Nitrate-responsive NarX-NarL represses arginine-mediated induction of the *Pseudomonas aeruginosa* arginine fermentation *arcDABC* operon

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Denitrification and arginine fermentation are major parts of the anaerobic metabolism of *Pseudomonas aeruginosa*, which is important for biofilm formation and infection. The two-component regulatory system NarX-NarL is part of the underlying network and is required for denitrifying growth. All target promoters identified so far are activated by NarL. In this study the effect of NarL on arginine fermentation was investigated using proteome, Northern blot and *lacZ* reporter gene analyses. NarL-dependent repression of the *arcDABC* operon was observed and the corresponding NarL-binding site in the *arcD* promoter region was functionally localized at –60 bp upstream of the transcriptional start site using site-directed promoter mutagenesis and reporter gene fusion experiments. The results clearly show that in the presence of nitrate NarL represses the arginine-dependent activation of the *arcDABC* operon mediated by ArgR. It does not influence the oxygen-tension-dependent activation via Anr. Thus, the anaerobic energy metabolism of *P. aeruginosa* is coordinated via NarX-NarL activity. In the presence of nitrate the highly efficient denitrification is preferred over the less attractive arginine fermentation.

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INTRODUCTION

In *Pseudomonas aeruginosa* anaerobic metabolism is important for biofilm growth and for the infection of the cystic fibrosis lung involving biofilm-like *P. aeruginosa* microcolonies (Barraud *et al.*, 2006; Filiatrault *et al.*, 2006; Hassett *et al.*, 2002; Palmer *et al.*, 2007; Platt *et al.*, 2008; Sauer *et al.*, 2002; Van Alst *et al.*, 2007; Worlitzsch *et al.*, 2002; Xu *et al.*, 1998). In the absence of oxygen *P. aeruginosa* grows either by denitrification or by arginine fermentation (Carlson & Ingraham, 1983; Vander Wauven *et al.*, 1984; Zumft, 1997). The expression of genes which encode enzymes for denitrification in *P. aeruginosa* are controlled by the regulatory proteins Anr and Dnr, as well as the nitrate-responsive two-component system NarX-NarL (Arai *et al.*, 1997; Schreiber *et al.*, 2007; Ye *et al.*, 1995). Enzymes required for arginine fermentation are encoded by genes which are organized in the operon *arcDABC* (Luthi *et al.*, 1990). Anaerobic expression of *arcDABC* has been shown to be Anr-dependent and further

stimulated by the arginine-responsive regulator ArgR (Lu *et al.*, 1999). The global oxygen-sensing regulator Anr is an *Escherichia coli* Fnr homologue and is essential for fermentative and denitrifying growth in the absence of oxygen (Ye *et al.*, 1995). Moreover, Anr controls the expression of *dnr*, which encodes the nitrogen-oxide-sensing regulator Dnr (Arai *et al.*, 1997). Dnr finally induces expression of the genes encoding the nitrite, nitric oxide and nitrous oxide reductases required for denitrification (Arai *et al.*, 1999, 2003). Mutant and gene expression studies in *P. aeruginosa* and *Pseudomonas stutzeri* showed that the two-component system NarX-NarL is additionally required for denitrifying growth (Härtig *et al.*, 1999; Schreiber *et al.*, 2007). The *P. aeruginosa* NarX protein is a sensor kinase and shares 38 % and 34 % sequence identity with NarX from *P. stutzeri* and *E. coli*, respectively (Härtig *et al.*, 1999). The NarL protein is the corresponding response regulator to NarX and shares amino acid identity of 63 % and 58 % with NarL from *P. stutzeri* and *E. coli*, respectively (Härtig *et al.*, 1999). In addition to NarX-NarL, *E. coli* harbours a second two-component system named NarQ-NarP (Chiang *et al.*, 1992; Rabin & Stewart, 1993). However, so far no obvious *narQ* homologue has been functionally identified in the *P. aeruginosa* genome (Stewart, 2003).

