JOURNAL OF BACTERIOLOGY, Aug. 2005, p. 5097–5107 Vol. 187, No. 15
0021-9193/05/$08.00+0 doi:10.1128/JB.187.15.5097–5107.2005

Involvement of AlgQ in Transcriptional Regulation of Pyoverdine Genes in *Pseudomonas aeruginosa* PAO1

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Received 15 February 2005/Accepted 29 April 2005

In response to iron limitation, *Pseudomonas aeruginosa* produces the fluorescent siderophore pyoverdine. Transcription of pyoverdine biosynthetic (*pvd*) genes is driven by the iron starvation sigma factor PvdS, which is negatively regulated by the Fur-Fe(II) holorepressor. We studied the effect of AlgQ, the *Escherichia coli* Rsd orthologue, on pyoverdine production by *P. aeruginosa* PAO1. AlgQ is a global regulatory protein which activates alginate, ppGpp, and inorganic polyphosphate synthesis through a cascade involving nucleoside diphosphate kinase (Ndk). AlgQ is also capable of interacting with region 4 of RpoD. In a reconstituted *E. coli* system, PvdS-dependent transcription from the *pvdA* promoter was doubled by the multicopy *algQ* gene. The *P. aeruginosa* Δ*algQ* mutant exhibited a moderate but reproducible reduction in pyoverdine production compared with wild-type PAO1, as a result of a decline in transcription of *pvd* genes. PvdS expression was not affected by the *algQ* mutation. Single-copy *algQ* fully restored pyoverdine production and expression of *pvd* genes in the Δ*algQ* mutant, while *ndk* did not. An increased intracellular concentration of RpoD mimicked the Δ*algQ* phenotype, whereas PvdS overexpression suppressed the *algQ* mutation. *E. coli rsd* could partially substitute for *algQ* in transcriptional modulation of *pvd* genes. We propose that AlgQ acts as an anti-sigma factor for RpoD, eliciting core RNA polymerase recruitment by PvdS and transcription initiation at *pvd* promoters. AlgQ provides a link between the pyoverdine and alginate regulatory networks. These systems have similarities in responsiveness and physiological function: both depend on alternative sigma factors, respond to nutrient starvation, and act as virulence determinants for *P. aeruginosa*.

Iron is an essential nutrient for almost all forms of life (2), but only small amounts are bioavailable in aerobic environments at neutral pH. To fulfill their nutritional iron requirement, most unicellular organisms and some plants have developed the ability to produce and/or acquire from the environment iron-chelating molecules called siderophores (2).

Under iron-limiting conditions, the opportunistic bacterial pathogen *Pseudomonas aeruginosa* is capable of acquiring iron bound to a variety of exogenous and endogenous chelators (40). One of these chelators, pyoverdine, is the principal iron uptake option for *P. aeruginosa* both in natural environments and in infected hosts (reviewed in reference 61). Pyoverdine is a peptidic siderophore composed of a fluorescent chromophore (a quinoline derivative) linked to a variable amino acid arm (5). Pyoverdine has a high affinity for Fe(III) (K_f, $\sim 10^{32}$ M^{-1}) and is capable of promoting *P. aeruginosa* infection and virulence in various animal models (reviewed in reference 61). A number of genes involved in the biosynthesis of pyoverdine$_{PAO1}$ have been identified to date in the *P. aeruginosa* PAO1 chromosome and have been found to be clustered in the *pvd* locus, which also encompasses the *fpvA* gene encoding the ferripyoverdine$_{PAO1}$ outer membrane receptor FpvA (43). Pyoverdine$_{PAO1}$ synthesis occurs in response to iron limitation (Fe concentration, <1 μM) and is shut off under iron-replete conditions to prevent potential iron overload and toxicity. Transcription of the pyoverdine biosynthesis and transport genes (i.e., *pvd* and *fpvA*) is repressed by binding of the dimeric Fur-Fe(II) holorepressor to the promoter-operator DNA regions (Fur boxes) of master regulatory genes, namely, *pvdS*, *fpvI*, and *fpvR* (44, 61). When there is an iron shortage, Fur repression is relieved, which gives free access to the σ^{70} (RpoD)-dependent RNA polymerase holoenzyme (RNAP) for transcription of *pvdS* (9, 37) and *fpvI* (3, 44), both of which encode extracytoplasmic function (ECF) sigma factors belonging to the iron starvation (IS) subgroup of the RpoD family (30, 63), as well as the *fpvR* gene, which encodes a cognate inner membrane-bound anti-sigma factor (27). PvdS recognizes a conserved DNA sequence called the IS box within target promoters and directs transcription of several genes, namely, the genes implicated in pyoverdine biogenesis (*pvd* genes) and in the expression of proteases and exotoxin A (40, 61). In contrast, FpvI controls only *fpvA* transcription (3, 44). The activity of both IS sigma factors, PvdS and FpvI, is under the control of a signaling mechanism that involves the FpvA receptor and the pyoverdine$_{PAO1}$ molecule itself. In the absence of ferripyoverdine$_{PAO1}$, the activity of both PvdS and FpvI is antagonized by FpvR (3). Binding of ferripyoverdine$_{PAO1}$ to FpvA initiates a signal transduction cascade that involves FpvR and causes the activation of both PvdS and FpvI, which direct the transcription of target genes (3, 27). This sophisticated mechanism ensures that pyoverdine$_{PAO1}$ is produced only when it is effective in delivering iron to the cell through productive engagement of FpvA (3).

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We previously reported that the activity of *pvdA*, *pvdD*, and *pvdE* promoter fusions was significantly lower in the heterologous host *Escherichia coli* overexpressing PvdS than in the homologous PAO1 system, and we suggested that additional regulatory factors could be implicated in the positive control of the *pvd* gene system (30). Interestingly, reduced siderophore levels were observed in the stable alginate-producing *P. aeruginosa* 8830 strain carrying a mutation in the *algQ* gene, also known as *algR2* (49, 60). Alginate is a critical virulence factor in chronic lung infections sustained by *P. aeruginosa* since it confers a typical mucoid phenotype which insulates bacteria from the host defenses (16, 18). The AlgQ protein positively regulates alginate synthesis, as well as the expression of enzymes related to nucleoside triphosphate (NTP) formation, namely, nucleoside diphosphate kinase (Ndk) and succinyl-coenzyme A synthetase. Hence, AlgQ modulates the levels of alginate, GTP, guanosine 3′,5′-bispyrophosphate (the ppGpp alarmone), and inorganic polyphosphate (polyP) (25, 48). Accordingly, the defect in synthesis of these metabolites in the *algQ* mutant 8830R2::Cm can be reversed by complementation with either the *algQ* or *ndk* gene in *trans* (25). AlgQ is 58% identical to the product of the pyoverdine$_{WCS358}$ regulatory gene *pfrA* of *Pseudomonas putida* WCS358 (60). Cross-complementation studies with the *algQ* gene of *P. aeruginosa* and the *pfrA* gene of *P. putida* WCS358 first established the link between pyoverdine and alginate production (60). Indeed, it was demonstrated that PfrA could fully restore alginate production in a *P. aeruginosa* 8830 *algQ* mutant, while AlgQ could partially restore pyoverdine$_{WCS358}$ synthesis in a *P. putida* WCS358 *pfrA* mutant (10, 60). More recently, production of alginate by *P. aeruginosa* PAO1 has been related to iron depletion, tightening the link between the iron starvation response and the regulation of alginate synthesis (24).

It is noteworthy that AlgQ also exhibits 55% similarity (87 of 157 amino acid residues) with the *E. coli* Rsd (regulator of sigma D) protein, which controls the level of functional RpoD subunits by forming a 1:1 complex with RpoD (20, 65). Like Rsd, AlgQ was shown to make contact with the C-terminal region of *P. aeruginosa* RpoD (12). On the basis of these findings, it has been hypothesized that AlgQ can also function as an anti-sigma factor for RpoD, increasing the amount of core RNAP (RNAPc) available for binding by alternative sigma factors (12). At the regulatory level, the *algQ* promoter shows basal expression during the exponential phase and significant activation during the stationary phase (25), recalling the growth phase-dependent expression of *rsd* in *E. coli* (20).

