The Fur-Regulated Gene Encoding the Alternative Sigma Factor PvdS Is Required for Iron-Dependent Expression of the LysR-Type Regulator PtxR in *Pseudomonas aeruginosa*

MICHAEL L. VASIL,[1]* URS A. OCHSNER,[1] ZAIGA JOHNSON,[1] JANE A. COLMER,[2]
AND ABDUL N. HAMOOD[2]

*Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262,[1]
and Department of Microbiology and Immunology, Texas Tech University
Health Sciences Center, Lubbock, Texas 74030[2]*

We previously identified a novel regulator of the exotoxin A gene (*toxA*) in *Pseudomonas aeruginosa*, PtxR, that belongs to the LysR family of prokaryotic regulatory proteins. Preliminary data also suggest that PtxR affects the expression of siderophores in *P. aeruginosa*. Because *toxA* expression and siderophore production in this organism are coordinately regulated by the ferric uptake regulator (Fur) and the Fur-regulated alternative sigma factor PvdS, regulation of *ptxR* itself in the context of these regulators was examined. RNase protection analyses of *ptxR* transcription revealed that there are two independent transcription initiation sites (T1 and T2). While transcription from the promoter of T1 is constitutive throughout the growth cycle of PAO1, transcription from the second promoter (P2) is negatively affected by iron. Transcription from the P2 promoter is constitutive in a *fur* mutant under microaerobic conditions but still iron regulated during aerobic growth. High concentrations (>100 nM) of the ferric uptake regulatory protein (Fur) failed to bind to either of the promoter regions of *ptxR* in either gel mobility shift assays or DNase I footprint experiments. These results indicate that Fur indirectly regulates the iron-dependent expression of *ptxR*. Iron-regulated transcription of *ptxR* from the P2 promoter, but not constitutive expression from the P1 promoter, was dependent on the Fur-regulated alternative sigma factor gene *pvdS*, even under aerobic conditions. Consequently, there are two levels of iron-regulated expression of *ptxR*. The iron-regulated expression of *ptxR* under microaerobic conditions from the P2 promoter of *ptxR* is mediated indirectly by Fur through the iron-regulated expression of *pvdS*. In contrast, *pvdS*-mediated iron regulation of *ptxR* under aerobic conditions is Fur independent.

Exotoxin A is one of a growing number of virulence factors produced by *Pseudomonas aeruginosa* that are regulated by multiple environmental factors (7, 14, 18, 20). Not only is the *toxA* gene influenced by the environmental levels of iron, but it is also affected by the presence of certain nucleotides in the growth medium, as well as oxygen tension and temperature (7, 18, 20). Several genes that have an impact on the regulation of exotoxin A production by *P. aeruginosa* have now been described. These genes include *regA*, *vfr*, *fur*, and *pvdS* (1, 3, 6, 9, 16, 19). However, some of these genes have differential effects on the iron-dependent expression of *toxA* and siderophores. Barton et al. reported that Fur affects the iron-regulated expression of *toxA* only under microaerobic conditions (1). That is, a Fur mutant of *P. aeruginosa* was unaffected in iron-regulated expression of *toxA* under aerobic growth conditions, but expression of *toxA* under microaerobic conditions was constitutive in this mutant. In contrast, expression of siderophores was constitutive under aerobic conditions in Fur mutants.

Recently we described a gene, *ptxR*, that also appears to regulate exotoxin A production in *P. aeruginosa* at the transcriptional level (4). The presence of a multicopy plasmid carrying *ptxR* in *P. aeruginosa* results in a four- to fivefold increase in exotoxin A production. Some preliminary evidence suggests that *ptxR* enhances *toxA* transcription through *regA*, a gene encoding a positive regulator of *toxA*. Nonetheless, it is not

clear whether these effects are direct or indirect. PtxR has substantial homology to several members of the LysR family of transcriptional activators (13). There is a helix-turn-helix motif typical of LysR regulators (and DNA binding proteins in general) at the amino terminus of PtxR. Since PtxR, however, has not yet been purified, DNA binding studies have not yet been done on the *regA* or *toxA* promoters. It is also not clear whether *ptxR* is involved in the iron-regulated phase of *toxA* expression (2). No studies on the regulation of the *ptxR* gene itself have been reported. Preliminary observations from our laboratory indicate that multiple copies of *ptxR* also have an impact on siderophore expression in *P. aeruginosa* (5). Previously, we cogently demonstrated that the iron-directed, coordinate expression of *toxA* and siderophores occurs, at least in part, through the ferric uptake regulator (Fur) and the Fur-regulated *pvdS* gene, which encodes an alternative sigma factor (1, 9). Consequently, it is worthwhile to delineate the impact that the levels of environmental iron might have on the expression of *ptxR* itself and to assess whether Fur and PvdS influence the expression of this regulatory gene.

