvdS*) were subtracted from all cell extract samples. Cell extracts from *P. aeruginosa* PAO1 were included in each experiment for normalization. Each value is the average from at least four experiments. Statistical analyses were carried out using the SPSS13 software package (SPSS Inc., Chicago, Ill.).

In order to quantify the amount of PvdS, various amounts of hPvdS and cell extract prepared from *P. aeruginosa* PAO1 (9.4×10^7 cells) in late exponential growth phase (optical density at 600 nm between 2.0 and 2.5) were applied to a nitrocellulose membrane by slot blotting and immunodetection was carried out. Relative signal intensities were determined as described above. Standard curves were constructed with the values obtained with hPvdS. The amounts of PvdS in cell extracts were determined using the standard curves, and the average amount over six independent experiments was calculated.

RESULTS

Proteolysis of PvdS. The effect of the transmembrane signaling pathway on proteolysis of PvdS was examined using mutant strains affected in different parts of the pathway. Cell extracts were prepared, and PvdS was analyzed by Western blotting using a monoclonal antibody; the results are shown in Fig. 1. Similar results were obtained with bacteria in stationary phase and at the end of exponential growth phase (Fig. 1; also data not shown). No proteins were detected in a *P. aeruginosa* *pvdS* mutant (data not shown).

All samples contained full-length PvdS. Pvd-negative and FpvA-negative strains in which PvdS-dependent gene expression is suppressed due to FpvR (22) also contained a smaller fragment of PvdS that most likely arises due to proteolysis. This fragment was not seen in a pyoverdine mutant when exogenous pyoverdine was added to the growth medium, a situation that restores PvdS-dependent gene expression (22).

A PAO1 *fpvR* mutant did not have the PvdS subfragment, and a *pvdD fpvR* double mutant, unlike a *pvdD* FpvR-positive strain, did not contain the subfragment (Fig. 1). Conversely, overexpression of *fpvR* from pUCP::fpvR resulted in the ap-

FIG. 2. Relative amounts of PvdS in signaling mutants. (A) The bacterial strains shown, with pyoverdine added as indicated, were grown to late exponential phase, and cell extracts were prepared and analyzed by slot blotting followed by immunodetection of PvdS using monoclonal antibody mAb1308. The amounts of PvdS relative to the amount for strain PAO1 are shown with standard errors. FpvR⁺⁺, *P. aeruginosa* PAO1 containing pUCP::fpvR. (B) Expression of *pvdS*. β -Galactosidase assays were carried out for *P. aeruginosa* strains containing the pMP190::PpvdS reporter gene plasmid, and enzyme units are shown. All values are the means from at least four independent experiments, with standard errors shown.

pearance of the PvdS subfragment even in Pvd-positive bacteria. These findings mirror those obtained for gene expression, where mutation of *fpvR* does not affect PvdS-dependent gene expression in a wild-type background and increases it to wild-type levels in a pyoverdine-deficient strain, and overexpression of FpvR suppresses PvdS-dependent gene expression. They also indicate that FpvR is required for formation of the subfragment.

Influence of pyoverdine signaling pathway on amounts of PvdS. The above data suggested that in the absence of pyoverdine, FpvR directs PvdS into a proteolytic degradation pathway, with the subfragment of PvdS resulting from an early step in that process. The role of the pyoverdine signaling pathway in determining amounts of PvdS was therefore determined. The

amounts of PvdS in different strains were quantified by slot blotting since this permits processing of samples from different strains, with multiple replicates, simultaneously. The results are shown in Fig. 2A. The data show that less PvdS is present in strains lacking pyoverdine (PvdD-negative, PvdF-negative) or the FpvA receptor (FpvA-negative), and this correlates with the occurrence of the PvdS subfragment (Fig. 1). As the monoclonal antibody reacts with a subfragment of PvdS that is presumably inactive, the amount of full-size PvdS protein must be less than the total reactive protein shown in Fig. 2A. Addition of pyoverdine to pyoverdine-deficient mutants but not to the FpvA mutant restored the amount of PvdS to (or above) wild-type levels, consistent with the effect of pyoverdine in suppressing proteolysis (Fig. 1). Overproduction of FpvR reduced the

FIG. 3. Quantification of PvdS. The amounts of PvdS were quantified for *P. aeruginosa* PAO1 in late exponential phase. Cell extracts from strain PAO1 and from PAO1 *pvdS* were analyzed by slot blotting and immunodetection using mAb1308, along with known amounts of purified recombinant PvdS, and a representative blot is shown.

amount of PvdS, consistent with the proteolysis observed in Fig. 1. A mutation in *fpvR* also reduced the amount of PvdS. This mutation did not result in the occurrence of the PvdS subfragment (Fig. 1) but caused reduced expression of *pvdS* (22), and this is the likely reason for the reduced amount of PvdS observed for this strain.

Assays were carried out with a *pvdS::lacZ* reporter construct to determine whether the reduced amounts of PvdS in other strains were also due to reduced *pvdS* expression (Fig. 2B). The results show that expression of *pvdS* is increased relative to that for wild-type bacteria in strains lacking pyoverdine or FpvA. The finding that *pvdS* expression is increased whereas the amount of PvdS is reduced in these strains is consistent with the amount of PvdS being affected by proteolysis.