This work was undertaken to address the role of AlgQ in the regulation of pyoverdine biosynthesis by *P. aeruginosa* PAO1. We observed that *pvdA* transactivation in *E. coli* MC4100 carrying a multicopy *pvdS* gene was doubled by the presence of *algQ* in *trans*. We found that the reduced level of pyoverdine$_{PAO1}$ in a *P. aeruginosa* PAO1 *algQ* null mutant correlated with diminished transcription of the *pvdA*, *pvdD*, and *pvdE* biosynthetic genes, while PvdS expression was not affected. No differences in either pyoverdine$_{PAO1}$ production or expression of *pvd* biosynthetic genes were observed upon complementation of the *P. aeruginosa* PAO1 *algQ* null mutant with the *ndk* gene. Increasing the RpoD levels in wild-type *P. aeruginosa* mimicked the *algQ* mutation, while increasing the PvdS levels suppressed the *algQ* mutant phenotype. *E. coli rsd* could partially substitute for *algQ* in transcriptional modulation of *pvd* genes. Hence, we propose that AlgQ is the functional homolog of *E. coli* Rsd. According to our results, AlgQ modulates the expression of *pvd* genes by interacting with free RpoD subunits, enabling PvdS to compete effectively with RpoD for RNAPc binding, thereby favoring transcription initiation at *pvd* promoters.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. The *P. aeruginosa* PAO1 *algQ* null mutant, designated PAO1Δ*algQ*, was a generous gift from M. Foglino (Centre National de la Recherche Scientifique, Marseille, France). This mutant has a deletion extending from bp 5916921 to bp 5917397 of the *P. aeruginosa* PAO1 chromosome, precisely matching the *algQ* coding sequence (data not shown). *E. coli* was routinely grown in LB medium or in M9 minimal medium (46). To reduce iron availability, the iron chelator 2,2′-dipyridyl was added to M9 minimal medium at a concentration of 150 μM. *P. aeruginosa* was grown in NYB or SM9 (64) or in cetrimide agar (Pseudosel; Target). DCAA was used as the low-iron medium for *P. aeruginosa* (62). Media were solidified with 1.2% agar N.1 (Unipath). Antibiotics were used in selective media at the following concentrations: tetracycline, 12.5 μg/ml for *E. coli* and 150 μg/ml for *P. aeruginosa*; chloramphenicol, 30 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; ampicillin, 100 μg/ml for *E. coli*; carbenicillin, 500 μg/ml for *P. aeruginosa*; kanamycin, 25 μg/ml; nalidixic acid, 20 μg/ml; and streptomycin, 25 μg/ml for *E. coli*.

DNA manipulation and genetic techniques. All procedures used for the handling of recombinant DNA have been described previously (46). Transfer of plasmids from *E. coli* to *P. aeruginosa* was performed by triparental mating with the helper plasmid pRK2013 (14). Recombinant plasmids carrying *pfrA*, *algQ*, *rsd*, or *ndk* were obtained by gene amplification and cloning, as indicated below. A 728-bp DNA fragment encompassing the entire *pfrA* gene and its putative promoter was obtained by PCR amplification with primers FW*pfrA* (5′-GGG GCT CGA GCC AGG GGG TAT TAA ACA CT-3′) and RV*pfrA* (5′-GGG GGA TCC TGA TCG CAC CCT ATA CG-3′), corresponding to nucleotides (nt) −171 to −190 and nt 520 to 537 relative to the ATG translation start codon of the *pfrA* gene, respectively, using pASR1A as the template (60). The XhoI and BamHI restriction sites (underlined) were included to make directional cloning of the amplicon into the SalI and BamHI restriction sites of pACYC184 possible, which yielded pACYC*pfrA*. An 896-bp DNA fragment encompassing the entire *algQ* gene and its promoter region (23) was obtained by PCR amplification with primers FW*algQ* (5′-TAT CTC GGC TTC TCC ATC GT-3′) and RV*algQ* (5′-GGG GGA TCC TTC GAG ATC GAC CTG CTG-3′), corresponding to nt −321 to −301 and nt 556 to 575 relative to the ATG translation start codon of the *algQ* gene, respectively, using PAO1 genomic DNA as the template. The *algQ* amplicon was digested with BamHI (site underlined in RV*algQ*) and XhoI (249 bp upstream of the start codon) for directional cloning into the SalI and BamHI restriction sites of pACYC184 and pUCP19, yielding pACYC*algQ* and pUCP*algQ*, respectively. An 848-bp DNA fragment encompassing the entire *rsd* coding region and its indigenous promoter (21) was obtained by PCR amplification with primers FW*rsd* (5′-GCT CTA GAC TAA CCA AAC AGG TTC CCC-3′) and RV*rsd* (5′-TTC CGC GGA TCC CGA ATA AA-3′), corresponding to nt −264 to −238 and nt 565 to 584 relative to the *rsd* start codon, respectively, using *E. coli* MC4100 genomic DNA as the template. The *rsd* amplicon was digested with XbaI and BamHI (sites underlined in the primer sequences), cloned in pUCP19, and then excised by SalI-BamHI digestion and transferred into pACYC184, yielding pACYC*rsd*. The pACYC-derived constructs were individually used to transform *E. coli* MC4100 (7) carrying both pBRXB and the pMP220::P*pvdA* transcriptional fusion (9, 29). To express *algQ* and *rsd* genes from a low-copy-number vector, individual amplicons were ligated as XbaI-BamHI digests into the same sites of pJB785TT (47), yielding pJB*algQ* and pJB*rsd*, respectively. A 708-bp DNA fragment encompassing the entire *ndk* gene and its promoter region (56) was obtained by PCR amplification with primers FW*ndk* (5′-CCG GAA TTC GAG GAA CGG GAC TAG CC-3′) and RV*ndk* (5′-CGC GGA TCC TCA CCC ACG CTC GAT CA-3′), corresponding to nt −260 to −241 and nt 429 to 445 relative to the ATG translation start codon of the *ndk* gene, respectively, using PAO1 genomic DNA as the template. The *ndk* amplicon was digested with EcoRI and BamHI (sites underlined) for directional cloning into pUCP19, yielding pUCP*ndk*. A 2,085-bp DNA fragment encompassing the entire *rpoD* gene and its promoter was obtained by PCR amplification with primers FW*rpoD* (5′-CGG AAT TCA GAG AGC ACT ACA

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or relevant characteristics	Source or reference
P. aeruginosa strains		
PAO1 (ATCC 15692)	Prototroph	American Type Culture Collection
PAO1Δ*algQ*	*algQ* null mutant	M. Foglino
E. coli strains		
MC4100	*araD139 rpsL150 relA1 flbB5301 deoC1 pstF25 rbsR* Δ(*lacZYA-argF*)*U169*, Str^r	7
DH5αF′	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* [Φ80d*lacZ*ΔM15] Nal^r	31
Plasmids		
pRK2013	Helper plasmid; ColE1 replicon, Km^r Mob$^+$ Tra$^+$	14
pUCP18/19	*E. coli-Pseudomonas* shuttle vector derived from pUC18/19; ColE1, pRO1600 replicon *lacZ*α *bla*, Ap^r Cb^r	51
pUCP*algQ*	896-bp PCR-generated fragment, encompassing the entire *algQ* gene and its promoter region XhoI/BamHI digested and the resulting 647-bp fragment ligated to the SalI-BamHI sites of pUCP19	This study
pUCP*ndk*	708-bp PCR-generated fragment, encompassing the entire *ndk* gene and its promoter region, ligated to the EcoRI-BamHI sites of pUCP19	This study
pUCP*rpoD*	2.1-kb PCR-generated fragment, encompassing the entire *rpoD* gene and its promoter region, ligated to the EcoRI-PstI sites of pUCP19	This study
pUCP*pvdS*	700-bp XhoI-HindIII fragment from pQE*pvdS* (29), encompassing the entire *pvdS* gene and the P_{T5} *lacO* control region, ligated to the SalI-HindIII sites of pUCP18	This study
pPV226	1.1-kb BglII fragment of pPV5 ligated to pUCP18	29
pJB785TT	Broad-host-range, low-copy-number vector (one copy/cell in *P. aeruginosa*; five to seven copies/cell in *E. coli*); RK2 replicon, *luc bla* Ap^r Cb^r	47 and references therein
pJB*rsd*	848-bp XbaI-BamHI fragment, encompassing the entire *rsd* gene and its promoter region, ligated to pJB785TT	This study
pJB*algQ*	896-bp XbaI-BamHI fragment, encompassing the entire *algQ* gene and its promoter region, ligated to pJB785TT	This study
pMP190	Broad-host-range, low-copy-number promoter-probe vector; IncQ replicon, *lacZ* Cm^r Tra$^-$	53
pMP220	Broad-host-range, low-copy-number promoter-probe vector; IncP replicon, *lacZ* Tc^r Tra$^-$	53
pMP220::P*pvdA*	300-bp SphI/BglII fragment, encompassing the entire *pvdA* promoter, ligated to pMP220 in the same orientation as the reporter *lacZ* gene (formerly designated pPV51)	29
pMP220::P*pvdS*	587-bp BglII-EcoRI fragment, encompassing the entire *pvdS* promoter, ligated to pMP220 in the same orientation as the reporter *lacZ* gene	1
pMP190::P*pvdD*	Promoter of *pvdD* pyoverdine biosynthesis gene directionally cloned into pMP190	9
pMP190::P*pvdE*	Promoter of *pvdE* pyoverdine biosynthesis gene directionally cloned into pMP190	9
pACYC184	p15A replicon, Cm^r Tc^r	46
pACYC*algQ*	647-bp PCR-generated fragment, encompassing the entire *algQ* gene and its promoter region, ligated to the SalI-BamHI sites of pACYC184	This study
pACYC*pfrA*	728-bp PCR-generated fragment, encompassing the *pfrA* gene and its putative promoter region, ligated to the SalI-BamHI sites of pACYC184	This study
pACYC*rsd*	848-bp PCR-generated fragment, encompassing the entire *rsd* gene and its promoter region, ligated to the SalI-BamHI sites of pACYC184	This study
pBR322	ColE1 replicon, Ap^r Tc^r	46
pBRXB	1.8-kb XhoI-BamHI fragment containing *pvdS* ligated to pBR322	9
pASR1A	9.3 kb KpnI fragment containing the *P. putida* WCS358 *pfrA* gene	60

