JOURNAL OF BACTERIOLOGY, Jan. 1999, p. 161–166 Vol. 181, No. 1
0021-9193/99/$04.00+0

Kinetics of *nirS* Expression (Cytochrome cd_1 Nitrite Reductase) in *Pseudomonas stutzeri* during the Transition from Aerobic Respiration to Denitrification: Evidence for a Denitrification-Specific Nitrate- and Nitrite-Responsive Regulatory System

ELISABETH HÄRTIG AND WALTER G. ZUMFT*

Lehrstuhl für Mikrobiologie der Universität zu Karlsruhe, Karlsruhe, Germany

Received 25 June 1998/Accepted 29 October 1998

After shifting an oxygen-respiring culture of *Pseudomonas stutzeri* to nitrate or nitrite respiration, we directly monitored the expression of the *nirS* gene by mRNA analysis. *nirS* encodes the 62-kDa subunit of the homodimeric cytochrome cd_1 nitrite reductase involved in denitrification. Information was sought about the requirements for gene activation, potential regulators of such activation, and signal transduction pathways triggered by the alternative respiratory substrates. We found that *nirS*, together with *nirT* and *nirB* (which encode tetra- and diheme cytochromes, respectively), is part of a 3.4-kb operon. In addition, we found a 2-kb monocistronic transcript. The half-life of each of these messages was approximately 13 min in denitrifying cells with a doubling time of around 2.5 h. When the culture was subjected to a low oxygen tension, we observed a transient expression of *nirS* lasting for about 30 min. The continued transcription of the *nirS* operon required the presence of nitrate or nitrite. This anaerobically manifested *N*-oxide response was maintained in nitrate sensor (NarX) and response regulator (NarL) knockout strains. Similar mRNA stability and transition kinetics were observed for the *norCB* operon, encoding the NO reductase complex, and the *nosZ* gene, encoding nitrous oxide reductase. Our results suggest that a nitrate- and nitrite-responsive regulatory circuit independent of NarXL is necessary for the activation of denitrification genes.

Denitrification is a facultative trait whose manifestation depends on environmental factors. Physiological studies have shown that the expression of denitrification genes usually occurs in the absence of oxygen, or at least at a low oxygen tension (pO_2), and requires the simultaneous presence of an *N*-oxide (reviewed in reference 33). The expression of denitrification in the strict sense is sequential in *Pseudomonas stutzeri* with respect to nitrate respiration, since activation of the *narGHJI* operon occurs at a higher partial oxygen pressure than that at which the other reductase genes are activated (24, 25). By monitoring the production of *narH* and *nirS* transcripts of *Paracoccus denitrificans*, it has been shown that induction of nitrate reductase precedes that of nitrite reductase (8).

In *Escherichia coli*, the transcription factor FNR mediates the oxygen response while NarL, as part of a NarXL two-component sensor-response regulator system, mediates the nitrate signal. Both FNR and NarL are required for the expression of the *narGHJI* operon. The numerous studies directed at these transcription factors have provided a detailed mechanistic picture of anaerobic and nitrate-dependent gene activation in a nitrate-respiring bacterium (reviewed in references 19 and 20). Moreover, a crystal structure for the nitrate response regulator NarL has been reported recently (7).

It is unknown which *N*-oxide-sensitive regulatory system controls the genes encoding the reductases of denitrifying bacteria that act on the substrates nitrite, nitric oxide, and nitrous oxide and to what extent their activation depends on coordinately and/or sequentially acting regulators. Advances in mRNA methodology prompted us to study gene expression in *P. stutzeri* by monitoring the kinetics of individual transcripts rather than by using reporter gene fusions. In the present study, we investigated the signal and regulator requirements for the transcriptional activation of *nirS*, *norCB*, and *nosZ* (i.e., the structural genes for the three reductases involved in nitrite denitrification) following a shifting of the respiratory metabolism from oxygen to nitrate or nitrite. By studying *narX* and *narL* deletion mutants, we obtained evidence for the existence of a second nitrate- and nitrite-responsive regulatory system in *P. stutzeri* that is specific for denitrification. A preliminary account of this work has appeared previously (21).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type traits of *P. stutzeri* ATCC 14405 are represented in this study by the spontaneously streptomycin-resistant strain MK21 (36). *P. stutzeri* was grown on synthetic asparagine-citrate medium at 30°C (36). For aerated cultures, 500 ml of medium in a 1-liter flask was inoculated with an overnight culture to an optical density at 660 nm of about 0.2 and incubated in a gyratory shaker at 240 rpm. Denitrification was induced by adding sodium nitrate (0.1%) or sodium nitrite (0.05%) and simultaneously shifting to strongly O_2-limited conditions by reducing the shaker speed to 120 rpm. When necessary, kanamycin, ampicillin, or streptomycin was added at a final concentration of 50, 100, or 200 $\mu g\ ml^{-1}$, respectively. Nitrite in the culture medium was measured as an azo dye (32).