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Abbreviations: MU, Miller units; RuBPS, ruthenium(II)-tris(bathophenanthroline disulfonate).

Recently, the NarL regulon of *E. coli* was reassessed in a comprehensive microarray experiment (Constantinidou *et al.*, 2006). This approach revealed the activation of 51 operons and the repression of 41 operons by NarL. In *E. coli* the NarX-NarL system is involved in the coordination of anaerobic energy metabolism by inducing nitrate respiration and repressing less efficient modes of energy metabolism including fermentation processes (Constantinidou *et al.*, 2006; Stewart & Rabin, 1995). In pseudomonads only a few genes have been experimentally confirmed to be targets of NarL activation. In *P. stutzeri* these are *narG*, *narK* and *dnrE* and in *P. aeruginosa* *hemA*, *narK*, *nirQ* and *dnr* (Härtig *et al.*, 1999; Krieger *et al.*, 2002; Schreiber *et al.*, 2007; Vollack *et al.*, 1999). However, so far no gene has been shown to be repressed by NarL. The effect of NarL on the transcription of genes involved in other, less favourable energy-generating systems than denitrification is unknown. In this study we investigated the effect of NarL on arginine fermentation in more detail using a combination of 2D gel electrophoresis, Northern blot analysis and *lacZ* reporter gene fusion experiments.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in LB medium (per litre: 10 g tryptone, 5 g yeast extract and 5 g NaCl) or modified AB minimal medium (Heydorn *et al.*, 2000; Schreiber *et al.*, 2006) as indicated. For anaerobic growth 50 mM KNO₃ was added. If appropriate, the antibiotic tetracycline was added at final concentrations of 5 µg ml⁻¹ for *E. coli* and 100 µg ml⁻¹ for *P. aeruginosa*. All incubations were carried out at 37 °C.

Proteome analysis. *P. aeruginosa* cells were grown anaerobically in LB containing 50 mM KNO₃. Cultures in the exponential growth phase were mixed with twice the volume of ice-cold potassium

phosphate buffer (100 mM; pH 7.4) and allowed to cool for 5 min. Cells were centrifuged at 8000 g for 20 min at 4 °C and washed twice with potassium phosphate buffer. Sedimented *P. aeruginosa* cells were resuspended in a small volume of potassium phosphate buffer and protein isolation was done as described previously (Schreiber *et al.*, 2006). The protein concentration was determined in sample buffer using the PlusOne 2D Quant kit (Amersham Biosciences). The 2D gel electrophoresis was performed using immobilized pH gradient (IPG) strips of 17 cm length covering the pH range 5–8 (Bio-Rad). The IPG strips were rehydrated overnight in rehydration buffer containing 500 µg protein. Isoelectric focusing (IEF) was carried out at 20 °C under mineral oil in a PROTEAN IEF Cell (Bio-Rad) for a total of 110 kV h. The focused IPG strips were reduced for 15 min in an SDS equilibration solution containing 15 mM DTT and afterwards alkylated twice for 15 min in the same buffer containing 150 mM iodoacetamide prior to SDS-PAGE. The IPG strips were transferred to 10% polyacrylamide gels (25.5 × 20.5 cm). SDS-PAGE was performed at a constant temperature of 20 °C with 2 W per gel for approximately 20 h. All gels were stained with ruthenium(II)-tris(bathophenanthroline disulfonate) (RuBPS), as described before (Schreiber *et al.*, 2006). Gels were documented with an FX-Scanner (Bio-Rad). Analysis and quantification of differential protein spot patterns was performed using the Software Z3 (Compugen). Two independent replicates were used for analysis. Proteins were identified by mass spectrometry as described previously (Schreiber *et al.*, 2006).