GTG TTG-3′) and RV*rpoD* (5′-GAA CTG CAG GCG GTC TCG CGG TGG A-3′), corresponding to nt −105 to −126 and nt 1941 to 1959 relative to the ATG translation start codon of the *rpoD* gene, respectively, using PAO1 genomic DNA as the template. EcoRI and PstI restriction sites (underlined) were included to make directional cloning of the amplicon into the same restriction sites of pUCP19 possible, yielding pUCP*rpoD*. The *pvdS* gene under the transcriptional control of the T5 promoter (P_{T5}) was excised from vector pQE*pvdS* (29) by XhoI-HindIII digestion and ligated into the SalI-HindIII sites of pUCP18, yielding pUCP*pvdS*. Recombinant pUCP derivatives were individually introduced into *P. aeruginosa* PAO1 cells.

Pyoverdine$_{PAO1}$ determination and β-galactosidase assays. *P. aeruginosa* strains were grown for 48 h at 37°C on cetrimide agar plates. Isolated colonies were suspended in saline, the A_{600} was adjusted to ~1.0, and the preparations were diluted 1:50 in prewarmed DCAA. The cultures were grown at 37°C with vigorous aeration (250 rpm in a New Brunswick 25 orbital shaker). Pyoverdine$_{PAO1}$ was quantified by measuring the absorbance at 405 nm of culture supernatants diluted 1:2 in 100 mM Tris-HCl (pH 8.0), using a Perkin-Elmer LS50 spectrophotometer (64).

The *pvdA*, *pvdD*, *pvdE*, and *pvdS* promoters cloned upstream of the *lacZ* reporter gene in plasmids pMP220::P*pvdA*, pMP190::P*pvdD*, pMP190::P*pvdE*, and pMP220::P*pvdS*, respectively, have been described previously (1, 9, 29). For reporter gene activity measurements, *P. aeruginosa* strains harboring the *pvd*::*lacZ* transcriptional fusions were grown for 14 h at 37°C in DCAA supplemented with the appropriate antibiotics. The cultures were then diluted 1:100 in the same medium with or without 100 μM $FeCl_3$ and subcultured for 4 to 6 h with shaking until the A_{600} was ~0.4. *E. coli* MC4100 carrying both pMP220::P*pvdA* and pBRXB plus pACYC*algQ*, pACYC*pfrA*, or pACYC*rsd* were grown for 18 h at 37°C in M9 minimal medium containing 100 μg/ml ampicillin, 10 μg/ml

tetracycline, and 30 μg/ml chloramphenicol. The cultures were then diluted 1:100 in the same medium containing either 100 μM $FeCl_3$ or 150 μM 2,2′-dipyridyl for an additional 12 h of growth at 37°C (final A_{600} in low-iron medium, ~0.8; final A_{600} in high-iron medium, 1.2). The β-galactosidase (LacZ) activity was determined spectrophotometrically using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. The activity was normalized to the A_{600} of the bacterial culture and was expressed in Miller units (36). The results of β-galactosidase assays were expressed as means of at least six independent experiments performed in duplicate. To overcome the experimental fluctuations resulting from variable traces of iron in different DCAA batches, β-galactosidase levels were also expressed as percentages relative to the level obtained for *P. aeruginosa* carrying the control vectors (pUCP19 or pJB785TT), which was considered 100%. Standard deviations were calculated for each raw data set and are reported below for mean percent values. The chi-square test was used to assess the statistical significance of differences between β-galactosidase activity values (with $P \leq 0.05$).

Production of a mouse polyclonal antiserum against PvdS. PvdS was purified as previously reported (30). Five BALB/c mice were immunized by intramuscular injection of 20 μg of protein dissolved in saline emulsified 1:1 in complete Freund's adjuvant (Sigma). The mice were given a booster immunization consisting of 10 μg of protein emulsified 1:1 with Freund's incomplete adjuvant (Sigma) 14 and 28 days later. On day 45 after the first immunization, the mice were bled, and the antibody titer in each serum was determined. Animal experiments were performed according to ethical guidelines for the conduct of animal research (D.L.vo 116/92).

SDS-polyacrylamide gel electrophoresis, immunoblotting, and densitometry. Bacterial cultures were harvested by centrifugation and suspended in saline for protein content determination. The protein concentration was determined using a *DC* protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Samples containing known amounts of protein were suspended in gel loading buffer (0.25 M Tris-HCl, 2% sodium dodecyl sulfate [SDS], 10% 2-mercaptoethanol, 20% glycerol), heated at 100°C for 5 min, and separated on a 0.1% SDS–15% polyacrylamide gel as described by Laemmli (26). After electrophoresis, gels were stained with Coomassie brilliant blue (46), or resolved proteins were electrotransferred onto a nitrocellulose filter (Hybond C extra; Amersham) using a semidry transfer unit (Hoefer Scientific Instruments) for 1 h at 150 mA. The filters were blocked with 2× TBST (100 mM Tris-HCl [pH 8.0], 1.0 M NaCl, 0.1% Tween 20) containing 1% bovine serum albumin, washed with 2× TBST, and incubated with polyclonal anti-PvdA (41) or anti-PvdS mouse antisera or with monoclonal anti-RpoD antibodies (Neoclone) diluted 1:100, 1:500, and 1:1,000 with 2× TBST, respectively. Proteins were detected by using secondary anti-mouse antibodies conjugated with either alkaline phosphatase (Promega) or horseradish peroxidase (Calbiochem). Final development was performed with the 5-bromo-4-chloro-3-indoylphosphate (BCIP) and nitroblue tetrazolium chloride reagents for colorimetric determinations (Promega) or with the Amersham ECL chemiluminescent reagents (Amersham Biosciences), followed by exposure to X-ray film (Kodak) for autoradiography. Densitometric measurements of band intensities were obtained by use of the Quantity One software and a Gel Doc 2000 charge-coupled device camera (Bio-Rad).

