Transcriptional regulation of nitrate assimilation in *Pseudomonas aeruginosa* occurs via transcriptional antitermination within the nirBD–PA1779–cobA operon

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Bioinformatic approaches employed to analyse intergenic regions of *Pseudomonas aeruginosa* O1 (PAO1) for small RNAs (sRNAs) revealed a putative RNA gene encoded upstream of the nitrate assimilation operon nirBD–PA1779–cobA. Here, we show that this RNA, termed nitrogen assimilation leader A (NaLA), represents the leader RNA of the nirBD–PA1779–cobA operon, and that nalA transcription is δ54- and NtrC-dependent. A PAO1 nalA deletion strain and a strain bearing a deletion in ORF PA1785 failed to grow on nitrate. PA1785 was identified as a homologue of the *Azotobacter vinelandii* nasT gene, the product of which is required for transcription of the *A. vinelandii* nitrate/nitrite reductase operon. Collectively, these studies reveal that transcriptional antitermination of the leader RNA NaLA is required for expression of the PAO1 nitrate assimilation operon, and that this process is governed by conserved functions in PAO1 and *A. vinelandii*.

**INTRODUCTION**

Nitrate can be used by Bacteria as a terminal electron acceptor (nitrate respiration) to maintain the redox balance (nitrate dissimilation) and as a nutrient (nitrate assimilation). Reduction of nitrate to nitrite is a common step in all three pathways (Moreno-Viviani et al., 1999).

Nitrate respiration occurs, for instance, in *Pseudomonas aeruginosa* biofilms colonizing the lungs of cystic fibrosis patients, where low oxygen and high nitrate concentrations favour anaerobic respiration (Yoon et al., 2002; Schobert & Jahn, 2010). Three operons are involved in nitrate reduction to gaseous nitrogen (Vollack et al., 1998). The *narK1K2GHJI* operon encodes the nitrate reductase required for nitrate reduction to nitrite (Schreiber et al., 2007). Detoxification of nitrite to nitric oxide (NO), and further to nitrous oxide (N₂O), requires functions encoded by the *nirERS–norCB* locus (Kawasaki et al., 1997). Finally, the functions of the *nosRZDFYL* operon (Zumft et al., 1990) are required for reduction of nitrous oxide to inert gaseous nitrogen. The expression of all three loci occurs under anaoxic conditions, and is mediated by the regulatory network composed of the regulators Anr, NarXL, Dnr and NirQ (Ye et al., 1995; Arai et al., 1997; Schreiber et al., 2007).

In contrast to nitrate respiration, in which the nitrate reduction cascade results in ATP synthesis, periplasmic nitrate dissimilation mediated by the *napEFDABC* operon does not lead to energy production (Bedzyk et al., 1999; Van Alst et al., 2009). In *Pseudomonas* spp., the periplasmic nitrate reductase is apparently used for denitrification during the aerobic to anaerobic transition, as well as to maintain the redox balance by dissipating the accumulated reducing power into the reduction of nitrate to nitrite (Bedzyk et al., 1999).

The Nas enzymes (for nitrate assimilation) convert inorganic nitrate to a biologically useful form via the reduction of nitrate to nitrite and consecutively to ammonium (Moreno-Viviani et al., 1999; Richardson et al., 2001). The latter step is catalysed by the sirohaem-dependent NADH-nitrite reductase in various bacteria (Lin & Stewart, 1997), including *Mycobacterium tuberculosis*, *Klebsiella oxytoca* and *Azotobacter vinelandii* (Toukdarian & Kennedy, 1986; Cali et al., 1989; Malm et al., 2009).

In *Klebsiella* spp., expression of the nitrate assimilation (*nasFEDCBA*) operon (Fig. 1a) is subject to dual regulation.
Transcription of the operon is driven by a σ54-dependent promoter and mediated by phosphorylated NtrC. Full transcription further requires the nitrate-responsive NasR protein for transcription antitermination within the nasF leader (Lin & Stewart, 1996; Chai & Stewart, 1998). This protein possesses an N-terminal nitrate/nitrite-sensing domain (Lin & Stewart, 1997; Chai & Stewart, 1998) and a C-terminal AmiR and NasR transcription antitermination regulation (ANTAR) domain (Shu & Zhulin, 2002) for antitermination of transcription (Lin & Stewart, 1997). Hence, regulation of nitrate assimilation is controlled by both a general nitrogen regulation system depending on the presence of a preferred nitrogen source and by the availability of nitrate.