RNase protection assays were used to determine whether transcription of *ptxR* is affected by the same relative levels of iron that negatively regulate the expression of *toxA*, the alternative sigma factor gene *pvdS*, and siderophores in *P. aeruginosa*. The riboprobes required for these assays were synthesized by using suitable oligonucleotide primers. The primers were utilized to amplify certain parts within either the *ptxR* open reading frame or its upstream region. The probe for the 5′ region of *ptxR* comprises bp 52 to 607 of the *ptxR* sequence entered in GenBank (accession no. U35068), while the internal

* Corresponding author. Mailing address: Department of Microbiology, Box B175, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Denver, CO 80262. Phone: (303) 315-8627. Fax: (303) 315-6785. E-mail: Mike.Vasil@uchsc.edu.
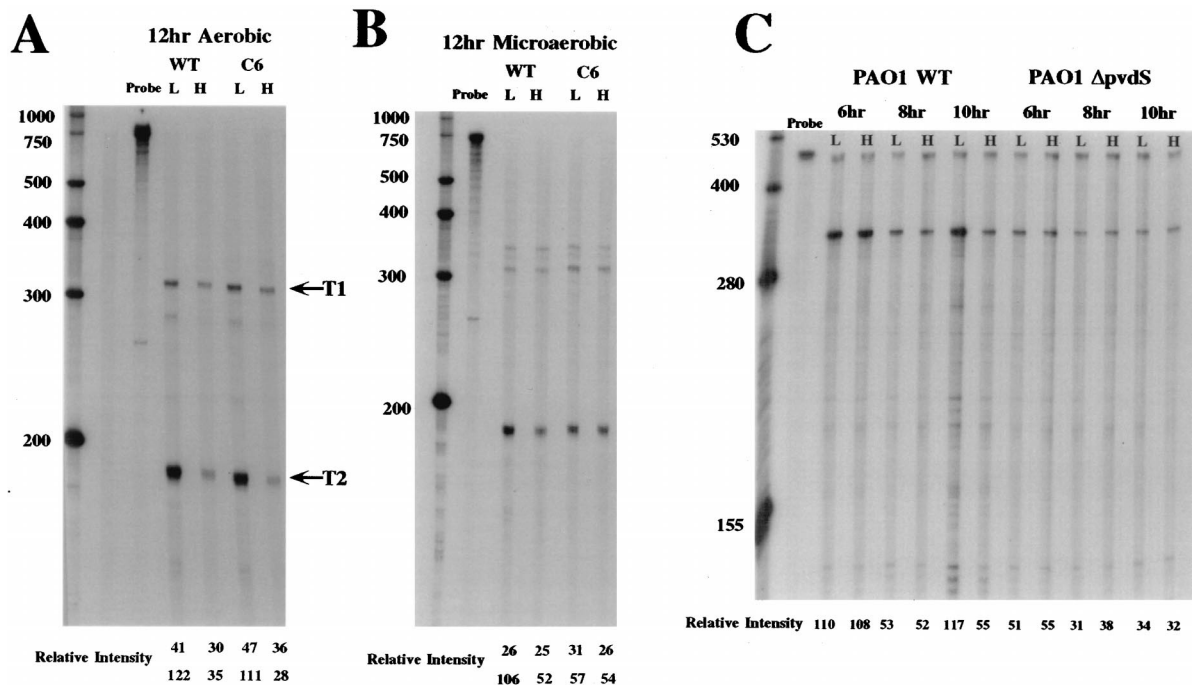
FIG. 1. RNase protection assays of *ptxR* transcription. (A) RNase protection analysis of *ptxR* transcription in the PAO1 wild type and the Fur mutant PAO1C6 grown under aerobic conditions as previously described (1). RNA was isolated at 12 h from cells grown under low (L)- or high (H)-iron conditions (1). The riboprobe used in these experiments encompasses the 5′ region of *ptxR* plus its translational initiation codon. This probe detects both the T1 and T2 transcripts. (B) RNase protection analysis of *ptxR* transcription in the PAO1 wild type and the Fur mutant PAO1C6 grown under microaerobic conditions as previously described (1). (C) RNase protection analysis of *ptxR* mRNA in the PAO1 wild type and its *pvdS* mutant, PAO1Δ*pvdS*. The *ptxR* internal RNA probe was used in these experiments. The higher band in each lane represents an excess of undigested probe. The lower band in each panel represents *ptxR* mRNA, including both T1 and T2 transcripts. The cultures were grown aerobically with (H) and without (L) iron. Samples were obtained at the times indicated. The RI for each band was determined by using NIH Image software, version 1.55. An RNA size standard ladder is on the left of each panel. The lanes labeled probe contain the labeled probe used in these studies that was not digested with the RNase cocktail. WT, wild type; C6, PAO1C6.