Northern blot analysis. For RNA extraction, cells were grown under oxygen-limited conditions in LB medium at 37 °C to an OD₅₇₈ of 0.2. RNA was prepared as described by Boes *et al.* (2006). Ten micrograms of RNA was separated electrophoretically on a 1% agarose gel containing 5% formaldehyde and then transferred to a nylon membrane. For hybridization, a digoxigenin-labelled *arcA* probe was used and the typical *arcABC*, *arcAB* and *arcA* transcripts were detected (Gamper *et al.*, 1992). The *arcA* probe was generated using the primer pair *arcA*-NO-for (5'-CTGACCGAGACCATCCAGAA-3') and *arcA*-NO-rev (5'-CTAATACGACTCACTATAGGGAGACAGAGGTGTTGGTGTAGG-3'). DNA was labelled using the Digoxigenin RNA Labelling kit (Roche). CDP-Star (NEB) was used for detection.

Table 1. Strains and plasmids used in this study

Bacterial strain or plasmid	Genotype or phenotype	Reference
<i>P. aeruginosa</i>		
PAO1	Wild-type	Dunn & Holloway (1971)
PAO9104	<i>narL::cat</i> , Cm ^R	Krieger <i>et al.</i> (2002)
BB43	PAO1 <i>attB::</i> (<i>P</i> _{arcD} - <i>lacZ</i>)	This work
BB45	PAO9104 <i>attB::</i> (<i>P</i> _{arcD} - <i>lacZ</i>)	This work
BB46	PAO1 <i>attB::</i> (<i>P</i> _{arcDΔNarL} - <i>lacZ</i>)	This work
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80d <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ ⁻ <i>rpsL</i> <i>nupG</i>	GibcoBRL (Invitrogen)
S17 λ- <i>pir</i>	<i>pro thi hsdR</i> ⁺ T ^p Sm ^r ; chromosome::RP4-2 Tc::Mu-Km::Tn7/λ <i>pir</i>	de Lorenzo & Timmis (1994)
SM10	<i>thi-1 thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu (Km ^R)	de Lorenzo & Timmis (1994)
Plasmids		
mini-CTX- <i>lacZ</i>	Tc ^R ; promoterless <i>lacZ</i> gene	Becher & Schweizer (2000)
pFLP2	Ap ^R ; source of FLP recombinase	Hoang <i>et al.</i> (1998)
pDH11	Tc ^R ; mini-CTX- <i>lacZ</i> containing a 1005 bp <i>Pst</i> I– <i>Bam</i> HI fragment encoding the <i>arcD</i> promoter	Dana Heldt
pBB20	Tc ^R ; pDH11 with the mutated NarL-binding site	This work

Construction of chromosomal *arcD* promoter-*lacZ* reporter gene fusions and corresponding reporter gene assay. The complete *arcD* promoter region (Fig. 3) was fused to *E. coli lacZ*. A 1005 bp PCR product was generated using the primer pair ArcDfor_A (5'-AACTGCAGGCTGCCGTGGCTCATGAT-3') and ArcDrev_B (5'-CGGGATCCCTTTGCGGGAGGGAAGA-3'); the recognition sequences for *Pst*I and *Bam*HI are underlined. The resulting DNA fragment was digested with *Pst*I and *Bam*HI and cloned into the *Pst*I/*Bam*HI-digested mini-CTX-*lacZ* vector to generate pDH11 (Table 1). This plasmid was integrated into the *attB* site of the *P. aeruginosa* PAO1 genome and the corresponding *narL* mutant strain to generate strains BB43 and BB45, respectively (Table 1). Transfer of plasmids into *P. aeruginosa* was carried out by a diparental mating as described before (Schreiber *et al.*, 2006). In the mutant strains BB43, BB45 and BB46, parts of the mini-CTX-*lacZ* containing the tetracycline-resistance gene were deleted using a FLP recombinase encoded on the pFLP2 plasmid (Hoang *et al.*, 1998). The β -galactosidase activities of the strains carrying *lacZ* reporter gene fusions were determined in the early exponential phase at an OD₅₇₈ of 0.2 and are given in Miller units (MU) (Schreiber *et al.*, 2006). Data are the result of at least three independent experiments.