RNA purification and primer extension. Total RNA for primer extension analysis (100 μg for each reaction) was extracted from *P. aeruginosa* and *E. coli* cultures grown in DCAA (A_{620}, ~0.6) and M9 minimal medium (A_{620}, ~0.8), respectively, as previously described (29). For primer extension analysis, 1 pmol of oligonucleotide RV*P*pa (5′-GGC GGT TGC AGT TGC CTG AGT CAT-3′; complementary to the coding strand of the *pvdA* gene from the eighth codon to the ATG translation start site) was end labeled with [γ-^{32}P]ATP (Amersham Biosciences) and used for the reverse transcription reaction as described elsewhere (29). The unlabeled primer was used to sequence the DNA region upstream of the *pvdA* gene from plasmid pPV226, using a T7 sequencing kit (Pharmacia) and [α-^{32}P]dATP. Primer extension products were run in parallel in the sequencing reactions to map the start sites of the transcripts.

In silico sequence and genome analysis. Searches for *P. aeruginosa* PAO1 AlgQ homologues were performed by the BLASTP network service (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). Sequences were also retrieved from the Clusters of Orthologous Groups (COG) database (COG3160; http://www.ncbi.nlm.nih.gog/COG/new). Genomic contexts were obtained from http://www.pseudomonas.com and http://genome.jgi-psf.org/microbial/.

RESULTS

AlgQ, PfrA, and Rsd increase the PvdS-dependent activity of the *pvdA* promoter in the heterologous *E. coli* host. To analyze the involvement of the *algQ*, *pfrA*, and *rsd* gene products in the transcriptional regulation of pyoverdine genes, we performed a heterologous expression assay with *E. coli* MC4100 carrying both the pMP220::P*pvdA* reporter gene system and plasmid pBRXB, which harbored the *pvdS* sigma factor gene under the control of its iron-regulated promoter (9, 29). Plasmids pACYC*algQ*, pACYC*pfrA*, and pACYC*rsd*, containing the *algQ*, *pfrA*, and *rsd* coding sequences with their indigenous promoters, respectively, were individually introduced into *E. coli* MC4100(pMP220::P*pvdA*, pBRXB) for measurement of β-galactosidase activity under iron-depleted and iron-replete conditions (Table 2). Compared with the *E. coli*(pACYC184, pBRXB) control strain, the iron-regulated P*pvdA* activity was doubled by the presence of either *algQ* or *rsd* and was trebled by *pfrA*. In the absence of pBRXB (*pvdS*), no P*pvdA* activity was detected in *E. coli* MC4100, irrespective of the presence of transactivating genes. This indicates that the *algQ*, *pfrA*, and *rsd* gene products, in concert with PvdS, contribute to the positive control of *pvdA* expression in the heterologous *E. coli* system.

TABLE 2. Effect of *algQ*, *pfrA*, and *rsd* on P*pvdA*::*lacZ* activity in *E. coli* MC4100 expressing the *pvdS* gene

Plasmids	LacZ activity[a]	
	M9 medium + 2,2′-dipyridyl[b]	M9 medium + $FeCl_3$[b]
pMP220::P*pvdA* + pBR322	35	31
pMP220::P*pvdA* + pBRXB	256	27
pMP220::P*pvdA* + pBRXB + pACYC184	214	29
pMP220::P*pvdA* + pBR322 + pACYC*algQ*	32	33
pMP220::P*pvdA* + pBR322 + pACYC*pfrA*	35	30
pMP220::P*pvdA* + pBR322 + pACYC*rsd*	33	31
pMP220::P*pvdA* + pBRXB + pACYC*algQ*	450	40
pMP220::P*pvdA* + pBRXB + pACYC*pfrA*	621	51
pMP220::P*pvdA* + pBRXB + pACYC*rsd*	419	48

[a] β-Galactosidase (LacZ) activity is expressed in Miller units (36). The values are means of three independent determinations. The standard deviation is <13% of each value.

[b] The M9 minimal medium was supplemented with 150 μM 2,2′-dipyridyl and with 100 μM $FeCl_3$ to reduce and increase iron availability, respectively.

Pyoverdine$_{PAO1}$ production is reduced in a *P. aeruginosa* PAO1 *algQ*-defective background. To investigate the effect of AlgQ on pyoverdine$_{PAO1}$ production, we monitored pyoverdine$_{PAO1}$ levels during the whole growth cycle of wild-type *P. aeruginosa* PAO1 strains [PAO1(pUCP19) and PAO1(pJB785TT)], of *algQ* null mutants [PAO1Δ*algQ*(pUCP19) and PAO1Δ*algQ*(pJB785TT)], and of *algQ* null mutants complemented with either a single copy or multiple copies of *algQ* [PAO1Δ*algQ*(pUCP*algQ*) and PAO1Δ*algQ*(pJB*algQ*), respectively]. The growth rates in low-iron medium (DCAA) were comparable for all strains tested throughout the experiment (data not shown). In spite of this, the Δ*algQ* mutants reproducibly exhibited an ca. 20% reduction in pyoverdine$_{PAO1}$ production compared with wild-type strain PAO1 carrying the control vectors (shown in Fig. 1 only for pUCP19). Pyoverdine$_{PAO1}$ production was totally restored upon complementation of the Δ*algQ* mutation with a single copy of the *algQ* gene [PAO1Δ*algQ*(pJB*algQ*)] (Fig. 1). Introduction of either pUCP*algQ* or pJB*rsd* into PAO1Δ*algQ* increased pyoverdine$_{PAO1}$ production to ca. 90% of the wild-type levels (Fig. 1 and data not shown). Comparatively, the multicopy plasmid pUCP*algQ*

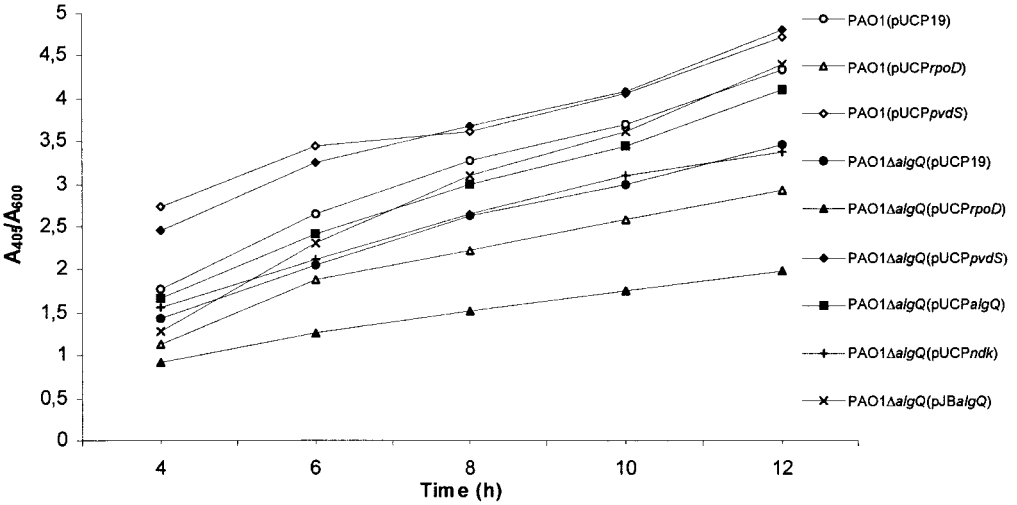

FIG. 1. Effect of the *algQ* mutation on pyoverdine yields in iron-depleted cultures of *P. aeruginosa* PAO1 carrying a single copy of *algQ* and multiple copies of *algQ*, *ndk*, *pvdS*, or *rpoD*. Bacterial growth in DCAA was monitored by measuring the absorbance at 600 nm of the cultures at different times. At each time, pyoverdine$_{PAO1}$ levels were determined by measuring the absorbance at 405 nm of cell-free culture supernatants diluted 1:2 in 100 mM Tris-HCl (pH 8.0). Data are expressed as relative fluorescence levels (A_{405}/A_{600}). All measurements were performed in duplicate and in multiple experimental trials with different batches of DCAA. The standard deviations are <8% of the values.

was more effective in complementation during early growth, whereas the single-copy plasmid pJB*algQ* had a delayed effect (Fig. 1). Since synthesis of alginate, GTP, ppGpp, and polyP is restored in a *P. aeruginosa algQ* mutant by complementation with the *ndk* gene (25), we measured pyoverdine$_{PAO1}$ production in the *P. aeruginosa* PAO1 *algQ* null mutant harboring plasmid pUCP*ndk* (Fig. 1). Comparable pyoverdine$_{PAO1}$ levels were produced by PAO1Δ*algQ*(pUCP19) and PAO1Δ*algQ* (pUCP*ndk*). Thus, AlgQ is required for optimal production of pyoverdine$_{PAO1}$ in *P. aeruginosa* PAO1, but it cannot be replaced by Ndk for this function.