In A. vinelandii, nitrate assimilation has been shown to require the nitrate and nitrite reductases NasA and NasB (Fig. 1a), respectively (Ramos et al., 1993). Similarly to Klebsiella, the expression of the Azotobacter operon nasAB is controlled by an antitermination mechanism mediated by the antiterminator protein NasT, the activity of which is negatively controlled by the nitrate-sensitive regulator NasS (Gutierrez et al., 1995; Stülke, 2002). In the absence of nitrate, transcription terminates in the nasAB leader as NasS inactivates NasT. In contrast, binding of nitrate to NasS seems to lead to NasT-mediated antitermination within the nasAB leader, and consequently to nasAB expression (Lin & Stewart, 1997).

Due to homologies with functions encoded in the known nitrate assimilation operons of Klebsiella spp. (Lin et al., 1993; Wu & Stewart, 1998), A. vinelandii (Gutierrez et al., 1995), Bacillus subtilis (Ogawa et al., 1995) and Propionibacterium freudenreichii (Sattler et al., 1995), the P. aeruginosa O1 (PAO1) loci PA1778–PA1786 were inferred to encode functions required for nitrate assimilation in
PAO1 (Stover et al., 2000). The product of PA1779 (Fig. 1a) was later shown to display nitrate reductase activity (Noriaga et al., 2005). The nirBD genes have been inferred to encode the two subunits of nitrate reductase (Stover et al., 2000; Fig. 1a) based on their homology with the Klebsiella pneumoniae and K. oxytoca nasB (Fig. 1a) and nasC genes, respectively (Winsor et al., 2009). However, in contrast to A. vinelandii and K. oxytoca, the molecular mechanisms underlying regulation of nitrate assimilation have not been studied in PAO1.

The PAO1 RNA nitrate assimilation leader A (NalA) has been independently identified in this study and by Livny et al. (2006). We show that (i) NalA represents the leader RNA of the nirBD–PA1779–cobA operon, (ii) transcription of nala is driven by σ54 and requires NtrC, and (iii) expression of the downstream nitrate assimilation operon requires transcriptional antitermination within the NalA leader, which is mediated by the A. vinelandii NasT homologue PA1785.

**METHODS**

**Bioinformatics.** The search algorithm described by Lenz et al. (2004) was used with some modifications to unravel novel small RNAs (sRNAs), transcription of which should be driven by a σ54-dependent promoter. First, the *P. aeruginosa* intergenic regions were downloaded from http://www.pseudomonas.com (Intergenic DNA, *P. aeruginosa* PAO1, version 2004-01-14). Next, it was assumed that the putative sRNAs comprise Rho-independent terminators. A total of 524 *P. aeruginosa* rho-independent terminators were downloaded from http://www.tigr.org/software/transterm.html, of which 502 were found in intergenic regions. Using PATSER (http://rsat.ulb.ac.be/ tutorials/tut_patser.html), the *P. aeruginosa* intergenic regions were then screened for potential σ54-binding sites with a weight matrix constructed from a compiled set of approximately 180 σ54-binding sites from multiple bacterial species (Dombrecht et al., 2002). All matches with a score higher than or equal to the cut-off score 8.9 (Dombrecht et al., 2002) were considered as potential hits. Accounting for a distance between a putative sRNA start and the next ORF of at least 50 nt the number of these 214 potential hits was further reduced to 168. Combining these 168 hits with the list of Rho-independent terminators resulted in a list of 22 intergenic loci, which contained both a putative σ54-binding site and a putative Rho-independent terminator. Among those 22 candidates only one candidate RNA emerged, in which the σ54-binding site and the terminator sequences were in an appropriate sequential arrangement. The putative sRNA mapped in the intergenic region between the ORFs PA1782 and PA1781 (Fig. 1a).