probe encompasses bp 740 through 1066. The amplified DNA fragments were cloned into the pCRII vector (Invitrogen Corporation, Carlsbad, Calif.). RNA probes were then generated from these fragments by runoff transcription from either the SP6 or T7 promoter using the Riboprobe kit (Promega). The riboprobe for mapping the transcriptional start sites of *ptxR* extended from 504 bp 5′ to the *ptxR* initiation codon to the first 61 bp of *ptxR* and carries an additional ∼100 bases of pCRII vector sequences. An additional riboprobe used in some of these studies (see Fig. 1C) included the region that encodes an internal portion of PtxR as well as additional pCRII vector sequences. RNase protection experiments were done as previously described (1, 9). Autoradiographs of the dried gels were scanned and imported into Adobe Photoshop for adjustment of brightness and contrast (Adobe Systems Incorporated, San Jose, Calif.). Quantitative analysis of the autoradiographs was performed with NIH Image software, version 1.55.

RNase protection experiments were first performed with the 565-bp *ptxR*-specific RNA probe that extends 5′ to the translational initiation codon of *ptxR*. Ostensibly, this probe extends beyond the transcriptional initiation site and promoter region of *ptxR*. In Fig. 1A, detection of two protected fragments in the 12-h RNA sample from cells grown aerobically under low-iron conditions suggests that *ptxR* has two transcriptional initiation sites under these conditions, T1 and T2, but at this point it was also possible that T2 was a product of the processing of the T1 transcript. Another RNase protection analysis of RNA isolated from 6- and 10-h samples corroborated these data (data not shown). Transcription from the T1 site, the putative P1 promoter, does not appear to be strongly influenced by iron and appears constitutive during all growth phase periods (i.e., 6, 8,

10, and 12 h) examined. Based on the size of the protected fragment in these experiments, the T2, iron-dependent, transcription start site is ∼166 ± 3 bp 5′ to the *ptxR* translation initiation codon.

Next, the issue of whether iron-regulated expression of *ptxR* is dependent on Fur was addressed by examination of *ptxR* expression in the well-characterized *P. aeruginosa* Fur mutant, PAO1C6 (1). Figure 1A shows that the iron-regulated pattern of *ptxR* expression under aerobic conditions from the P2 promoter in the Fur mutant is essentially identical to that observed in the wild-type strain (PAO1WT) (relative intensities [RIs] of PAO1WT of 122 [low iron] versus 35 [high iron]; compared to RIs of PAO1C6 of 111 [low iron] versus 28 [high iron]). In contrast, in the PAO1C6 Fur mutant grown under microaerobic conditions, iron-dependent transcription of *ptxR* from this promoter was constitutive (RIs of 57 [low iron] versus 54 [high iron] [Fig. 1B]) but still significantly influenced by iron in PAO1WT (RIs of 106 [low iron] versus 52 [high iron]). These data suggest that the T1 and T2 transcripts are independent and that the T2 transcript is not merely processed from the T1 transcript since the T2 transcript is differentially expressed during both the growth phase and in response to the levels of environmental iron, as well as to Fur.

The initial observations regarding the iron-regulated expression of *ptxR* were supported by examining the expression of β-galactosidase from a *ptxR::lacZ* translational fusion carried on a plasmid in PAO1WT and in the Δ*pvdS* mutant (PAO1Δ*pvdS*). The level of β-galactosidase activity expressed from this fusion was determined as previously described (8, 9). However, for reasons that are not clear at the present time, the level of expression of β-galactosidase in these studies was too

low to be completely certain that there was a significant difference between expression of the *ptxR::lacZ* fusion in the wild-type strain and the Δ*pvdS* mutant. Nevertheless, there was a reproducible greater-than-twofold higher level of β-galactosidase expression in PAO1WT carrying the *ptxR::lacZ* fusion when it was grown under iron-limiting conditions than there was in PAO1Δ*pvdS* carrying this fusion when it was grown under the same conditions. There was no difference in the levels of β-galactosidase in these strains when they were grown in iron-replete medium (data not shown). These data correlate well with the RNase studies described in this report and together with the RNase protection experiments support our contention that there is a PvdS-dependent iron-regulated expression of *ptxR* and a PvdS-independent expression of this gene which is not iron regulated.