Isolation of RNA and Northern blot analysis. Total RNA was extracted from batch cultures by the hot-phenol method, with 10- or 20-ml samples being taken at appropriate time intervals during the transition from aerobic to denitrifying growth conditions (1). Twenty micrograms of RNA from each sample was denatured by glyoxal-dimethyl sulfoxide treatment and separated on a 1.2% agarose gel (26). Equal loading of samples onto the gel was verified by acridine orange staining of the rRNA. Transfer of RNA to a positively charged nylon membrane (Boehringer Mannheim) was achieved by upward capillary action. DNA probes were labeled with dUTP-digoxigenin by random priming.

The *nirS* probe was derived by *Kpn*I digestion of plasmid pNIR44, a 1.9-kb *Pst*I-*Bgl*II fragment of plasmid pNOR161 served as the *norCB* probe, and the *nosZ* probe was obtained as a 500-bp *Pst*I fragment from plasmid NS220. Details of these probes are described elsewhere (31). DNA labeling and detection kits were from Boehringer Mannheim; they were used in accordance with the specifications of the manufacturer. The dioxetane reagent CDP-Star [disodium

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Universität Karlsruhe, PF 6980, D-76128 Karlsruhe, Germany. Phone: 49 (721) 60 80. Fax: 49 (721) 60 84 920. E-mail: dj03@rz.uni-karlsruhe.de.

2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate] was used as the chemiluminescent substrate for membrane-based detection of alkaline phosphatase conjugates. Biomax MR (Kodak) exposures were quantitated by scanning laser densitometry with an ImageMaster scanner and software (Pharmacia).

Primer extension analysis. The 5′ end of the *nirS* transcript was mapped by primer extension (6). Reverse transcription was initiated from the γ-^{32}P 5′-labeled primer 5′-ATCAGGCCAGCCAGGATAGG-3′, which was complementary to the coding strand at positions 267 to 286 of the published *nirS* sequence (23). The nucleotide sequence was obtained by the dideoxy chain termination method, using a Thermo-Sequenase kit (United States Biochemical Corp.), [^{35}S]dATP (Amersham), and the same primer. The primer extension and sequencing reaction products were analyzed on a 6% denaturing polyacrylamide gel.

Construction of *narL* and *narX* deletion strains. The *narXL* gene region was obtained from cosmid clones of *Sau*3A-digested genomic DNA of *P. stutzeri* by using the vectors pJA1 (12) and SuperCos1 (Stratagene). An 892-bp fragment of cosmid c164 with the complete *narL* gene (22) was amplified with the primers 5′-GCA**AAGCTT**CGGCCTGAAGAACAGCG-3′ and 5′-TC**GAATTC**TTGAG CGATTGCGCACAG-3′. The primers added *Hin*dIII and *Eco*RI restriction sites (nucleotides in boldface) to allow cloning into pUC18. The pUC derivative with the *narL* gene was designated pUCnarL. The kanamycin resistance (Km^r) cassette was excised from plasmid pBSL15 (2) and used to replace a 358-bp *Ava*I-*Hin*cII fragment in *narL*, resulting in vector pUCnarL::Km^r. The *narX*-carrying plasmid pBSXL was constructed by inserting a 2.4-kb *Ava*I fragment of the *narX* cosmid g279 into pBluescriptII SK(−). The *narX* locus was mutated by replacing a 520-bp internal *Eco*47III-*Hin*dIII fragment with the Km^r cassette from pBSL15 to give plasmid pBSXL::Km^r. Plasmids pUCnarL::Km^r and pBSXL::Km^r were transferred to MK21 by electroporation (Gene Pulser; Bio-Rad). The *narL* mutant MRL118 and the *narX* mutant MRX119, resulting from double-crossover events, were obtained by selection for kanamycin resistance and ampicillin sensitivity. Mutational inactivation of the genes was verified by sequencing and Southern hybridization. MRL118 was negative with the *Ava*I-*Hin*cII *narL* fragment as a probe and gave a single hybridizing 2.8-kb fragment with the Km^r probe in a genomic *Pst*I digest. The mutated 3.1-kb *narX* fragment (wild-type size, 2.4 kb) was detected in a genomic *Ava*I digest of DNA from MRX119 by hybridization, using a 153-bp PCR fragment generated from the 3′ end of the gene as the probe (primer pair 5′-CAAGCATGCGGAAGCGAAC C-3′ and 5′-GGCGCGTTCTTGCAGG-3′).