AlgQ modulates *pvd* gene expression at the transcriptional level. To gain further insight into the role of AlgQ in the transcriptional control of *pvd* genes, the promoter activities of the *pvdA*::*lacZ*, *pvdD*::*lacZ*, *pvdE*::*lacZ*, and *pvdS*::*lacZ* transcriptional fusions were compared for wild-type strain PAO1 and the Δ*algQ* mutant with or without the *algQ*, *rsd*, or *ndk* gene in *trans* under iron-deficient and iron-sufficient conditions. Under low-iron conditions, the β-galactosidase levels expressed by *pvdA*, *pvdD*, and *pvdE* transcriptional fusions were reduced by ca. 25 to 50% in the *algQ* mutant compared with wild-type strain PAO1, depending on the fusion (Table 3). Introduction of the single-copy pJB*algQ* plasmid into PAO1Δ*algQ* restored the wild-type activity of *pvd* promoters, while the multicopy construct pUCP*algQ* had weaker effects, as also observed for the heterologous *rsd* gene in pJB*rsd* (Table 3). No significant difference in promoter activity was detected between the *algQ* null mutant carrying pUCP19 and the *algQ* null mutant carrying pUCP*ndk* (Table 3). As expected, no promoter activity was observed in iron-rich cultures (Table 3). These results indicate that AlgQ positively affects the transcription of several coregulated genes of the *pvd* biosynthetic cluster in an Ndk-independent manner. Remarkably, the activities of the *pvdS*::*lacZ* transcriptional fusion were similar in *algQ*-deficient and -proficient backgrounds (Table 3).

Next, we examined the effect of AlgQ on the expression levels of both PvdA and PvdS. Total proteins from iron-starved *P. aeruginosa* cell lysates were separated by SDS-polyacrylamide gel electrophoresis and probed with either anti-PvdA or anti-PvdS mouse polyclonal sera (41; this study). Quantitative estimation of band intensities in the Western blot assays demonstrated that PvdA expression by *P. aeruginosa* PAO1Δ*algQ* was reduced by ca. 60% compared with the expression by wild-type strain PAO1 and was fully restored upon complementation with pJB*algQ* (Fig. 2A). Also, in this case both pUCP*algQ* and pJB*rsd* increased PvdA expression without reaching the wild-type level (Fig. 2A and B). Interestingly, PvdS levels were comparable in wild-type strain PAO1 (pUCP19), PAO1Δ*algQ*(pUCP19), and PAO1Δ*algQ*(pUCP *algQ*) (Fig. 2C). These results are in line with the observed regulation of *pvdA*::*lacZ* and *pvdS*::*lacZ* transcriptional fusions; in fact, they demonstrate that AlgQ affects the extent of PvdA expression without influencing PvdS levels. Because expression of PvdA and expression of PvdS were similar in PAO1Δ*algQ*(pUCP19) and PAO1Δ*algQ*(pUCP*ndk*) (data not shown), the *algQ*-complementing activity of *ndk* was not investigated further.

Effect of AlgQ on the transcription initiation pattern at the *pvdA* promoter. The following two transcription start sites were previously identified upstream of the *pvdA* gene: a major start site (T1) and a minor site (T2) located 68 and 43 nt upstream from the A of the *pvdA* start codon (29). To evaluate the effect of AlgQ on the transcription initiation pattern of *pvdA*, primer extension analysis of total RNA extracted from iron-deficient cells of wild-type strain PAO1(pUCP19) and its *algQ* null derivative PAO1Δ*algQ* carrying either pUCP19 or pUCP*algQ* was performed (Fig. 3). The transcription initiation profiles were similar in the wild-type and *algQ*-defective backgrounds, although the amount of a primer extension product(s) was smaller in the *algQ* mutant (Fig. 3, lanes 2 and 3, respectively). Introduction of pUCP*algQ* into the *algQ* mutant improved the yield of a primer extension product(s) (Fig. 3, lane 4). As

TABLE 3. Effect of the *algQ* mutation on the activities of different *pvd*::*lacZ* transcriptional fusions in *P. aeruginosa* PAO1 cells carrying *rpoD*, *pvdS*, *algQ*, *ndk*, and *rsd* genes in *trans*

Strain	LacZ activity[a]							
	P*pvdA*::*lacZ*		P*pvdD*::*lacZ*		P*pvdE*::*lacZ*		P*pvdS*::*lacZ*	
	−Fe(III)	+Fe(III)	−Fe(III)	+Fe(III)	−Fe(III)	+Fe(III)	−Fe(III)	+Fe(III)
PAO1(pUCP19)	7,973	45	6,402	52	5,504	52	4,180	58
PAO1(pUCP*rpoD*)	5,956 (75 ± 5)[b]	28	3,559 (56 ± 4)	31	3,242 (59 ± 4)	29	4,431 (106 ± 4)	51
PAO1(pUCP*pvdS*)	8,771 (110 ± 8)	4,868	8,963 (140 ± 6)	5,333	6,808 (123 ± 8)	4,289	4,013 (96 ± 5)	52
PAO1Δ*algQ*(pUCP19)	5,980 (75 ± 8)	25	4,049 (63 ± 4)	29	3,821 (69 ± 8)	26	4,270 (102 ± 4)	62
PAO1Δ*algQ*(pUCP*rpoD*)	3,572 (45 ± 6)	33	2,681 (42 ± 3)	27	2,708 (49 ± 5)	32	4,598 (110 ± 7)	47
PAO1Δ*algQ*(pUCP*pvdS*)	8,124 (101 ± 4)	4,471	8,771 (137 ± 8)	4,959	6,671 (121 ± 7)	4,032	3,929 (94 ± 6)	54
PAO1Δ*algQ*(pUCP*algQ*)	7,554 (95 ± 5)	32	5,013 (81 ± 14)	28	4,933 (93 ± 3)	35	4,318 (103 ± 8)	43
PAO1Δ*algQ*(pUCP*ndk*)	6,177 (77 ± 7)	31	4,081 (64 ± 4)	30	3,928 (71 ± 7)	33	4,265 (102 ± 6)	55
PAO1(pJB785TT)	7,863	47	6,513	48	5,623	36	4,092	52
PAO1Δ*algQ*(pJB785TT)	5,740 (73 ± 7)	35	4,364 (67 ± 4)	46	3,655 (65 ± 3)	43	4,174 (102 ± 8)	61
PAO1Δ*algQ*(pJB*algQ*)	7,627 (97 ± 5)	36	6,448 (99 ± 3)	43	5,848 (104 ± 6)	52	3,969 (97 ± 5)	54
PAO1Δ*algQ*(pJB*rsd*)	6,684 (85 ± 6)	48	5,275 (81 ± 4)	37	4,554 (81 ± 5)	49	3,928 (96 ± 4)	53

[a] β-Galactosidase activities were determined in lysates of *P. aeruginosa* PAO1 cultures grown for 6 h in DCAA [−Fe(III)] and in DCAA supplemented with 100 μM $FeCl_3$ [+Fe(III)]. The values, expressed in Miller units (36), are the means of six independent determinations. The standard deviations are <15% of the means.

[b] The values in parentheses (means ± standard deviations) are percentages based on the value obtained for *P. aeruginosa* PAO1 carrying the control vector (either pUCP19 or pJB785TT), which was considered 100%.