We also performed additional RNase protection experiments using RNA preparations from different times in the growth phase of PAO1 and an independent riboprobe composed only of *ptxR* coding sequences. This *ptxR*-specific RNA probe contains the region encoding ~110 amino acids of PtxR. The experimental results shown in Fig. 1C were obtained with RNA samples taken at 6, 8, and 10 h from cells grown aerobically in the presence or absence of added iron. Quantitative analysis of the autoradiograph revealed no significant differences in the amount of *ptxR* mRNA detected in cells grown for 6 or 8 h under high- or low-iron conditions (RI at 6 h, 110 [low iron] versus 108 [high iron]; RI at 8 h, 53 [low iron] versus 52 [high iron]). However, at the 10-h time point, cells grown under iron-limiting conditions produced levels of *ptxR*-specific mRNA more than twofold higher than those from cells grown under iron-replete conditions (RI, 117 [low iron] versus 55 [high iron]). It should be noted that the internal probe used in these studies does measure both the T1 and T2 transcripts detected in the previous experiments. Accordingly, at least some of the transcriptional activity detected at 10 h in the cells grown in iron-replete medium is likely the result of expression from the P1 promoter. To confirm and more precisely identify the transition to iron-regulated transcription, additional RNA samples were obtained from cells grown for 6 and 12 h (RNase protection autoradiograph not shown). Again, at 6 h, no difference in the amount of *ptxR* mRNA was detected in cells grown in the presence or absence of iron (RI at 6 h, 120 [low iron] versus 114 [high iron]). By contrast, at 12 h, significantly higher levels of *ptxR*-specific message were detected in cells grown in low-iron medium than in cells grown in high-iron medium (RI, 121 [low iron] versus 52 [high iron]). These results confirm the RNase protection studies using the 5′ probe, which indicated that there are most likely two independent transcriptional initiation sites for this gene. One is constitutively expressed throughout the growth cycle, while the other is negatively influenced by iron in the late logarithmic and early stationary phases of growth.

Additional data strongly supporting these assertions are shown in Fig. 2. In these experiments, we used the probe that encompasses both promoters of the *ptxR* gene (see above). While there were no significant differences in the expression of either the T1 or T2 transcript at the 6-h time point with respect to whether the cells were grown under iron-deficient or iron-replete conditions (data not shown), there was a significant difference in the amount of the T2 transcript detected in the PAO1 wild-type strain grown for 12 h under iron-limiting conditions or iron-replete conditions (RI, 102 [low iron] versus 42 [high iron]). In contrast, there was no significant difference in the amount of the T2 transcript detected in the Δ*pvdS* mutant grown under iron-replete or iron-deficient conditions (RI, 33 [low iron] versus 44 [high iron]). Also in support of our hy-
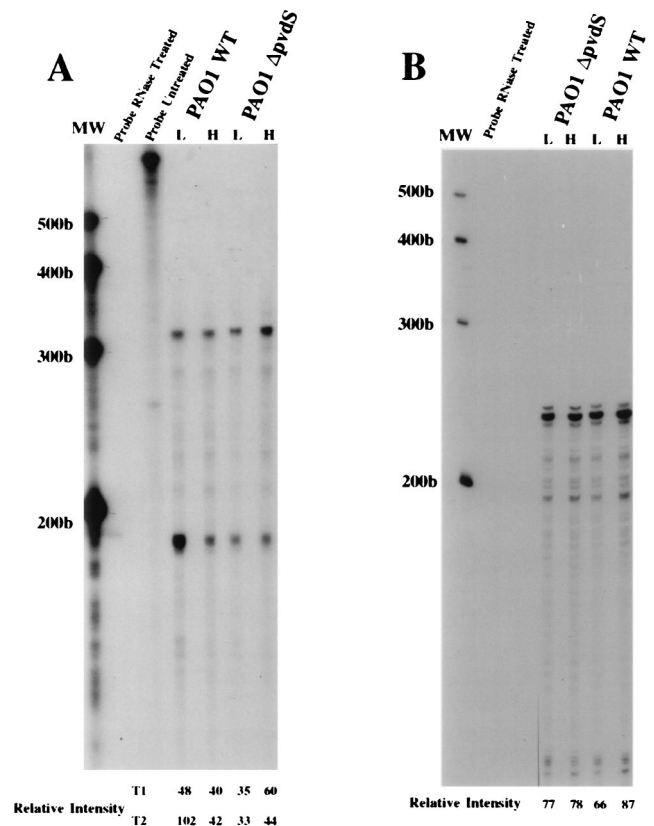