RESULTS

***nirS* is part of an operon.** We identified the *nirS* transcript in the total-RNA fraction of cells grown under denitrifying conditions to monitor the expression pattern of nitrite reductase at the mRNA level. A 0.5-kb *Kpn*I fragment from the central region of *nirS* was used as hybridization probe (Fig. 1). In Northern blot analyses, two transcripts, of 2 and 3.4 kb, were found which were absent from aerobic cells. We interpret them to be the monocistronic transcript of the *nirS* gene and the polycistronic message from the *nirSTB* operon. Transcription upstream of *nirS*, beginning with *nirQ*, proceeds in the opposite direction (Fig. 1); thus, the large transcript cannot originate from upstream genes. The monocistronic transcript may result from early termination of transcription or from posttranscriptional processing of the polycistronic message. Several inverted repeats, possible stem-loop-forming mRNA structures, at the 3′ ends of *nirS* and *nirB* (23) are candidates for affecting transcription and/or processing. The resulting increase of the *nirS* transcript relative to that of *nirTB* may be necessary to control the amounts of NirS, NirT, and NirB proteins so that they are in the appropriate balance for the denitrification process. The genes *nirT* and *nirB* encode tetraheme and diheme *c*-type cytochromes, respectively. Mutagenesis of *nirT* demonstrated the requirement of the encoded heme protein for an in vivo-functional nitrite-reducing system (23). NirT is similar to the NapC proteins encoding the putative electron-transferring *c*-type cytochromes of periplasmic nitrate reductases (33). Homologs of NirT have also been found in nondenitrifying bacteria and ascribe a broader significance to this protein (11, 13, 18, 27). Transcription of *nirB* together with *nirS* indirectly provides support for a role for the encoded diheme protein in nitrite reduction. A proteolytically shortened NirB was shown to have peroxidase activity (17).

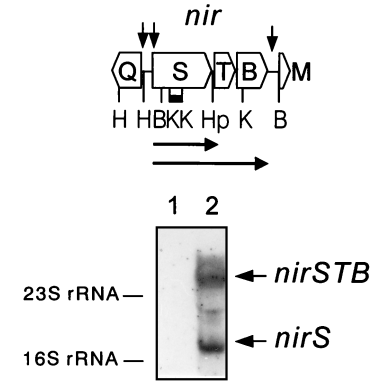

FIG. 1. *nirS* is transcribed as an operon with two downstream *nir* genes. Shown at the top are the physical map and organization of the *nir* operon of *P. stutzeri*. The horizontal arrows indicate the extents of the two *nirS* transcripts. The black bar shows the position of the 0.5-kb *Kpn*I fragment used as the *nirS* probe in Northern blotting. Vertical arrows point to the location of FNR boxes. Restriction site abbreviation are as follows: B, *Bam*HI; H, *Hin*dIII; Hp, *Hpa*I; and K, *Kpn*I. At the bottom of the figure is a Northern blot of 20-μg quantities of total RNA from cells aerobically cultivated without nitrate addition (lane 1) and from nitrate-denitrifying cells (lane 2). Hybridization was done with the digoxigenin-labeled *Kpn*I fragment. The 2.0-kb monocistronic *nirS* transcript and the 3.4-kb transcript of the *nirSTB* operon are marked by arrows; the 16S and 23S rRNA species served as standards.