Mutation of the putative NarL-binding site. A potential NarL-binding site in the *arcD* promoter (pDH11) was mutated using the crossover PCR technique (Ho *et al.*, 1989). The putative binding motif in the *arcD* promoter region for the regulatory protein NarL was detected using the Virtual Footprint tool of the PRODORIC database (Müch *et al.*, 2005). Mutation of the NarL box (TACTCAA→CATTCAA) was based on the published NarL consensus binding sequence (Tyson *et al.*, 1993). The mutation was introduced using the primer pair oBB42 (5'-AATAGCTTCC-CATTCAAAGTAATTAGAT-3') and oBB43 (5'-ATCTAATTACT-TTGAATGGGAAGCTATT-3'; the mutated NarL-binding site is underlined) to generate pBB20. Nucleotide exchanges were verified by DNA sequence determination. The corresponding vector pBB20 was integrated into the *attB* site of the *P. aeruginosa* strain PAO1 chromosome to generate strain BB46 (Table 1).

RESULTS

Proteome analysis of the *narL* mutant strain

2D gel electrophoresis of water-soluble proteins was employed to monitor the NarL-dependent production of proteins in *P. aeruginosa*. We compared the protein patterns of the *P. aeruginosa narL* mutant PAO9104 with that of the wild-type PAO1. Seven proteins were found differentially synthesized in the *narL* mutant (Fig. 1, Table 2). We detected increased protein concentrations of NarH in wild-type cells. The NarH protein is the soluble, cytoplasmic subunit of the membrane-bound nitrate reductase, encoded by the *narK₁K₂GHJI* operon (Palmer *et al.*, 2007; Schreiber *et al.*, 2007; Sharma *et al.*, 2006). This is in agreement with earlier investigations, which described that in *P. aeruginosa* and *P. stutzeri* the NarX-NarL two-component system activates transcription of the nitrate reductase operon (Härtig *et al.*, 1999; Schreiber *et al.*, 2007). The second protein found in increased concentrations in wild-type cells was porin E1 (OprE). The porin protein E1 gene *oprE* has been described before to be expressed in response to anaerobiosis (Yamano *et al.*, 1993). Furthermore, the two-component class II ribonu-

cleotide reductase subunits NrdJa and NrdJb showed an increased concentration in wild-type cells, suggesting a NarL-dependent induction of the corresponding genes (Torrents *et al.*, 2005). Both proteins were recently found induced under anaerobic conditions in the presence of nitrate (Platt *et al.*, 2008). Only three proteins with increased concentration in the *narL* mutant compared to PAO1 wild-type were found, suggesting a repression of the corresponding genes by NarL. MALDI-TOF analysis identified these proteins as arginine deiminase (ArcA), catabolic ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC) (Table 2). All three proteins are encoded by genes of the *arcDABC* operon and represent the arginine fermentation pathway for ATP generation.

Northern blot analysis of the *arcDABC* mRNA

No gene repression by NarL has previously been described for pseudomonads. Consequently, we focused our investigation on the observed decrease in ArcA, ArcB and ArcC protein concentration. In order to determine whether the regulation occurs on the mRNA or on the protein level, we performed Northern blot analyses using a digoxigenin-labelled *arcA* probe. The detection of three abundant transcripts, *arcABC*, *arcAB* and *arcA*, has been described before (Gamper *et al.*, 1992). First, we compared RNA levels prepared from *P. aeruginosa* wild-type cells grown under oxygen limitation in LB medium either with or without 50 mM nitrate. Strong signals corresponding to the *arcABC*, *arcAB* and *arcA* transcripts were detected only for RNA prepared from wild-type cells grown without nitrate (see Fig. 2). We did not detect an *arcDABC* transcript. However, its low abundance was described before (Gamper *et al.*, 1992). Interestingly, the probing of RNA prepared from the *narL* mutant strain PAO9104 grown with 50 mM nitrate resulted in almost identical signal intensities. These results clearly indicate that NarL represses arginine fermentation on the transcriptional level.