FIG. 2. RNase protection analysis in the PAO1 wild type (PAO1WT) and its *pvdS* mutant, PAO1Δ*pvdS*. (A) The probe from the promoter region of the *ptxR* gene described in the text was used in these experiments. Included are the probe without any added mRNA, digested with the RNase cocktail, and the undigested probe. The cultures were grown aerobically with (H) and without (L) iron. All samples were obtained at 12 h. (B) The RNA samples were examined with the probe generated from the constitutively expressed lipoprotein gene (*oprX*) described in the text and elsewhere (1) and entered in GenBank under accession no. AF050676. Although included in the analysis, the undigested probe is not shown in this figure. The RI for each band was determined by using NIH Image software, version 1.55. MW, molecular size standards (measured in bases).

pothesis that the *ptxR* gene has an iron-dependent and an iron-independent promoter, the levels of the T1 transcript remained relatively constant regardless of the level of iron. There does appear to be a slightly increased expression of the T1 transcript in the Δ*pvdS* mutant, but this occurs under iron-replete conditions only. In the experiment illustrated in Fig. 2B, we also provided a control for the amount of mRNA in the samples analyzed in this study.

We previously reported that a gene encoding an outer membrane-associated lipoprotein (*oprX*; GenBank accession no. AF050676), which is divergently transcribed from the *fur* gene, is constitutively expressed in *P. aeruginosa* with regard to growth phase and to levels of iron in the growth media (1). In this assay, the same RNA samples used to generate the results shown in Fig. 2A were examined by RNase protection assays using the probe derived from the constitutively expressed lipoprotein gene. As shown in Fig. 2B, the relative levels of this transcript are constant for each of the strains or conditions examined. (It is important to note that the order of the PAO1WT and PAO1Δ*pvdS* samples are reversed in Fig. 2A and B.) The data from these RNase protection assays using the probe from the constitutively expressed lipoprotein gene strongly indicate that the differences we have observed for the *ptxR* T2 transcript are not caused by errors in the determination of the

amount of RNA used in the different samples. Moreover, in support of the view that the band we designate as the T1 transcript is not the result of incomplete digestion of unhybridized RNA, we have provided data in Fig. 2 showing that the probes in the absence of any mRNA sample are completely digested by the RNase cocktail used in these experiments.

Based on these RNase protection data and our preliminary *ptxR*::*lacZ* fusion studies (see above), *ptxR* appears to be expressed at a low level in PAO1. This low level of expression is a characteristic feature of several genes encoding LysR-type regulators (6).

The ferric uptake regulator (Fur) in *P. aeruginosa* and in numerous other prokaryotic organisms is the central regulatory protein influencing the expression of iron-regulated genes. This is accomplished by iron-bound Fur interacting with an operator site (Fur box) in iron-regulated genes and directly repressing their synthesis by inhibiting transcription initiation. However, it is also recognized that Fur may exert its effect indirectly by altering the expression of activators or sigma factors required for the expression of iron-regulated genes. To test whether the former scenario applies to *ptxR* expression, we examined whether Fur purified from *P. aeruginosa* could directly bind to the promoter region of *ptxR* at biologically relevant concentrations (e.g., <1 μM). Both gel mobility shift assays and DNase I footprint assays performed as previously described were used for this purpose (1, 10, 11). A region that extends more than 500 bp 5′ to the initiation codon of *ptxR* was examined in these assays. This noncoding region does contain two sequences that have a match in 11 of 19 nucleotides with a consensus Fur box and we have previously shown that a match in 10 of 19 nucleotides can constitute a functional Fur binding operator in *P. aeruginosa* (11). Nevertheless, despite this similarity to a consensus Fur binding site, in both assays concentrations of Fur purified from *P. aeruginosa* as high as 1 μM failed to bind to any sequence in the entire intergenic region between *ptxR* and another gene 5′ to *ptxR* that is divergently transcribed from *ptxR* (12); in contrast, control DNA fragments that are known to bind Fur, including those containing synthetic consensus Fur binding sites, were always positive in these assays. These data suggest that Fur does not regulate the expression of *ptxR* under microaerobic conditions by directly binding to the region 5′ to *ptxR* and by blocking the access of RNA polymerase to either the P1 or P2 promoter.