Mapping the *nirS* promoter. The promoter of *nirS* was mapped by primer extension analysis (Fig. 2). The sequence TAGCAT at position −10 shows similarity to sigma factor σ^{70}-dependent promoters. A potential FNR-binding site, TTGAT-N_4-GTCAA, that is almost identical to the FNR-binding consensus sequence of *E. coli* is positioned at −43.5. FNR of *E. coli* binds to the partially palindromic nucleotide sequence TTGAT-N_4-ATCAA, known as the FNR box, of FNR-activated promoters that is centered preferentially −41.5 bp from the start site of transcription (20). Expression of *nirS* depends on DnrD (formerly termed FnrD), which is a member of the greater FNR-CRP family of regulators (30). We presume that the FNR box of the *nirS* promoter is the target of DnrD-dependent regulation. The second FNR box, located further upstream, is considered to be required for the expression of *nirQ*. In *Pseudomonas aeruginosa*, this gene was shown to be expressed anaerobically and to possibly be regulated from the single FNR box shared by the *nirS* and *nirQ* genes (4).

Stability of transcripts. The balance between mRNA synthesis and decay has a profound effect on prokaryotic and eukaryotic gene expression (10). A fast rate of turnover of mRNA is crucial for a rapid response with a pattern of gene expression altered by changing external growth conditions. The half-life of the *nirS* transcript was determined with MK21 which had been induced for denitrification over a 30-min period. Rifampin (200 μg ml^{-1} final concentration) was then added to prevent de novo mRNA synthesis, and the amount of *nirS* transcript remaining was determined by Northern blot analysis. We determined a half-life of approximately 13 min for both the monocistronic and polycistronic *nirS* messages (Fig. 3). Also, the transcripts of *norCB* and *nosZ* were monitored under the same conditions. The genes *norCB*, encoding the NO reductase complex, form an operon with a single 2-kb transcript (35). *nosZ*, the N_2O reductase structural gene, is transcribed from six promoters, but in each case as a monocistronic unit (14). The *norCB* and *nosZ* mRNAs each exhibited the same half-life as the *nirS* transcripts in the denitrifying cell (data not shown). Hence, a differential stability of transcripts is