Amount of PvdS per cell. We next determined the amounts of PvdS in cells of wild-type bacteria in late exponential growth phase. Purified recombinant hPvdS and cell extracts (9.4×10^7 cells per sample) prepared from *P. aeruginosa* PAO1 were analyzed by immunodetection following slot blotting and densitometry. A representative result is shown in Fig. 3. The amounts of PvdS present in different cell extracts were determined from standard curves constructed using values obtained with hPvdS. The average amount of PvdS was 730 molecules per cell (standard error, 120). This value is comparable to that reported recently by other researchers, 582, using a different growth medium (38).

DISCUSSION

The primary aim of this research was to investigate whether the pyoverdine transmembrane signaling system, and in particular the antisigma factor FpvR, influences proteolysis of the alternative sigma factor PvdS. Under all conditions in which PvdS-dependent gene expression occurred at wild-type levels (22), only full-size PvdS was detected in Western blotting (Fig. 1). However, in all samples in which gene expression was repressed, a subfragment of PvdS was also detected. The PvdS subfragment was detected in cells that overproduce FpvR and in Pvd-negative strains but not in a Pvd-negative, FpvR-negative double mutant, suggesting that FpvR is required for formation of the subfragment. Furthermore, less PvdS was

present in strains in which the subfragment was observed (Fig. 2A). Reporter gene assays (Fig. 2B) showed that reduced amounts of PvdS in strains lacking pyoverdine or FpvA are not due to reduced expression of the *pvdS* gene. Our data suggest that a likely reason for reduced amounts of PvdS is increased proteolysis following binding by FpvR. Genetic evidence has shown that the cytoplasmic part of FpvR interacts with PvdS (32). Collectively, these data are consistent with a model in which, in the absence of pyoverdine or FpvA, FpvR binds to PvdS and renders it susceptible to proteolytic degradation, with the subfragment observed in Fig. 1 resulting from an early step in the degradative process. In the presence of pyoverdine and FpvA, the antisigma activity of FpvR is suppressed, resulting in increased expression of PvdS-dependent genes (22) and also wild-type amounts of PvdS (Fig. 2).

Other antisigma factors bind to their sigma factor partners in a way that inhibits recognition of core RNA polymerase or promoter DNA by the sigma factor (7, 8, 34). Binding of σ^E by RseA and FliA (σ^{28}) by FlgM is reversible, and the sigma factor can be released to form holoenzyme complexes and initiate transcription (reviewed in references 2 and 9). It is likely that binding of PvdS by FpvR will prevent PvdS from interacting with core RNA polymerase or promoter DNA, as well as making it susceptible to proteolysis. A critical issue that has yet to be addressed is whether PvdS that is bound to FpvR can be released to form a holoenzyme with RNA polymerase or whether the only fate for bound PvdS is to undergo degradation.

While the manuscript was in preparation, an independent study investigating the intracellular levels of PvdS was published (38). As part of their study, these researchers investigated the influence of the pyoverdine signaling pathway on amounts of PvdS and did not detect differences in amounts of PvdS between wild-type bacteria and signaling mutants (38). One likely explanation for the detection of proteolysis of PvdS and of differences in amounts of PvdS between strains in this study (Fig. 1 and 2) but not in the study of Tiburzi et al. (38) is the growth phase at which bacteria were harvested. In the research reported here, samples were collected at late exponential phase ($t = 6.5$ h) or in stationary phase, whereas in the study of Tiburzi et al., cells were collected during midexponential phase ($t = 4$ h). Consistent with the findings of Tiburzi et al., we did not detect proteolysis of PvdS in bacteria collected in midexponential phase (data not shown). Other technical differences that may have contributed to different findings in the two studies are the different growth media used and the anti-PvdS antibodies that were used. We have used four different anti-PvdS monoclonal antibodies in Western blotting, and in each case we observed the subfragment of PvdS under conditions where the signaling pathway is not active (Fig. 1).

The amount of PvdS during late exponential phase is about 730 molecules per cell under our conditions. This is higher than has been reported for alternative sigma factors in *E. coli*, which are present at up to 350 molecules per cell (19), although it is consistent with data from other researchers (582 molecules per cell [38]). The primary sigma factor RpoD (σ^{70}) is present at 933 molecules per cell in *P. aeruginosa* in growth phase (38). *P. aeruginosa* contains about 21 PvdS-dependent promoters (28), an amount which is likely to be less than 1% of the promoters in the genome. Purification of RNA polymerase

holoenzyme from *P. aeruginosa* (38, 41) demonstrates that the majority of enzyme molecules contain RpoD rather than PvdS, despite the high number of PvdS molecules per cell. This may reflect binding of PvdS molecules by FpvR, inhibiting their association with RNA polymerase even in “signaling-on” cells, or may indicate that PvdS has a lower affinity for RNA polymerase core enzyme than does RpoD.

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