The *pvdS* gene encodes a putative alternative sigma factor that, in addition to regulating the production of the siderophore pyoverdine, regulates the expression of *toxA* and *regA* in *P. aeruginosa* (9). Expression of the *pvdS* gene appears to be directly influenced by the ferric uptake regulatory protein Fur, which binds to the *pvdS* promoter and blocks its transcription in cells grown under iron-replete conditions (9). Because Fur does not have a direct impact on the iron-regulated phase of *ptxR* expression by binding to either of its promoters, it seems likely that it could impart its effect indirectly through another gene, perhaps *pvdS*. This alternative scenario was examined by analyzing the transcription of *ptxR* in a wild-type PAO1 background and in a previously described mutant of PAO1 carrying a specific deletion in the *pvdS* gene. This PAO1 Δ*pvdS* mutant strain has growth curve characteristics identical to the PAO1 wild-type parental strain (9). The expression of *ptxR* in the PAO1 wild type and in the PAO1 Δ*pvdS* mutant was examined by RNase protection experiments and by analysis of β-galactosidase expression in these strains carrying the *ptxR*::*lacZ* fusion described above. Our data also offer compelling evidence that the iron-regulated P2 promoter of *ptxR* is dependent on the expression of *pvdS* and that Fur regulates the latter phase of *ptxR* expression indirectly in cells grown in a microaerobic

environment but not in *P. aeruginosa* PAO1 grown under aerobic conditions.

An increasingly complex regulatory circuit directing the coordinate expression of *toxA* expression and siderophore production in *P. aeruginosa* is emerging. At least five separate regulatory genes (*regA*, *fur*, *vfr*, *pvdS*, and *ptxR*) have been demonstrated to have a substantial (i.e., greater-than-fivefold) impact on the production of exotoxin A in this opportunistic organism, although it is not yet clear how all of these regulatory factors coordinately exert their effect on production of this virulence determinant or whether they do so all at the same time (1, 3, 6, 9, 16, 19). It has been known for a number of years that the iron-regulated phase of *regA*, which encodes a positive activator of *toxA* transcription, is intertwined with the iron-regulated phase of *toxA* (1–3, 6, 16, 17). More recently, we demonstrated that Fur is required for this iron-dependent control of *regA* and, ultimately, *toxA* (1–3, 6, 16, 17). However, we found that Fur regulates the expression of *toxA* only under microaerobic conditions; under aerobic conditions, the iron-regulated expression of *toxA* is unaffected in the Fur mutant employed in the present studies (see below). We conjectured that there is an additional iron-dependent regulator controlling the expression of these genes under aerobic conditions (1, 9). In support of this hypothesis, we discerned that the gene encoding the putative alternative sigma factor PvdS is situated between Fur and the iron-dependent regulation of *regA* and *toxA* in the regulatory pathway leading to optimal production of exotoxin A (1, 9). Concerning other possible regulators of *toxA*, West et al. demonstrated that the product of the *vfr* gene, which is highly homologous to the catabolite repressor protein (Crp) of *Escherichia coli*, is required for the optimal expression of both *regA* and ultimately *toxA* (19). Vfr seems to exert its effect by directly binding to the *toxA* and *regA* promoters, enhancing their transcription. However, the issue of whether environmental or physiological conditions (e.g., levels of cyclic nucleotides) exert an effect on binding and regulation by Vfr is not clear at the present time. There does not appear to be any significant direct association between Vfr and the iron-regulated expression of *regA* or *toxA*.