**Bacterial strains, plasmids and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37 °C in Luria–Bertani medium (Miller, 1972) supplemented with appropriate antibiotics. Antibiotics were added to final concentrations of 50 μg gentamicin ml⁻¹, 100 μg ampicillin ml⁻¹ (Escherichia coli) and 300 μg carbenicillin ml⁻¹ (PAO1). Minimal salt medium (40 mM K₂HPO₄, 22 mM KH₂PO₄, 0.5 mM MgSO₄, 10 μM FeSO₄, pH 7.0) was used for gene expression analysis. Sodium succinate (20 mM) was added as a carbon source. (NH₄)₂SO₄, KNO₃, glutamate or glutamine was added as a nitrogen source. Strains were grown in Luria–Bertani at 37°C under aerobic conditions.

**Table 1.** Strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
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<td>Haas Laboratory</td>
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<td>Broad-host-range promoter-probe plasmid, GmR</td>
<td>Rist &amp; Kertes (1998)</td>
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source to a final concentration of 10 mM, where specified. NaN₂₃ was added to a final concentration of 5 mM.

**RNA preparation and Northern blot analysis.** Total RNA was purified using the hot phenol method (Lin-Chao & Bremer, 1986). The steady-state levels of NalA were determined by Northern blot analyses using 10 μg total RNA. The RNA was denatured for 5 min at 65 °C in loading buffer containing 50% formamide, separated on 8% polyacrylamide/8 M urea gels and then transferred to nylon membranes by semi-dry electroblotting. The RNAs were cross-linked to the membrane by exposure to UV light. The membranes were hybridized at 40 °C with gene-specific 32P end-labelled oligonucleotides (NalA, M50; 5S rRNA, I26; see Table S1 available with the online version of this paper), and the hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

**Determination of the 5’ and 3’ ends of NalA.** Simultaneous mapping of the 5’ and 3’ ends of NalA RNA was performed as follows. Total RNA was purified from PAO1(pMEnalA) (Table 1) grown in LB medium to OD₆₀₀ 2.5. The RNA was treated with tobacco alkaline phophatase (TAP) (Epicentre Biotechnologies), which removes 5’-pyrophosphates. The 5’ and 3’ ends of the TAP-treated RNA were ligated using T4 RNA ligase (Fermentas). First-strand cDNA, second-strand synthesis and final product amplification were performed using the OneStep RT-PCR kit (Qiagen) and 250 ng total RNA. The primer used for first-strand cDNA synthesis was M50. Primer N49 (Table S1) was added after first-strand cDNA synthesis in addition to primer M50. A non-TAP-treated, non-ligated RNA was used as a negative control. The reaction products were loaded on a 12% polyacrylamide gel, and the expected product was sliced out of the gel, eluted and finally cloned into the pGEM-T Easy vector (Promega) using standard procedures. The inserts of several plasmid clones were sequenced to reveal the 5’–3’ junction.

**Construction of the PAO1 nalA deletion strain.** The sequence upstream (positions −691 to −5 with respect to the transcriptional start of nad) and the sequence downstream (positions +63 to +814) of nalA were amplified by PCR using the oligonucleotide pairs L49/M49 and N49/O49 (Table S1), respectively, and chromosomal PAO1 DNA as template. The 752 bp downstream fragment was cloned into the PvuII and EcoRI site of plasmid pSUP202, resulting in pSUPNalAdown. The 687 bp upstream fragment was cloned into the EcoRI and NcoI site of pSUPNalAdown, resulting in pSUPNalA. Deletion of nalA in the plasmid was confirmed by DNA sequencing (Fig. S1a), and pSUPNalA was subsequently transformed into E. coli S17-1 and transferred by conjugation to PAO1. In order to ensure that a double crossover had occurred we scored for tetracycline sensitivity of the PAO1ΔnalA mutant. The 68 nt chromosomal deletion (Fig. S1a) spanning nucleotides −5 to +63 of NalA (the transcriptional terminator comprising nucleotides +65 to +106 was retained) was further confirmed by PCR using genomic DNA from PAO1ΔnalA and primer pair U46/I47 (Table S1) followed by sequencing of the obtained PCR product.

**Construction of plasmids and site-directed mutagenesis.** All cloning procedures were carried out in E. coli strain Top10 and the relevant DNA segments of all newly constructed plasmids were verified by DNA sequencing.

For NalA overproduction, plasmid pMEnalA was constructed. The *nalA* fragment (comprising the PAO1 genome sequence 192837–1928656; Winsor et al., 2009) was amplified by PCR using PAO1 chromosomal DNA together with the forward primer I29 containing a XbaI site and the reverse primer J29 containing an EcoRI site (Table S1). The obtained 205 nt fragment was inserted into the corresponding sites of plasmid pME4510, giving rise to pMEnalA.