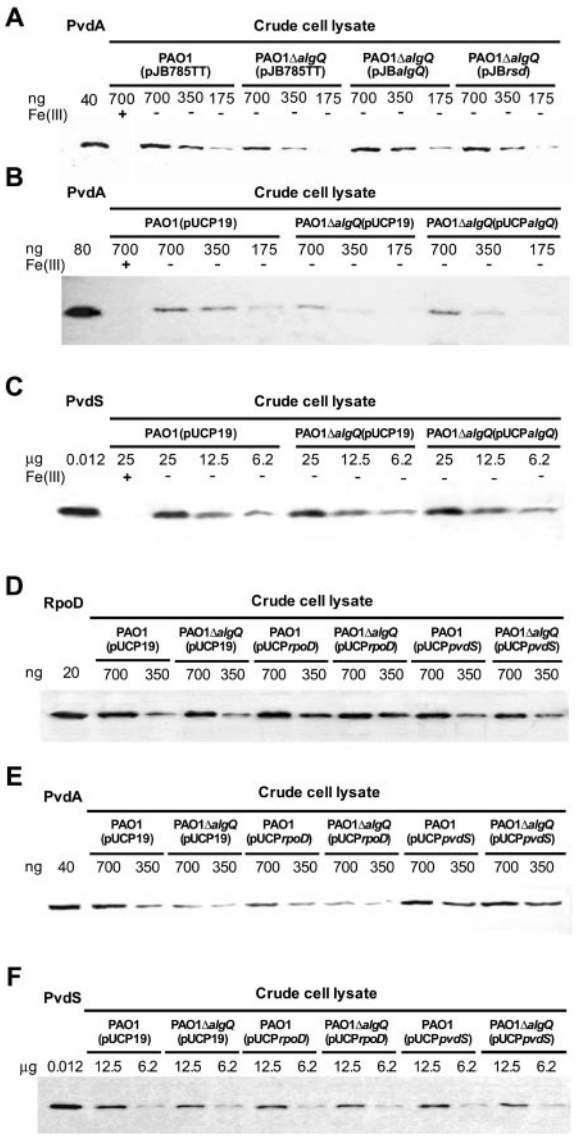

FIG. 2. Western blot analysis of PvdA, PvdS, and RpoD expression in *P. aeruginosa* PAO1 and PAO1Δ*algQ* carrying different plasmids. Whole-cell lysates obtained from iron-poor (DCAA) [−Fe(III)] and iron-rich (DCAA plus 100 μM $FeCl_3$) [+Fe(III)] cultures were probed with anti-PvdA (A, B, and E) or anti-PvdS (C and F) polyclonal mouse antisera and with commercial monoclonal anti-RpoD antibodies (D). The strains used and amounts of total proteins from each extract are indicated in each panel. The purified proteins (PvdA, 47.7 kDa; PvdS, 21.2 kDa; RpoD, 70.2 kDa) were used as positive controls. For details, see Materials and Methods.

expected, no primer extension product(s) was detected with total RNA from iron-replete *P. aeruginosa* cells (Fig. 3, lane 1).

Primer extension analysis was also performed with total RNA extracted from iron-deficient cells of *E. coli* MC4100 carrying the *pvdA*::*lacZ* transcriptional fusion plus plasmid pBRXB and, alternatively, either pACYC*algQ* or pACYC*pfrA*. In the heterologous *E. coli* system, *pvdA* transcription from the major T1 start point was enhanced by *algQ* and, to a greater extent, by *pfrA* (Fig. 3, lanes 5 and 6, respectively). Interest-

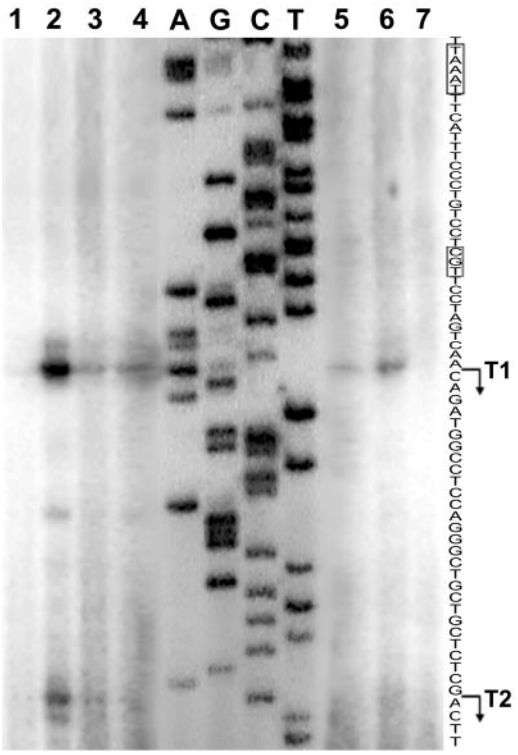

FIG. 3. Effect of the *algQ* mutation on the initiation pattern of *pvdA* transcripts in *P. aeruginosa* PAO1 *algQ*-proficient and -defective backgrounds and in the heterologous host *E. coli*. The primer extension reaction was carried out with the 5′-end-labeled oligonucleotide RV*P*pa and equal amounts of total RNA isolated from *P. aeruginosa* and *E. coli* cells grown in DCAA and M9 medium, respectively. The single-stranded *pvdA* promoter sequence is shown on the right, and consensus motifs recognized by PvdS are enclosed in boxes. The previously identified 5′ ends of T1 and T2 transcripts (29) and the direction of transcription are indicated by bent arrows. Lanes A, G, C, and T, *pvdA* sequencing ladder generated from pPV226 with the same oligonucleotide (RV*P*pa): lane 1, primer extension analysis of total RNA extracted from *P. aeruginosa* PAO1(pUCP19) iron-rich cultures (DCAA plus 100 μM $FeCl_3$); lanes 2 to 4, primer extension analysis with total RNA from *P. aeruginosa* PAO1(pUCP19) (lane 2), PAO1Δ*algQ*(pUCP19) (lane 3), and PAO1Δ*algQ*(pUCP*algQ*) (lane 4); lanes 5 to 7, primer extension analysis with total RNA from *E. coli* MC4100(P*pvdA*::*lacZ*; pBRXB) carrying pACYC*algQ* (lane 5), pACYC*pfrA* (lane 6), and pACYC184 (lane 7). Lane cutting and pasting were needed to visualize the sequencing ladder and primer extension products, which required different exposure times.

ingly, no T2 product was detectable in *E. coli*, even after prolonged autoradiography (Fig. 3).

Multicopy *rpoD* mimics the *algQ* mutation in wild-type *P. aeruginosa* strain PAO1. Because both AlgQ and Rsd bind RpoD (12), the effect of AlgQ on the expression of *pvd* genes could be explained by an anti-sigma factor mechanism. We hypothesized that AlgQ could increase the availability of free RNAPc and facilitate the formation of the PvdS-dependent RNAP holoenzyme, thereby allowing transcription of PvdS-dependent genes to occur more efficiently. To verify this hypothesis, we tested the effect of an overdose of RpoD on PvdS-dependent transcription of *pvd* genes. The multicopy plasmid pUCP*rpoD*, carrying the entire *rpoD* coding sequence and its own promoter (15), was introduced into wild-type and Δ*algQ P. aeruginosa* strains. The presence of the multicopy *rpoD* gene increased the levels of RpoD by ca. 1.5-fold, as determined by Western blot analysis with monoclonal anti-RpoD antibodies (Fig. 2D). Interestingly, the pyoverdine$_{PAO1}$ yields were diminished to the same extent in PAO1(pUCP*rpoD*) and PAO1Δ*algQ*(pUCP19) and were further decreased in PAO1Δ*algQ*(pUCP*rpoD*) (Fig. 1). Accordingly, expression of P*pvdA*::*lacZ*, P*pvdD*::*lacZ*, and P*pvdE*::*lacZ* transcriptional fusions was significantly reduced in both wild-type and Δ*algQ* strains carrying pUCP*rpoD* (Table 3). The results of a comparison of PvdA expression in PAO1(pUCP*rpoD*) and PvdA expression in PAO1Δ*algQ*(pUCP*rpoD*) mirrored the transcriptional response observed for the P*pvdA*::*lacZ* fusion (Fig. 2E and Table 3). Furthermore, transformation of the *algQ* mutant with pUCP*pvdS*, a multicopy plasmid driving constitutive *pvdS* expression, restored or even increased the pyoverdine$_{PAO1}$ yields, P*pvdA*::*lacZ*, P*pvdD*::*lacZ*, and P*pvdE*::*lacZ* promoter activities, and PvdA expression levels (Fig. 1 and 2E and Table 3). Expression of the P*pvdS*::*lacZ* transcriptional fusion was not significantly affected by multicopy *rpoD* and *pvdS* in either wild-type or Δ*algQ* strains (Table 3), and Western blot analysis of PAO1(pUCP*rpoD*) and PAO1Δ*algQ*(pUCP*rpoD*) lysates failed to detect appreciable differences in the PvdS levels (Fig. 2F). Altogether, these results argue for an anti-RpoD mechanism in AlgQ transcriptional regulation of *pvd* biosynthetic genes.