In our previous and present reports, we provide insight into another level of complexity in the processes regulating exotoxin A expression. Previous experimental data suggest that *ptxR* also imparts a significant effect on the transcription of *toxA* (4). Optimal enhancement of *toxA* transcription by *ptxR* is detectable in *P. aeruginosa* as soon as the early to mid-logarithmic phase of growth, a pattern that is similar to the enhancement of *toxA* transcription by *regA* (2, 3). We therefore examined how *ptxR* expression might be affected by the environmental levels of iron and by proteins known to play key roles in the iron-regulated expression of *toxA*, PvdS and Fur. Such experiments could help us understand the possible role that *ptxR* might play in the cascade of events leading to the production of exotoxin A. The pattern of regulation of *ptxR* expression by iron and these regulators parallels the pattern of the response of *regA* to these same factors; that is, there is a biphasic pattern to the expression of *ptxR* similar to that observed with *regA* (2, 3). The early phase of transcription from the *ptxR* P1 promoter is constitutive. However, during the late logarithmic stages of growth, transcription of *ptxR* becomes negatively influenced by iron to a significant degree (Fig. 1 and 2). Interestingly, the timing of this biphasic pattern is virtually coincidental with the biphasic pattern of *regA* expression. That is, at some point during the late logarithmic phase to the early stationary phase of growth (at 6 to 10 h), the expression of transcription of both *ptxR* and *regA* becomes significantly influenced by the environmental levels of iron in the growth

medium. However, in contrast to the late expression of *regA*, which is not detectable in iron-replete medium, a low constitutive level of *ptxR* transcription is detected during the later stages of the growth cycle.

Because we had previously implicated Fur and the alternative sigma factor PvdS in the iron-regulated pathways for the expression of *regA* and *toxA*, we postulated that either or both of these factors could also influence the expression of *ptxR*. The data presented in this report clearly link PvdS, and indirectly Fur, to the iron-regulated expression of *ptxR*, although the mechanism by which *ptxR* exerts its effect on the expression of *regA* and *toxA* is still obscure. PtxR also appears to have an effect on siderophore production. Multiple copies of *ptxR* abrogate iron-regulated expression of siderophores on chrome azure S plates. Moreover, *ptxR* is located immediately 3′ to an operon involved in the production of the chromophore of pyoverdine, although it is transcribed in the opposite direction from this operon (15) (Genbank accession no. AF002222).

Determination of the factors that influence the DNA binding activity of this putative LysR-type regulator is a critical issue that needs to be addressed. While the members of this class of regulators are thought to bind DNA, via the DNA binding motif located in their amino-terminal regions, they apparently respond to different factors that have a crucial impact on their ability to bind to the promoter region of a target gene (13). Accordingly, until such a factor is identified for PtxR, it will be difficult to obtain a completely lucid image of the mechanism by which PtxR exerts its effects on the expression of *toxA* or other genes, including those involved in siderophore production. Examination of the ability of PtxR to bind to the promoters *toxA* and other iron-regulated genes will ultimately be required to understand how PtxR influences production of exotoxin A and siderophores. Moreover, although we demonstrated that *pvdS* is required for the iron-dependent phase of *ptxR* expression, it is not clear from the available data whether PvdS directly regulates the P2 promoter of *ptxR* by binding and activating its transcription under iron-deficient conditions or whether there is yet another regulatory gene positioned between *pvdS* and *ptxR*. At the present time, there is not enough information about what constitutes a binding site for PvdS. In the case of the regulation of *toxA* by iron, it appears that an additional regulator (besides PvdS and Fur), perhaps PtxR, is required for the iron-regulated production of exotoxin A under aerobic conditions. In this regard, it is interesting that we found that Fur affects the iron-regulated expression of both *toxA* and *ptxR* only under microaerobic conditions but that Fur has an effect on siderophore expression under aerobic conditions (1, 9).

The data presented in this paper add to the already complicated mechanism for the regulation of exotoxin A production by iron and by oxygen levels. Why such a complex circuitry might be needed for the expression of a single extracellular virulence factor is the most perplexing aspect of this regulation. The answer to this question may be related to the complexity of *P. aeruginosa* and its remarkable ability to survive in diverse environments. It is of interest that *ptxR*, *regA*, and *toxA*, in contrast to *fur* and *pvdS*, apparently do not exist in the other pseudomonads most closely related to *P. aeruginosa*, i.e., *P. fluorescens* and *P. putida*. We performed Southern blot hybridization studies using probes from all of these genes against genomic DNA from diverse *P. aeruginosa* isolates as well as isolates of *P. fluorescens* and *P. putida*. While the probes derived from *fur* and *pvdS* strongly hybridize with genomic DNA from all the species mentioned above even under high-stringency conditions, probes derived from *ptxR*, *regA*, and *toxA* hybridized to genomic DNA from the *P.*

aeruginosa strains examined but failed to hybridize to the genomic DNA from either *P. fluorescens* or *P. putida*, even under low-stringency conditions (12). It is reasonable to suppose that those genes which contribute exotoxin A production are of some importance to *P. aeruginosa* in environments where other fluorescent pseudomonads fail to effectively compete, such as in a compromised mammalian host. It may be crucial for *P. aeruginosa* to maintain multiple activators and repressors for the expression of exotoxin A so that the variety of different environments (e.g., aerobic and microaerobic) that this organism may encounter in such a host do not completely abrogate expression of this virulence determinant. Further experimental analysis of the molecular processes by which the increasing number of regulatory genes such as *ptxR* and *pvdS* interact may provide additional insight into these interesting questions.