For construction of the transcriptional *pnaLA–lacZ* fusion, the region comprising positions −231 to +33 with respect to the transcriptional start of NalA was amplified by PCR using PAO1 genomic DNA together with forward primer U46 containing a SalI site and reverse primer V46 (Table S1) containing a BamHI site. The PCR product was cloned into the corresponding sites of plasmid pQF50, resulting in pQFnalA.

Mutations at the −24 and the −12 site, respectively, of the putative σ₅₄ *nalA* promoter were introduced by PCR amplification of 1 ng pQFnalA plasmid using either the mutagenic primer M57 (−12 mutations) or O63 (−24 mutations) together with primer V46 (Table S1). The amplified linear pQF12mutnalA and pQF24mutnalA plasmids were circularized using T4 DNA ligase (Fermentas) and transformed into E. coli Top10. Background contamination by template plasmid was reduced by treatment with 10 U DpnI prior to transformation. The −12 and −24 mutations in the NalA promoter present in the respective plasmids were confirmed by DNA sequencing (Fig. S1b).

The two putative NtrC binding sites upstream of *nalA* (−128 to −113 and −108 to −93 with respect to the *nalA* transcriptional start) were deleted by amplification of 1 ng pQFnalA plasmid using the oligonucleotides A81/L80 (Table S1). The amplified linear plasmid pQFAntrCBSnalA was subsequently circularized with T4 DNA polymerase. Background contamination by template plasmid was reduced by treatment with 10 U DpnI prior to transformation. The correct deletion of the putative NtrC binding sites was confirmed by DNA sequencing (Fig. S1b).

For the transcriptional antitermination studies, the PAO1 genome fragment corresponding to basepairs 1928991–1928592 (Winsor et al., 2009) encompassing the *nalA* promoter, *nalA* or the *nalA* deletion (nucleotides −5 to +63 of NalA) and the first 12 codons of *nirB* (positions +1 to +36 with respect to the A of the *nirB* start codon) was PCR-amplified using genomic DNA derived from PAO1 or from PAO1ΔnalA together with primer pair U46/R64. The obtained fragments (410 bp for *pnaLA–nirB* and 342 bp for *pΔnalA–nirB*) were inserted into the SalI and BamHI sites of plasmid pQF50, resulting in pQFnalA–nirB and pQFΔnalA–nirB, respectively.

**β-Galactosidase assay.** The PAO1 strains were grown in minimal medium containing succinate (20 mM) and ammonium (10 mM) under aerated at 37 °C until they reached OD₆₀₀ 0.8. The cells were washed in PBS (140 mM NaCl, 2.7 mM KCl, 100 mM Na₂HP₂O₄, 2 mM KH₂PO₄, pH 7.4) and then resuspended either in fresh nitrogen-free minimal medium containing succinate (nitrogen depletion) or in succinate-containing minimal medium in the presence of the indicated nitrogen source. Samples were withdrawn when they reached OD₆₀₀ 1 or at the indicated time (hours) after shift to fresh medium. All experiments were done in triplicate. The β-galactosidase values were determined as described elsewhere (Miller, 1972).

**RT-PCR.** PAO1 was grown for 18 h in minimal medium containing succinate (20 mM) and ammonium (10 mM). The cells were washed in PBS and resuspended in nitrogen-free minimal medium containing succinate (BM2) or supplemented with nitrate where indicated. Samples were withdrawn 5 h following the shift to fresh medium. Purified total RNA (10 μg) was treated with 2 U RNase-free DNase I (Roche). For cDNA synthesis, 20 pmol of each of the primers N55/R64 (Table S1) were annealed to 5 μg RNA for 5 min at 65 °C. Upon cooling on ice, RNase-free AMV reverse transcriptase buffer (Promega) and dNTPs (10 mM) in a total volume of 20 μl were added. Then, 30 U AMV reverse transcriptase (Promega) was added, and the reaction was continued for 1 h at 50 °C. From this reaction 2 μl aliquots were used as templates in 25 μl PCR amplification reactions using GoTag Green Master Mix (Promega) and the primer pairs N55/R64 or Q64/R64 (Table S1), at a final concentration of 0.5 pmol each. The generated PCR fragments were analysed on 6%
RESULTS AND DISCUSSION