The *arcDABC* operon is repressed by NarL

We used transcriptional *P_{arcD}-lacZ* fusions to investigate the influence of NarL on the *arcDABC* promoter. We determined β -galactosidase activities of strains BB43 (wild-type) and BB45 (*narL* mutant) under identical conditions as described for the proteome analysis experiments. β -Galactosidase activities of *P_{arcD}-lacZ* increased more than threefold in the *narL* mutant (BB45, 1975 MU) compared to the wild-type (BB43, 550 MU) under anaerobic conditions in the presence of nitrate. This observation suggests that the absence of the NarL regulator leads to an increased transcription of *arcD*. Consequently, NarL is responsible for *arcDABC* repression.

Identification of the NarL-binding site in the *arcDABC* promoter

Next, we were interested to know if the observed decrease in the *arcDABC* mRNA level and corresponding *in vivo*

Fig. 1. 2D PAGE of crude cell-free extracts from the *P. aeruginosa narL* mutant (a) and the PAO1 wild-type strain (b) incubated under anaerobic conditions in LB medium containing 50 mM nitrate. Boxed areas in the gel images with significant changes in protein pattern are shown as enlarged images in (c). Boxed spots with numbers represent proteins that are synthesized at higher or lower amounts in the *narL* mutant strain. The numbers correspond to the numbers of the protein spots given in Table 2. Two representative gel images from three replicate gels are shown. Proteins were stained with RuBPS (see Methods).

promoter activity is mediated by direct repression of the *arcDABC* promoter via NarL. Earlier investigations showed that the *arcDABC* operon is expressed in response to oxygen limitation in an Anr-dependent manner (Galimand *et al.*, 1991) and that the presence of arginine increases transcription via the ArgR regulator (Lu *et al.*, 1999). The *arcDABC* promoter region contains an Anr-binding site at -41.5 nt (Gamper *et al.*, 1991) and an ArgR-binding site, which spans from -94 nt to -53 nt relative to the transcriptional start site (Lu *et al.*, 1999). We searched the *arcD* promoter for the presence of a NarL-binding site using the Virtual Footprint tool from the PRODORIC database (Münch *et al.*, 2005). This analysis revealed one conserved heptameric NarL-binding site located -60 nt upstream of the published transcriptional start site shown in Fig. 3 (Gamper *et al.*, 1991). The position of the NarL heptameric binding site at -60 nt

overlaps with the ArgR-binding site and suggests that NarL interferes with ArgR but not with the Anr regulator. In order to functionally confirm the bioinformatics prediction, the putative heptameric NarL-binding site was mutated (TACTCAA $\rightarrow$ CATTCAA) based on the published NarL consensus binding sequence from *E. coli* (TAC^C/T^NA/C^T) (Tyson *et al.*, 1993). Since the NarL box partly overlaps with the ArgR box we carefully selected positions for mutagenesis in order to avoid the change of nucleotides known to be important for ArgR binding (see Fig. 3 for details) (Lu *et al.*, 1999). We determined β -galactosidase activities of strain BB46 containing the mutated *P*_{arcD}^{NarL}-*lacZ* fusion in the wild-type strain. The activities of BB46 (1696 MU) were similar to those obtained for the *P*_{arcD}-*lacZ* in the *narL* mutant BB45 (1975 MU). This result confirms a direct repression of the *arcDABC* promoter via NarL.

Table 2. Identified proteins differentially synthesized in the *P. aeruginosa* wild-type compared to the *narL* mutant

Spot no.*	PA no.†	Protein†	Description†	Regulation‡ wt vs Δ <i>narL</i>
1	PA3874	NarH	Respiratory nitrate reductase β chain	45.73
2	PA3874	NarH	Respiratory nitrate reductase β chain	28.61
3	PA5496	NrdJb	Class II ribonucleotide reductase	12.36
4	PA5497	NrdJa	Class II ribonucleotide reductase	4.69
5	PA0291	OprE	Outer membrane porin OprE	5.31
6	PA0291	OprE	Outer membrane porin OprE	2.15
7	PA0291	OprE	Outer membrane porin OprE	1.54
8	PA5173	ArcC	Carbamate kinase	0.69
9	PA5171	ArcA	Arginine deiminase	0.13
10	PA5171	ArcA	Arginine deiminase	0.09
11	PA5172	ArcB	Catabolic ornithine carbamoyltransferase	0.12

*Spot number refers to Fig. 1.