REFERENCES

1. **Barton, H. A., Z. Johnson, A. I. Vasil, and M. L. Vasil.** 1996. Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. Mol. Microbiol. **21:**1001–1017.
2. **Frank, D. W., and B. H. Iglewski.** 1988. Kinetics of *toxA* and *regA* mRNA accumulation in *Pseudomonas aeruginosa*. J. Bacteriol. **170:**4477–4483.
3. **Frank, D. W., D. G. Storey, M. S. Hindahl, and B. H. Iglewski.** 1989. Differential regulation by iron of *regA* and *toxA* transcript accumulation in *Pseudomonas aeruginosa*. J. Bacteriol. **171:**5304–5313.
4. **Hamood, A. N., J. A. Colmer, U. A. Ochsner, and M. L. Vasil.** 1996. Isolation and characterization of a *Pseudomonas aeruginosa* gene, *ptxR*, which positively regulates exotoxin A production. Mol. Microbiol. **21:**97–110.
5. **Hamood, A. N., J. A. Colmer, U. A. Ochsner, and M. L. Vasil.** 1998. Unpublished observations.
6. **Hedstrom, R. C., C. R. Funk, O. R. Pavlovskis, and D. R. Galloway.** 1986. Cloning of a gene involved in regulation of exotoxin A expression in *Pseudomonas aeruginosa*. Infect. Immun. **51:**37–42.
7. **Liu, P. V.** 1973. Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. J. Infect. Dis. **128:**506–513.
8. **Miller, J. H.** 1972. Experiments in molecular genetics, 1st ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Ochsner, U. A., Z. Johnson, I. L. Lamont, H. E. Cunliffe, and M. L. Vasil.** 1996. Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated alternative sigma factor PvdS. Mol. Microbiol. **21:**1019–1028.
10. **Ochsner, U. A., A. I. Vasil, and M. L. Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J. Bacteriol. **177:**7194–7201.
11. **Ochsner, U. A., and M. L. Vasil.** 1996. Gene repression by the ferric uptake regulator (FUR) in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc. Natl. Acad. Sci. USA **93:**4409–4414.
12. **Ochsner, U. A., and M. L. Vasil.** 1998. Unpublished observations.
13. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
14. **Shortridge, V. D., A. Lazdunski, and Vasil.** 1992. Osmoprotectants and phosphate regulate expression of phospholipase C in *Pseudomonas aeruginosa*. Mol. Microbiol. **5:**2823–2831.
15. **Stintzi, A., P. Cornelis, D. Hohnadel, J. M. Myer, C. Dean, K. Poole, S. Kourambas, and V. Krishnapillai.** 1996. Novel pyoverdine biosynthesis gene(s) of *Pseudomonas aeruginosa*. Microbiology **142:**1181–1190.
16. **Storey, D. G., D. W. Frank, M. A. Farinha, A. M. Kropinski, and B. H. Iglewski.** 1990. Multiple promoters control the regulation of the *Pseudomonas aeruginosa regA* gene. Mol. Microbiol. **4:**499–503.
17. **Storey, D. G., T. L. Raivio, D. W. Frank, M. J. Wick, S. Kaye, and B. H. Iglewski.** 1991. Effect of *regB* on expression from the P1 and P2 promoters of the *Pseudomonas aeruginosa regAB* operon. J. Bacteriol. **173:**6088–6094.
18. **Vasil, M. L., R. W. Prince, and V. D. Shortridge.** 1992. Exoproducts: Pseudomonas exotoxin A and phospholipase C, p. 59–77. *In* R. B. Fick (ed.), *Pseudomonas aeruginosa* the opportunist: pathogenesis and disease. CRC Press, Inc., Boca Raton, Fla.
19. **West, S., A. Sample, and L. Runyen-Janecky.** 1994. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. J. Bacteriol. **176:**7532–7542.
20. **Woods, D. E., and M. L. Vasil.** 1994. Pathogenesis of *Pseudomonas aeruginosa* infections. Infect. Dis. Ther. **12:** 21–50.