The NalA RNA represents the leader of the PAO1 nirBD–PA1779–cobA operon

As described in Methods, the bioinformatic search for sRNA candidates with a \( \sigma^{54} \)-dependent promoter predicted an sRNA gene upstream of the PAO1 nirB gene (Fig. 1a). The same putative sRNA was previously identified as P11 in a study by Livny et al. (2006). Synthesis of the candidate RNA was verified by Northern blot analysis (Livny et al., 2006; Fig. 1b). Given the genomic location of p11 upstream of the PAO1 nirBD–PA1779–cobA operon (Fig. 1a), we renamed P11 as nitrogen assimilation leader A (NalA). The predicted secondary structure contains three putative stem–loop structures (Fig. S2a), the distal one of which is followed by a string of U’s (T’s in Fig. 1c), which is a hallmark of Rho-independent transcriptional terminators (Platt, 1986).

NalA transcription is dependent on \( \sigma^{54} \) and NtrC

NalA was initially detected during growth in stationary phase (Livny et al., 2006). Therefore, total RNA purified from PAO1 grown to stationary phase was used to map the 5’ and 3’ ends of NalA as outlined in Methods. This analysis (Figs 1c and S3) revealed that NalA is 96 nt in length. Upstream of the NalA start a putative −12 (CTGCTT) and a −24 (TTGGCA) sequence motif (Fig. 1c) were identified that are typical signatures for \( \sigma^{54} \)-dependent promoters (Morett & Buck, 1989; consensus: TGG-N10-GCT). In addition, two putative binding sites for the response regulator NtrC (Ferro-Luzzi Ames & Nikaido, 1985; Hirschman et al., 1985; MacFarlane & Merrick, 1985) were recognized upstream of the putative \( \sigma^{54} \) promoter (Fig. 1c). Similarly, two NtrC binding sites have been identified upstream of the *K. oxytoca* nitrate assimilation operon (Chai & Stewart, 1999; Fig. S2b).

First, we tested whether \( \sigma^{54} \) is required for NalA synthesis. A transcriptional fusion comprising the putative \( \sigma^{54} \)-dependent *nalA* promoter abutted with the *lacZ* gene (*pNalA*–*lacZ*) was constructed, and the promoter activity was monitored by measuring the \( \beta \)-galactosidase activity conferred by plasmid pQF*nalA* in PAO1 and PAO1\( \Delta \)rpoN (a) and PAO1 and PAO1\( \Delta \)ntrC (b), respectively, was determined 4 h after nitrogen depletion. (c) Mutations in the −24 and −12 motifs (see Fig. 1c) of the \( \sigma^{54} \)-dependent promoter abolished promoter activation upon nitrogen depletion. The deletion of the NtrC binding regions upstream of *nalA* (Fig. 1c) resulted in decreased promoter activity. All experiments were performed in triplicate; error bars, SD.

![Fig. 2.](image-url) NalA expression is dependent on \( \sigma^{54} \) and NtrC upon nitrogen depletion. The strains were grown as described in the text, and the \( \beta \)-galactosidase activity conferred by plasmid pQF*nalA* in PAO1 and PAO1\( \Delta \)rpoN (a) and PAO1 and PAO1\( \Delta \)ntrC (b), respectively, was determined 4 h after nitrogen depletion. (c) Mutations in the −24 and −12 motifs (see Fig. 1c) of the \( \sigma^{54} \)-dependent promoter abolished promoter activation upon nitrogen depletion. The deletion of the NtrC binding regions upstream of *nalA* (Fig. 1c) resulted in decreased promoter activity. All experiments were performed in triplicate; error bars, SD.

As binding of \( \sigma^{54} \) to its target promoter results in the formation of a transcriptionally inactive closed complex, the response regulator of two-component systems is usually required for transcriptional activation of the downstream gene (Kustu et al., 1989; Merrick, 1993; Li & Lu, 2007; Hervás et al., 2009). Given the presence of putative NtrC binding sites upstream of the *nalA* start (Fig. 1c), we next asked whether the response regulator NtrC is required for activation of the \( \sigma^{54} \)-dependent *nalA* promoter. As shown in Fig. 2(b), the differential \( \beta \)-galactosidase activities observed with PAO1 and the PAO1\( \Delta \)ntrC strain strongly suggested that the \( \sigma^{54} \)-dependent *nalA* promoter requires NtrC for activation.