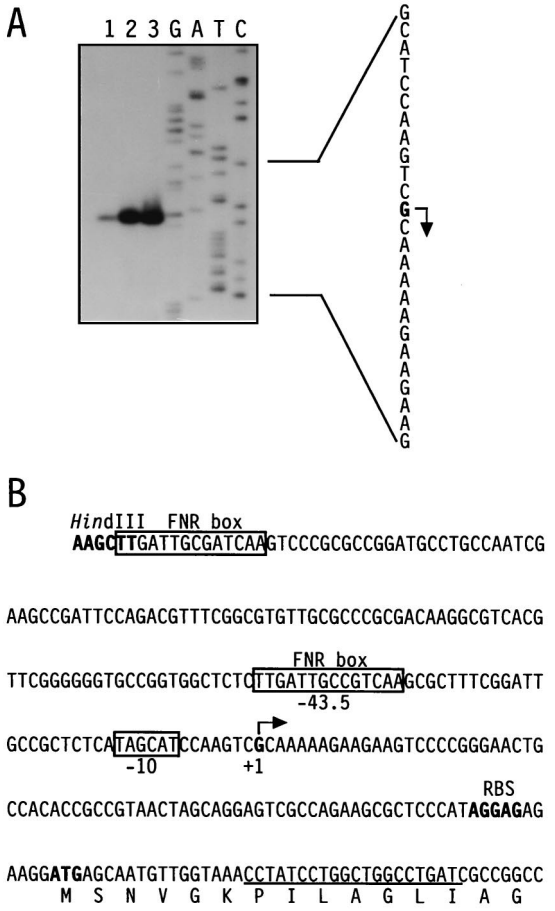

FIG. 2. The nirS promoter. (A) Primer extension analysis. Total RNA was extracted from cells cultured under nitrate-denitrifying conditions. The primer extension products from 10, 30, and 50 μg of RNA (lanes 1 to 3, respectively) were separated on a sequencing gel together with a sequence ladder generated with the primer shown in panel B. The complementary sequence of the transcription initiation site is represented; the 5′ end of the *nirS* transcript is indicated by the arrow. (B) The *nirS* promoter. The 5′ end of the transcript is labeled +1. Putative polymerase (−10) and FNR (−43.5) binding sites are boxed. The oligonucleotide that was used for primer extension is underlined. The first few N-terminal amino acids of the NirS protein are shown in one-letter code. RBS, ribosome-binding site.

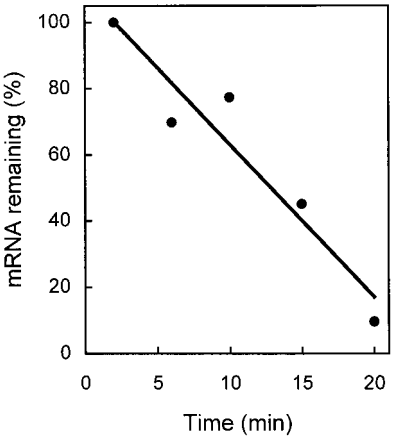

FIG. 3. Stability of *nirS* mRNA. A culture of *P. stutzeri* was induced for denitrification under O_2-limited conditions (shaker speed, 120 rpm) with sodium nitrate (1 g liter^{-1}). At 2, 10, 15, and 20 min after inhibition of transcription by addition of rifampin (200 μg μl^{-1} final concentration), total RNA was isolated and separated electrophoretically, and transcripts were detected by Northern blotting. Least-squares analysis gave a half-life of 12.6 min for the *nirS* mRNA.

apparently not an element in the regulation of the structural genes encoding the oxidoreductases of denitrification.

The stability of mRNA can be affected by the growth rate (28). A half-life of 13 min was determined for mRNA in denitrifying cells with a doubling time of about 2.5 h. Overall, this qualifies the transcripts of denitrification genes as being of intermediate to high stability. Reported half-life values for bacterial mRNAs range from 0.5 to 50 min, typically being between 2 and 4 min (9). An extremely long half-life of 5 to 7 h has been reported for *hoxS* mRNA (encoding a soluble hydrogenase) of *Ralstonia* (*Alcaligenes*) *eutropha* cells exhibiting a doubling time of 20 h (29). *ompA* mRNA, encoding a porin, is another example of a rather stable mRNA, with a half-life of 13 to 25 min in cells doubling every 40 min. However, this mRNA species becomes short-lived in slow-growing cells (28).

Temporal changes of *nirS* mRNA in the transition to denitrification. The time course of expression of *nirS* in response to nitrate and nitrite was monitored by mRNA analysis during the shift from aerobiosis to oxygen-limited and denitrifying conditions. We have previously shown that complete anaerobiosis is not required for expression of the denitrification system of *P. stutzeri* (25). The denitrification reductases appear when the level of saturation of the medium with air falls below 17% and an *N*-oxide is present. In a well-aerated culture (shaker speed, 240 rpm), no transcripts from the *nirS* operon were detected (Fig. 4). When the oxygen tension was subsequently lowered by reducing the shaker speed to 120 rpm and excluding the *N*-oxide, both *nirS* transcripts appeared within 15 min. At about 30 min after the onset of oxygen limitation, the *nirS* transcripts had nearly disappeared again, and they were not detected at subsequent sampling points extending to 3 h (Fig. 4A). This shows that although oxygen limitation alone causes a transient induction of *nirS* in *P. stutzeri*, it is not sufficient for long-term induction in the absence of an *N*-oxide. It is important to note that on addition of nitrate or nitrite to well-aerated cells, no activation of *nirS* transcription took place (data not shown).