†PA number, protein short name and description according to the *Pseudomonas* database (<http://www.Pseudomonas.com>) (Stover *et al.*, 2000).

‡Ratios of protein amount from a *P. aeruginosa* PAO1 wild-type culture to a *P. aeruginosa narL* mutant PAO9104 culture both incubated under anaerobic conditions in LB medium containing 50 mM KNO₃.

Arginine-dependent ArgR activation of *arcDABC* is repressed by nitrate-dependent NarL

To elucidate whether NarL interferes with the Anr or ArgR regulator at the *arcD* promoter, we extended our reporter gene experiments for the separate addition of nitrate and arginine to defined growth medium. The results are given in Table 3. The highest β -galactosidase activities of the *P*_{arcD}-*lacZ* fusion in the wild-type (BB43) and the *narL* mutant strain (BB45) were measured for anaerobic conditions in the presence of 20 mM arginine. The addition of nitrate decreased β -galactosidase activities by 55 % in the wild-type, but by only 24 % or 19 % in the *narL* mutant strain or the wild-type strain containing the *P*_{arcD} Δ NarL-*lacZ* fusion

with the mutated NarL-binding site in the *arcD* promoter region (Table 3). These results clearly show a NarL-dependent repression of the arginine-mediated activation of the *arcD* promoter via the proposed NarL-binding site.

DISCUSSION

Three enzymes of the arginine deiminase pathway, ArcA, ArcB and ArcC, were produced in higher amounts under anaerobic denitrifying conditions in the *P. aeruginosa narL* mutant strain, indicating a repression of the corresponding genes by NarL. It has been shown previously that the specific activity of the catabolic ornithine carbamoyltransferase (ArcB) of the arginine deiminase pathway is repressed by nitrate (Mercenier *et al.*, 1980). However, it was unknown whether this repression was mediated by the NarL regulator itself. Our Northern blot analysis and experiments with *P*_{arcD}-*lacZ* reporter gene fusions of the PAO1 wild-type and the PAO9104 mutant strains clearly indicated a repression at the transcriptional level.

A bioinformatics analysis of the *arcD* promoter region identified a putative heptameric NarL-binding site at -60 nt to the transcriptional start site. This putative binding site (TACTCAA) differs only in one position from the published *E. coli* NarL consensus binding sequence (TAC^C_TN^A_CT) (Tyson *et al.*, 1993). This putative NarL-binding site overlaps with the binding site of the ArgR regulator, which complicated mutagenesis of the NarL-binding site. However, we carefully selected positions for mutagenesis in order to avoid the change of nucleotides known to be important for ArgR binding (see Fig. 3 for details) (Lu *et al.*, 1999). The consensus sequence of the ArgR-binding site has been deduced from DNase I footprinting studies (Lu *et al.*, 1999, 2004). Control experiments in AB minimal medium with arginine confirmed that the mutation left the ArgR-binding site functional. It showed that β -galactosidase

Fig. 2. Northern blot analysis of transcripts from the *arcDABC* operon with RNA prepared from *P. aeruginosa* wild-type PAO1 grown under oxygen limitation in LB medium with 50 mM nitrate (lane 1), the wild-type PAO1 grown without nitrate addition (lane 2) and the *P. aeruginosa narL* mutant PAO9104 incubated in LB medium supplemented with 50 mM nitrate (lane 3). Total RNA (10 μ g) was isolated after 3 h incubation at 37 °C. The mRNAs were detected using an *arcA*-specific probe.