The \( \sigma^{54} \) - and NtrC-dependence of the *nalA* promoter was further verified by introducing mutations in the −12 and −24 regions of the putative \( \sigma^{54} \) promoter and by deletion of the two putative NtrC binding sites (Figs 1c and S1b).
Both modifications, in either the −12 or the −24 region, abrogated promoter activation 4 h after nitrogen depletion. Similarly, deletion of the NtrC binding sites resulted in a significant decrease of the promoter activity (Fig. 2c).

In Pseudomonas putida, open complex formation from σ54-dependent promoters depends on phosphorylated NtrC (Hervás et al., 2009). The phosphorylation status of NtrC depends on the nitrogen source; it is hardly phosphorylated in the presence of ammonium and glutamine, whereas it is phosphorylated in the presence of glutamate, nitrate and nitrite (Atkinson et al., 1994; Kamberov et al., 1995). We therefore monitored the β-galactosidase activity conferred by the pnaLA′-lacZ transcriptional fusion upon growth in different nitrogen sources. As anticipated, PAO1 grown in BM2 minimal medium supplemented with ammonium or glutamine, which are favoured nitrogen sources for PAO1 (Potts & Clarke, 1976; Janssen et al., 1981), displayed a very low promoter activity (Fig. 4b). In contrast, PAO1 grown in the presence of glutamate, nitrate or nitrite as sole nitrogen source showed an increase in the transcriptional activity of the pnaLA promoter (Fig. 4b). Taken together, these studies revealed that pnaLA transcription is mediated by a σ54- and NtrC-dependent promoter.

**NALA and nitrate and nitrite are required for transcription of genes located downstream of nalA**

As mentioned above, expression of the nitrate–nitrite assimilation operons in K. oxytoca and A. vinelandii occurs through transcriptional antitermination within the operon leader. Given the similarity in the genetic set up of the nitrate assimilation operons of P. aeruginosa and A. vinelandii (Fig. 1a), and the similarity of the nalA sequence to the proximal sequences of K. oxytoca nasF and A. vinelandii nasA respectively (Fig. 3), we next tested whether expression of the PAO1 nitrate assimilation operon requires antitermination within the nalA coding sequence. RT-PCR analysis of total RNA purified from PAO1 grown in BM2 minimal medium with or without nitrate revealed that co-transcription of nalA and nirB occurred only in the presence of nitrate (Fig. 4a). Moreover, in the presence of nitrate, β-galactosidase synthesis was only conferred by plasmid pQFnalA-nirB, comprising the nalA promoter, nalA and the first 12 codons of nirB abutted with the lacZ gene, but not by the variant plasmid pQFΔnalA-nirB, which contained the nalA deletion (Fig. 4b). Similarly, the PAO1ΔnalA strain failed to grow on nitrate as the sole nitrogen source, whereas PAO1 and the PAO1ΔnalA strain grew similarly in the presence of ammonium (Fig. 5a). Taken together, these data suggested that transcription of the PAO1 nitrate assimilation operon depends on antitermination within nalA.

**The A. vinelandii NasT homologue PA1785 is required for nitrate assimilation**

In K. oxytoca, antitermination occurs through binding of the antiterminator protein NasR to the upper portion of the 1:2 stem–loop structure of the nasF leader RNA (Chai & Stewart, 1999). In the PAO1ΔnalA mutant, which failed to grow on nitrate, the corresponding sequence required for NasR binding in K. oxytoca was deleted (Fig. 3), but the Rho-independent terminator was retained. Thus, the absence of the probable binding sequence for a putative PAO1 antiterminator protein but the retention of the terminator could readily explain the inability of PAO1ΔnalA to grow with nitrate (Fig. 5a). Similarly to K. oxytoca, transcriptional antitermination of the A. vinelandii nasAB operon requires the RNA-binding protein NasT (Gutierrez et al., 1995). These findings together with the studies described above...
prompted us to search for an *A. vinelandii* NasT homologue in PAO1. The product encoded by PA1785 showed 96% similarity to the *A. vinelandii* NasT protein (Fig. S5a). In addition, Pfam (Bateman et al., 2000) predicted both proteins to contain an ANTAR motif (Fig. S5a), which has been shown to be required for RNA binding and for antitermination of transcription by *K. oxytoca* NasR (Shu & Zhulin, 2002). Therefore, we tested whether the PA1875 product is necessary for utilization of nitrate as the sole nitrogen source. As shown in Fig. 5(b), the insertional inactivation of PA1875 in strain PW4148 resulted in cessation of growth upon shift to nitrate-containing minimal medium.