Addition of nitrate to oxygen-limited cells rapidly induced *nirS* transcription and cytochrome cd_1 synthesis (Fig. 4A). A short period of accumulation of nitrite in the culture medium accompanied the transition phase (Fig. 4C); this can be rationalized from the sequential induction of nitrate reduction and nitrite reduction (8, 24, 25). The level of *nirS* mRNA reached a maximum within 15 min and remained constant during the 3-h period of observation (Fig. 4A). Since the half-life of *nirS* mRNA is about 13 min, persistence of those transcripts for hours can occur only if the *nirS* operon is continuously activated. We repeated the experiment using nitrite instead of nitrate and found the same temporal response of the *nirS* transcripts (Fig. 4B). Toward the end of this experiment, the amount of *nirS* mRNA was decreased in the 3-h measurement, which coincided with the disappearance of nitrite (Fig. 4C). In parallel experiments lasting up to 5 h, we correlated the disappearance of the *nirS* transcripts with exhaustion of nitrite in the medium, i.e., lack of the inducer. The amount of *nir* message decreased within the time frame of the mRNA half-life.

We have performed the oxygen respiration-to-denitrification shift experiment in an identical manner with the probes for *norCB* and *nosZ*. The induction patterns observed for *norCB* and *nosZ* were the same as those seen for the *nirS* operon (data not shown). In both cases there was a short period of gene activation in response to lowering of the pO_2, whereas con-

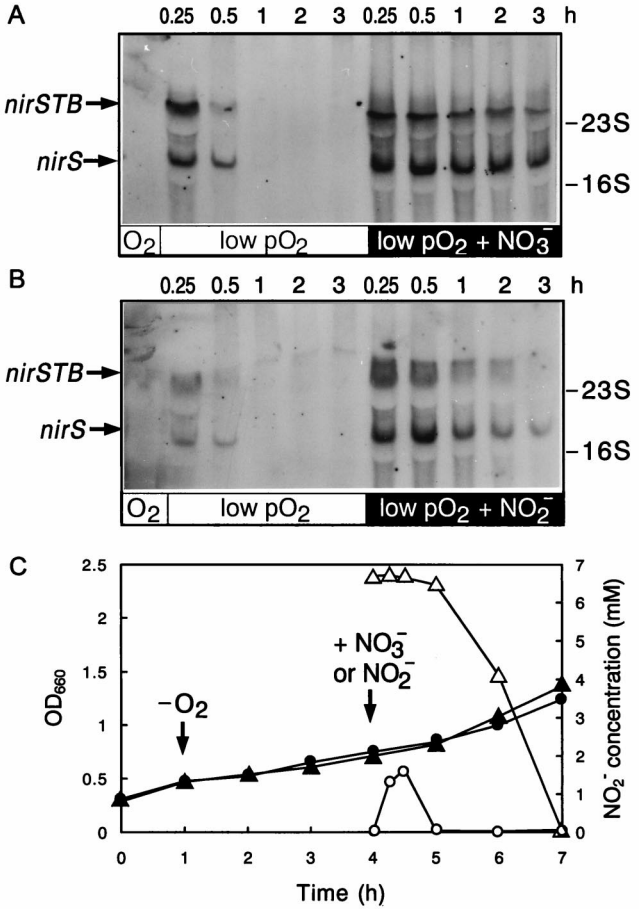

FIG. 4. Kinetics of *nirS* induction by nitrate or nitrite under low-oxygen-tension conditions. MK21 was grown aerobically (shaker speed, 240 rpm) and checked for the absence of *nirS* mRNA (left lanes, panels A and B). (A) The culture was shifted first to low-oxygen-tension conditions (shaker speed, 120 rpm; lanes labeled low pO_2 in panels A and B) and then induced for denitrification by addition of sodium nitrate (1 g $liter^{-1}$). (B) As in panel A, but induction was with sodium nitrite (0.5 g $liter^{-1}$). At the indicated time intervals after shifting to low-pO_2 conditions and adding the respiratory substrate, the appearance of *nirS* transcripts was monitored by Northern blot analysis. (C) Increases in cell mass, as determined by measurement of optical densities at 600 nm (closed symbols), and changes in nitrite concentrations (open symbols) were monitored in both experiments; data were plotted on the absolute time scale. Circles correspond to panel A; triangles correspond to panel B. Vertical arrows point to the effected changes in growth conditions.