DISCUSSION

As a rule, proteins with relevant biological functions have been conserved during evolution. This is also true for AlgQ, whose homologues (Rsd-like proteins) can be retrieved from several γ-*Proteobacteria* (COG3160; http://www.ncbi.nlm.nih.gov/COG/new/). Comparative analysis of the *algQ* genomic region in *P. aeruginosa* PAO1, *P. putida* KT2440, *Pseudomonas syringae* pv. tomato strain DC3000, *Pseudomonas fluorescens* PfO-1, and *Azotobacter vinelandii* revealed remarkable conservation of the whole locus (Fig. 4), as previously documented for the *pprA-pfrA-pprB* locus of *P. putida* WCS358 (59). These features suggest that AlgQ homologues have an important function(s) in *Pseudomonadaceae* and, more generally, in γ-*Proteobacteria*.

AlgQ was originally identified as a positive transcriptional regulator of the alginate biosynthetic gene *algD* (23) and later was shown to act as a pleiotropic regulatory protein capable of modulating the expression of several *P. aeruginosa* virulence factors, including extracellular proteases, rhamnolipid, neuraminidase, and a siderophore(s) (6, 25, 49, 60). However, the molecular mechanism(s) underlying the AlgQ function(s) in *P. aeruginosa* PAO1 remains largely unresolved. Here, we combined quantitative pyoverdine$_{PAO1}$ determination, *pvd* promoter-reporter gene assays, mRNA mapping, and immunoblot analysis of Pvd proteins to demonstrate that AlgQ finely modulates pyoverdine$_{PAO1}$ production through positive control of PvdS activity. We used this experimental approach to circumvent the intrinsic drawback of large-scale transcriptome profiling, which suffers from overlooking minor differences in mRNA levels.

Previous investigations on the effect of AlgQ on siderophore production were performed with the *algQ* mutant of *P. aerugi-*

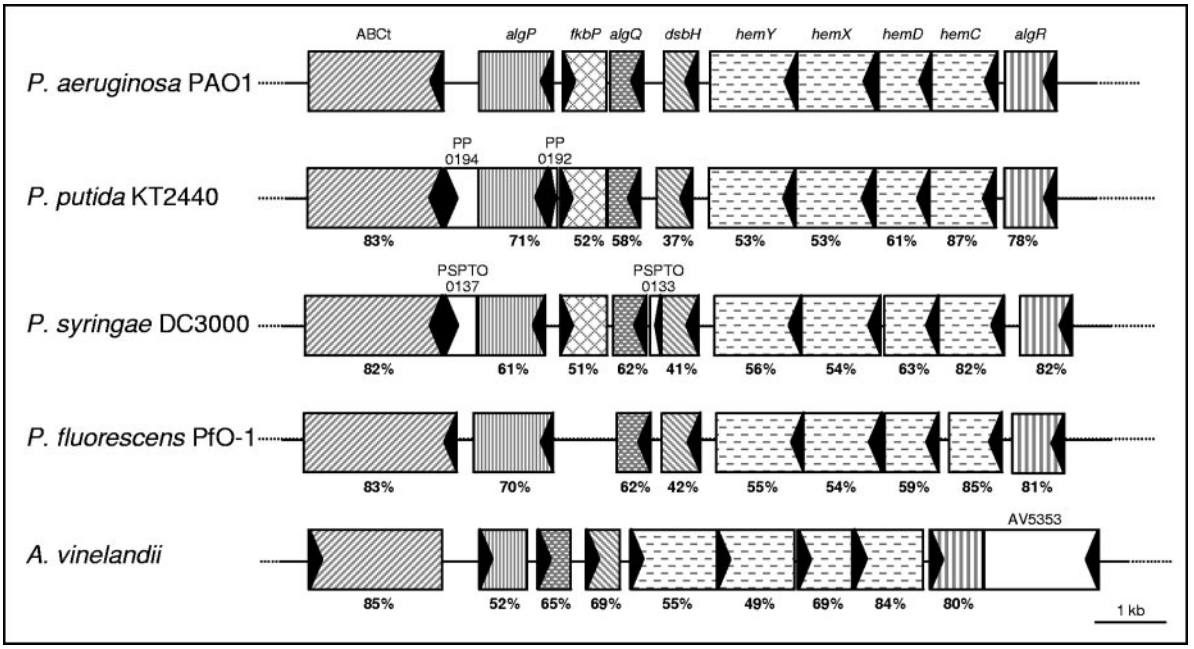

FIG. 4. Schematic diagrams of the genomic regions encompassing *algQ* homologues in *P. aeruginosa* PAO1, *P. putida* KT2440, *P. syringae* pv. tomato strain DC3000 (*P. syringae* DC3000), *P. fluorescens* PfO-1, and *A. vinelandii*. The designations of characterized and putative genes are indicated at the top. ABCt, ATP-binding transporter; *algP*, gene encoding the alginate regulatory protein; *fkbP*, gene encoding the peptidyl-prolyl *cis-trans* isomerase; *algQ*, gene encoding a regulatory protein; *dsbH*, gene encoding the DsbH family protein; *hemY*, gene encoding the HemY putative protein; *hemX*, gene encoding the uroporphyrin III C-methyltransferase; *hemD*, gene encoding the uroporphyrinogen III synthetase; *hemC*, gene encoding the porphobilinogen deaminase; *algR*, gene encoding a regulatory protein. Orthologues are indicated by the same pattern. The triangles indicate the direction of transcription. Annotation numbers of hypothetical open reading frames are indicated. The level of protein identity relative to *P. aeruginosa* PAO1 is indicated below each open reading frame.

nosa 8830 grown under ill-defined iron availability conditions (i.e., LB medium) (49). We noticed that both *P. aeruginosa* 8830 and its *algQ* mutant grow poorly under low-iron conditions (DCAA) compared with *P. aeruginosa* PAO1 (Ambrosi, unpublished results), while no differences between wild-type strain PAO1 and its isogenic Δ*algQ* derivative were found. As shown for the *E. coli* K-12 *rsd* mutant (21), *P. aeruginosa* PAO1Δ*algQ* apparently showed no distinct phenotype compared with the wild-type parental strain, as determined by growth kinetics, cell viability, and colony morphology in LB medium and DCAA. Thus, AlgQ is not essential for *P. aeruginosa* growth in laboratory medium under iron-depleted conditions.

The AlgQ-dependent stimulation of pyoverdine$_{PAO1}$ synthesis in *P. aeruginosa* PAO1 was paralleled by increased transcription of the *pvdA*, *pvdD*, and *pvdE* biosynthetic genes. This could not be ascribed to increased expression of PvdS. In fact, the *algQ* mutation neither affected *pvdS* expression nor altered PvdS levels (Table 3 and Fig. 2C). Hence, AlgQ acts as positive regulator of pyoverdine$_{PAO1}$ synthesis by acting at the post-transcriptional level on PvdS activity.

Experiments performed with the reconstituted *E. coli* system have demonstrated that PvdS-dependent transcription of *pvdA* is positively affected both by the Rsd-like proteins AlgQ and PfrA from *Pseudomonas* and by *E. coli* Rsd itself (Table 2). Related to this finding, AlgQ was previously shown to transactivate the AlgU-dependent *algD* promoter in *E. coli* cells grown under high-osmolarity conditions (23). Mapping of a *pvdA* mRNA(s) in the reconstituted *E. coli* system confirmed that transcription from the major T1 start point is enhanced by the presence of AlgQ and PfrA in *trans*. The absence of the minor T2 product and the inability of both AlgQ and PfrA to direct heterologous *pvdA* transcription at *P. aeruginosa* levels support our previous hypothesis (30) concerning the requirement of an as-yet-unidentified activating factor(s), besides PvdS and AlgQ, for full transcription at the *pvdA* promoter in *P. aeruginosa*.