In contrast to the *K. oxytoca* NasR protein, which apparently contains a domain for nitrate recognition and for antitermination (Goldman et al., 1994; Lin & Stewart, 1997), the NasT protein of *A. vinelandii* lacks the nitrate sensor domain (Gutierrez et al., 1995). However, the NasS protein of *A. vinelandii*, which binds nitrate (Gutierrez et al., 1995), shares similarities with the *P. aeruginosa* AmiC protein (Gutierrez et al., 1995). The AmiC protein is known to bind to and sequester the AmiR protein in the absence of the ligand amide (Wilson et al., 1996), thereby inhibiting AmiR-mediated antitermination of the *amiEBCRS* operon. In analogy, Lin & Stewart (1997) hypothesized that in *A. vinelandii* NasT is sequestered by NasS in the absence of nitrate, whereas nitrate binding to NasS relieves NasT inhibition and allows NasT to act as an

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*(Fig. 4.)* Transcription of the PAO1 *nirB* gene occurs in the presence of nitrate and requires NalA. (a) RT-PCR analysis of *nirB* transcription in PAO1 4 h after shift to succinate minimal medium (BM2) and BM2 supplemented with nitrate. Upper panel: location of primers used for the RT-PCR analysis and expected length of the PCR products. Lower panel: in lanes marked with a filled square and open square, the primer pairs N55/I47 and N55/R64, respectively, were used. –RT, no reverse transcriptase added. (b) β-Galactosidase activity was determined in PAO1(pQFnalA-nirB) and PAO1(pQFΔnalA-nirB) 4 h after shift to BM2 medium containing nitrate. The *nalA* deletion (positions −5 to +63) abolished *lacZ* expression. The experiment was performed in triplicate; error bars, SD.

*(Fig. 5.)* NalA and NasT are required for nitrate assimilation in PAO1. Growth of strains PAO1 (diamonds), PAO1ΔnalA (a, squares) and PW4148 (b, squares) in succinate minimal medium supplemented with ammonium (NH₄⁺, filled symbols) or nitrate (NO₃⁻, open symbols). The experiment was done in duplicate; error bars, SD.
antiterminator. We found 76% homology between PA1786 and the A. vinelandii NasS protein using CLUSTAL analysis (Larkin et al., 2007; Goujon et al., 2010; Fig. S5b). Given the homologies between the NasS/T proteins of both A. vinelandii and P. aeruginosa, we therefore suggest renaming the PAO1 ORFs 1785 and 1786 as nasT and nasS, respectively (Fig. 1a). In summary, this study strongly suggests that nitrate assimilation in PAO1 requires antitermination in the nalA leader when nitrate is present as a sole nitrogen source.

The phylogenetic relationships between several Pseudomonas spp. and A. vinelandii for several housekeeping functions has revealed that the A. vinelandii proteins are most closely related to the corresponding PAO1 orthologues. Therefore, Rediers et al. (2004) suggested that A. vinelandii belongs to the genus Pseudomonas. As nitrate assimilation in PAO1 and A. vinelandii is apparently governed by homologous cis elements and trans-acting factors (Fig. 6), this study adds support to the relationship between the two species at the level of conserved metabolic regulation.

Finally, as NalA was first described as a putative sRNA (Livny et al., 2006), it seems worth noting that every identified bacterial sRNA candidate should first be scrutinized for the possibility of functioning in cis, e.g. whether they are involved in transcriptional termination/antitermination or whether they are part of riboswitches.

ACKNOWLEDGEMENTS

We are grateful to Drs Dieter Haas, University of Lausanne, and C. Manoil, University of Washington, for providing strains. This work was supported by the Austrian Science Fund grant AF04311 and by the doctoral program ‘RNA Biology’ (W-1207).

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Edited by: A. S. Ball