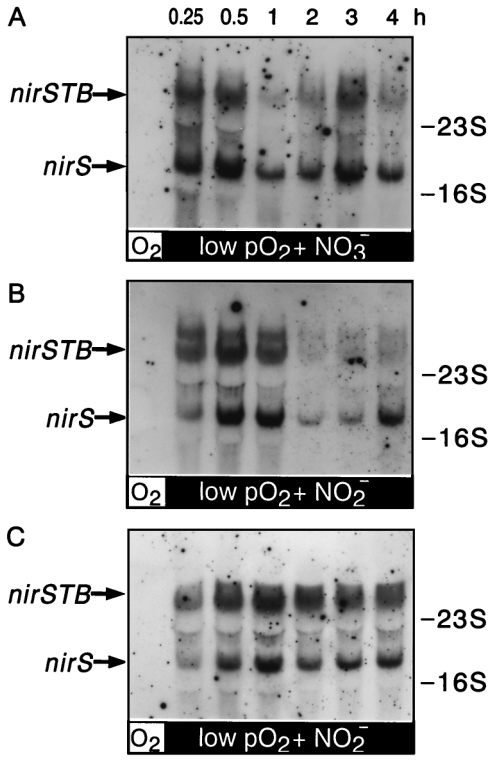

FIG. 5. Nitrate- or nitrite-induced *nirS* expression is independent of NarL or NarX. The nitrate response regulator mutant MRL118(Δ*narL*) (A and B) and the nitrate sensor mutant MRX119(Δ*narX*) (C) were grown aerobically (shaker speed, 240 rpm), checked for the absence of *nirS* transcripts by Northern blotting (data points labeled O_2), and then induced for denitrification by a shift to low pO_2 (shaker speed, 120 rpm) and the addition of nitrate (1 g of $NaNO_3$ per liter) (A) or nitrite (0.5 g of $NaNO_2$ per liter) (B and C). The production of transcripts were monitored in each case for 4 h after the shift.

tinuing transcription of *norCB* and *nosZ* again required the presence of nitrate or nitrite. Within the limits of the temporal resolution obtained with 15-min intervals of the initial sampling points, coordinate expression of *nirS*, *norCB*, and *nosZ* was observed. This is in agreement with the results from a previous immunochemical study (24).

Evidence for a regulatory system other than NarXL that mediates nitrate and nitrite induction of denitrification. We have recently found that *P. stutzeri* possesses a nitrate response regulator, NarL. This regulator acts in concert with the sensor NarX as part of a two-component system that mediates nitrate and nitrite response in the expression of respiratory nitrate reductase (22). The *narL* and *narX* genes were mutated to generate the deletion strains MRL118 and MRX119, respectively, as described in Materials and Methods. Under aerobic growth conditions, no *nirS* transcripts were detected, but under conditions of oxygen limitation, transcription of *nirS* was induced by nitrate or nitrite in both mutants (Fig. 5A and B). The nitrate-challenged *narL* strain MRL118 induced *nirS* mRNA within 15 min, and this mRNA then remained present at approximately the same level during the observation period of 4 h. The same result was obtained when the mutant was challenged with nitrite. The activation kinetics of *nirS* transcription in the *narL* mutant in response to nitrate or nitrite were indistinguishable from that of the wild type. Transcription of the *nirS* operon in the *narX* mutant MRX119 in response to nitrate and nitrite exhibited again the same kinetic pattern as the *narL* mutant or the wild type. Figure 5C shows exemplarily the activation of *nirS* transcription by nitrite in an experiment involving a shift from aerobic respiration to denitrification. Also, the expression of *norCB* and *nosZ* was not altered in the mutants MRL118 and MRX119. Since neither *narX* nor *narL* inactivation affected expression of the reductases involved in denitrification in the strict sense, we postulate the existence of one or more signal transduction pathways triggered by nitrate or nitrite, independent of the two-component sensor regulator system NarXL.