By what mechanism does AlgQ act as a positive regulator of pyoverdine biosynthetic genes? First, we hypothesized that AlgQ could indirectly affect pyoverdine$_{PAO1}$ yields through the up-regulation of Ndk, which enhances the formation of the ppGpp alarmone during nutritional starvation (25). We reasoned that the reduced levels of ppGpp in the *P. aeruginosa* *algQ*-defective background (25) could account for the diminished expression of the *pvd* biosynthetic genes. The ppGpp molecule bound to RNAPc has the potential (i) to alter promoter recognition and the kinetics of transcriptional initiation, either positively or negatively, (ii) to increase the competitiveness and association properties of alternative sigma factors for RNAPc (8, 22, 39), and (iii) to affect the induction profile of some genes controlled by alternative sigma factors (22, 58). However, an Ndk/ppGpp-dependent cascade of *pvd* promoter activation seems very unlikely, given that *ndk* did not suppress the *algQ* mutant phenotype at the level of pyoverdine$_{PAO1}$ synthesis and *pvd* transcription. The results of an immunoblot analysis of PvdA and PvdS also excludes a role for Ndk in improving the translation efficiency of *pvd* messengers through activation of elongation factor Tu and increased GTP synthesis

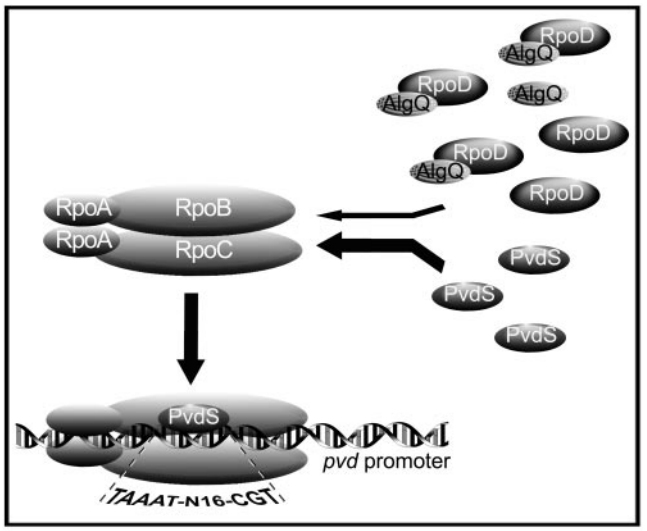

FIG. 5. Proposed model of AlgQ modulation of PvdS-dependent transcription. Under iron starvation conditions, the interaction between AlgQ and free RpoD subunits facilitates RNAPc recruitment by the IS sigma factor PvdS and hence transcription initiation at the *pvd* promoters. Canonical sequences recognized by PvdS are indicated.

(38). An intriguing observation was the partial complementation of the PAO1Δ*algQ* mutant by the multicopy *algQ* gene. Recovery of pyoverdine yields by complementation with low- and high-copy-number *algQ* exhibited a time-dependent profile (Fig. 1), suggesting that AlgQ must be present at appropriate concentrations at a given time to optimize expression of *pvd* genes. The reason for the dose-dependent effects of *algQ* on *pvd* gene expression is not known. However, evidence for incomplete complementation was also obtained in previous studies which showed that neither *algQ* nor *ndk*, under the control of the *tac* promoter, was able to fully restore alginate, ppGpp, and polyP synthesis in a *P. aeruginosa algQ* mutant (25, 56). Apart from this, it is evident that pyoverdine synthesis is modulated by the *algQ* gene but not by *ndk*.

Because *rsd* partially substituted for *algQ* in transcriptional regulation of *pvd* genes, we entertained the alternative idea that AlgQ could increase *pvd* expression by acting as an anti-sigma factor for RpoD (12). We tested this hypothesis by introducing either *rpoD* or *pvdS* on a high-copy-number plasmid into PAO1 and PAO1Δ*algQ*. As far as expression of *pvd* biosynthetic genes is concerned, multicopy *rpoD* mimicked and even exacerbated the Δ*algQ* phenotype in wild-type and *algQ* mutant strains, respectively, without altering the levels of RNAPc (13). Accordingly, multicopy *pvdS* suppressed the Δ*algQ* phenotype. A similar situation has been documented for the Rsd-dependent competition between RpoD and the alternative sigma factors RpoS and RpoH in *E. coli* (21). Based on the results described above, a model of AlgQ-dependent modulation of *pvd* expression was developed (Fig. 5). AlgQ could activate *pvd* genes by functioning as an anti-sigma factor of RpoD, consistent with its ability to interact with the RpoD subunit (12). The inhibition of an RpoD interaction with RNAPc would increase the opportunity for PvdS to bind RNAPc and to direct the RNAPc-PvdS holoenzyme to *pvd* promoters, thereby enhancing *pvd* gene expression. In *E. coli*, functional RNAPc is a limiting factor for the rate of transcription, and sigma subunits compete for a limited number of free RNAPc molecules (33, 52). Under iron-depleted conditions PvdS expression and activity increase rapidly, as measured by the rate of LacZ synthesis from both the P*pvdS*::*lacZ* and *pvd*::*lacZ* transcriptional fusions (Table 3) (29). While the increased intracellular level of PvdS is sufficient to direct *pvd* transcription, the positive effect of AlgQ on PvdS-RNAPc holoenzyme formation would ensure optimal transcription of *pvd* and possibly of the other PvdS-dependent genes, such as *prpL*, *toxA*, and *aprA* (40, 61). The lack of AlgQ modulation on *pvdS* expression is consistent with the general notion that genes whose products have a key role in cell survival under starvation conditions (e.g., iron-depleted conditions) are not affected by a reduction in RpoD-dependent RNAP levels (34).

In *P. aeruginosa*, transcription of the *algD* gene depends mainly on the alternative sigma factor AlgU, another member of the ECF subfamily of eubacterial sigma factors (11, 30, 32). AlgU activity is under negative control of the anti-sigma factor MucA, encoded by the *mucA* gene (35). However, it has been proposed that the responsiveness of *algD* to intracellular low iron levels (4, 17, 57) is AlgQ mediated (55). Given the minute amount of AlgU in stressed cells of *P. aeruginosa* PAO1 (50), the anti-RpoD activity of AlgQ could also enhance AlgU-dependent transcription.

Finally, it is also possible that AlgQ positively affects pyoverdine production via a direct effect on *pvd* promoters, as documented for *rhlR* and *lasR* promoters (28). Interestingly, it was recently shown that Rsd can also interact with RNAPc (19), likely directing RNAP to particular subsets of promoters. Whether this is also true for AlgQ remains to be proven.

Taken together, our data substantiate the connection between pyoverdine$_{PAO1}$ production, alginate biosynthesis, and energy metabolism (24). Alginate is known for being produced under nutrient-limited conditions or as a consequence of impaired energy metabolism. In particular, the *algQ* promoter responds to nutrient starvation (namely, phosphate limitation) by increasing Ndk expression to compensate for the decrease in NTP and deoxynucleoside triphosphate (dNTP) synthesis under phosphate-deficient conditions (25). On the other hand, NTP and dNTP deficiency can also result from a diminished supply of ATP from aerobic respiration, a primary metabolic drawback of iron depletion (42, 45, 54). This is in keeping with the observed correlation between iron limitation, poor oxygen transfer, and alginate synthesis under controlled bioreactor conditions (24). AlgQ might therefore contribute to the maintenance of the intracellular pool of NTPs and dNTPs directly through Ndk and indirectly by promoting pyoverdine-dependent iron uptake and hence formation of iron-containing respiratory enzymes and ATP synthesis from aerobic respiration. Moreover, the anti-RpoD activity of AlgQ could orient RNAP toward promoters governed by the alternative sigma factors PvdS and AlgU, causing stimulation of pyoverdine and alginate synthesis to protect bacteria from stressful situations, such as those encountered in vivo. The moderate effect of AlgQ on PvdS- and AlgU-dependent transcription (12, 66) is consistent with the hypothesis that it is a protein that is capable of antagonizing the activity of the major vegetative sigma subunit.

In conclusion, AlgQ seems to play a part in many regulatory processes, resulting in modulation of the expression of several

genes. As a global regulatory protein, AlgQ could participate in the transcriptional control of target genes within its regulon in concert with other factors, thereby allowing fine-tuning of gene expression in response to various environmental stimuli.

ACKNOWLEDGMENTS

We are grateful to M. Foglino for supplying the *P. aeruginosa* PAO1 *algQ* null mutant used in this study. We thank A. Merante and D. Perroni for assistance with artwork and V. Pallottini for animal house work.

This work was supported by MIUR-PRIN grant 2004060270_005 and ISPESL contract B98/DIPIA/03.

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