Fig. 3. Nucleotide sequence of the *arcDABC* promoter region. The previously determined transcriptional start site (Galimand *et al.*, 1991), the putative –10 region and the start of the *arcD* coding region are marked. Binding sites for the regulatory proteins ArgR, NarL and Anr are boxed. Bases identical to published consensus sequences (Galimand *et al.*, 1991; Lu *et al.*, 1999; Spiro & Guest, 1990) are marked by an asterisk. Compared to the ArgR box, the orientation of the NarL-binding site is in the opposite direction to the transcriptional start site (TACTCAA). The published consensus sequence used for NarL (Tyson *et al.*, 1993) reads TAC^C_TN^A_CT and differs in one position from that detected in the *arc* promoter.

activities of strain BB45, which carries the reporter gene fusion with the mutated NarL-binding site, increased fivefold in response to arginine compared to the reporter gene fusion with the intact NarL-binding site. The position of the putative NarL-binding site, as well as the results of the reporter gene fusion experiments, suggests the following model. The NarX-NarL regulatory system of *P. aeruginosa* is employed for activation of nitrate respiration and down-regulation of arginine fermentation under anaerobic conditions (Fig. 4). However, NarL does not completely abolish the expression of the *arcDABC* operon. In the presence of nitrate and arginine, NarL binding most likely prevents interaction of the arginine-dependent ArgR activator with its overlapping binding site and represses the further induction of *arcDABC* transcription by ArgR. No repression of *arcDABC* transcription by NarL was observed in the absence of arginine (Table 3). This finding is in agreement with the concept of a double role for NarL as an activator for nitrate reductase formation as well as a repressor of energetically less effective fermentative pathways.

Moreover, our findings are also in agreement with data recently published by Platt *et al.* (2008). The authors

observed expression of the arginine deiminase pathway under anaerobic denitrifying conditions in complex medium containing arginine. Under these conditions NarL prevents additional activation of *arcDABC* via ArgR, but not the basic expression mediated by the Anr regulator.

Our proteomics approach revealed four additional proteins with decreased concentration in the *narL* mutant strain. Only the corresponding gene of NarH, which is a part of the *narK₂GHJI* operon, has been shown to be under direct control of the NarL regulator (Schreiber *et al.*, 2007). We did not identify any highly conserved NarL-binding sites in the putative promoter regions of the genes for the remaining three proteins (data not shown). Therefore, a direct control via NarL seems unlikely. Moreover, proteins homologous to OprE, NrdJa and NrdJb are not part of the *E. coli* NarL regulon (Constantinidou *et al.*, 2006).

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Table 3. Expression of the *P_{arcD}-lacZ* reporter gene fusions in the *P. aeruginosa* wild-type and the *narL* mutant

<i>P. aeruginosa</i> strain	<i>lacZ</i> fusion	Strain background	β -Galactosidase activity (MU)*			
			–	–	+ Arg	+ Arg
			–	+ NO ₃ [–]	–	+ NO ₃ [–]
BB43	<i>P_{arcD}</i>	PAO1 (wt)	103	92	621	275
BB45	<i>P_{arcD}</i>	PAO9104 ($\Delta narL$)	233	305	666	505
BB46	<i>P_{arcD}</i> $\Delta narL$	PAO1 (wt)	469	431	2364	1916

*Bacteria were grown under oxygen-limited conditions in modified AB minimal medium. As indicated, 50 mM KNO₃ and 20 mM arginine were added. After 7 h incubation at 37 °C, samples were taken for the β -galactosidase assay. β -Galactosidase activities are the mean of at least three independent experiments. Standard deviations ranged between 3 and 15 % of the mean.

Fig. 4. Schematic representation of NarX-NarL-dependent regulation. The major regulator for anaerobic growth is Anr, which activates *arcDABC* even in the absence of arginine and nitrate. In the presence of arginine the ArgR regulator stimulates transcription of the *arcDABC* operon. If arginine and nitrate are available, the NarL regulator represses the ArgR-dependent *arcDABC* stimulation by binding to the corresponding promoter region. On the other hand, expression of the nitrate reductase operon *narK₁K₂GHJ*, which catalyses the first step in denitrification, is induced by the nitrate-response system NarX-NarL.

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