DISCUSSION

As shown in Fig. 4, oxygen withdrawal alone caused only a transient effect on the *nirS* operon, whereas its continuing expression and likewise that of the *norCB* and *nosZ* genes, required activation by nitrate or nitrite. Transcriptional activation of denitrification genes induced by low-oxygen conditions cannot as yet be ascribed to a distinct regulator in *P.*

stutzeri. Although FnrA, an FNR-type regulator, of this bacterium has been found earlier (15), it does not act as a global regulator, which would induce the structural genes for denitrification reductases in response to oxygen withdrawal, nor does it act on DnrD, which is also necessary for the expression of *nirS* and *norCB* (30). We presume that the FNR box of the *nirS* promoter (Fig. 2) is the target of DnrD. The *dnrD* gene itself is part of an operon that shows a complex pattern of transcriptional response to oxygen and nitrate (30). DnrD and its homologs from other bacteria belong into a separate phylogenetic branch within the greater FNR family. This group is unlikely to comprise oxygen-responsive elements, since all lack the cysteine residues needed for complexing the 4Fe-4S clusters of redox-active FNR proteins. In this respect, the situation in *P. stutzeri* is clearly different from that in *P. aeruginosa*, for which the FNR-type regulator ANR was suggested to respond to oxygen and function in a hierarchical relationship with the DNR regulator, targeting both *nirS* and *norCB* (5).

The observations made in the studies with the *narX* and *narL* mutants provide evidence for a pathway of nitrate and nitrite regulation which we ascribe to a new regulatory circuit. Since inactivation of *narX* did not produce a phenotype with respect to the induction of the denitrification genes proper, it is clear that NarX functions independently from this system; i.e., the latter has a nitrate- and nitrite-sensory element distinct from NarX. The genetic organization of *narXL* is suggestive of an operon structure (22), which means that the *narX* mutation is polar on *narL*, but this will not affect the conclusions noted above. Further work is needed to determine whether the postulated new system belongs to the two-component paradigm, which may comprise a system homologous to NarXL, such as NarQP of *E. coli* (16), but specific for denitrification, or whether it comprises a novel type of nitrate- and nitrite-responsive system.

In an immunochemical study, we have previously found that the mutant strain MK137, which lacks nitrate metabolism, requires nitrite for full induction of cytochrome cd_1. This observation suggests the presence of a nitrite-responsive element (34), which we can now attribute to the new regulatory system. The activator component of this system may directly recognize a promoter element of *nirS* or act indirectly via a further transcriptional regulator. Within this context, it is of interest that the *nirS* promoter of *P. aeruginosa* was demonstrated to be nitrite responsive by the use of *lacZ* fusions (3). The regulator for this effect and the cognate promoter element, however, were not identified. Experiments to isolate and characterize the genes encoding the nitrate- and nitrite-responsive components necessary for denitrification in *P. stutzeri* are in progress.

ACKNOWLEDGMENTS

We thank M. F. Alexeyev for kindly supplying plasmid pBSL15 and U. Schiek for providing a gene bank and the *narL* mutant.

The work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli*. J. Biol. Chem. **256:**11905–11910.
2. **Alexeyev, M. F.** 1995. Three kanamycin resistance gene cassettes with different polylinkers. BioTechniques **18:**52–55.
3. **Arai, H., Y. Igarashi, and T. Kodama.** 1991. Nitrite activates the transcription of the *Pseudomonas aeruginosa* nitrite reductase and cytochrome *c*-551 operon under anaerobic conditions. FEBS Lett. **288:**227–228.
4. **Arai, H., Y. Igarashi, and T. Kodama.** 1994. Structure and ANR-dependent transcription of the *nir* genes for denitrification from *Pseudomonas aeruginosa*. Biosci. Biotechnol. Biochem. **58:**1286–1291.
5. **Arai, H., T. Kodama, and Y. Igarashi.** 1997. Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. Mol. Microbiol. **25:**1141–1148.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
7. **Baikalov, I., I. Schröder, M. Kaczor-Grzeskowiak, K. Grzeskowiak, R. P. Gunsalus, and R. E. Dickerson.** 1996. Structure of the *Escherichia coli* response regulator NarL. Biochemistry **35:**11053–11061.
8. **Baumann, B., M. Snozzi, A. J. B. Zehnder, and J. R. van der Meer.** 1996. Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes. J. Bacteriol. **178:**4367–4374.
9. **Belasco, J. G.** 1993. mRNA degradation in prokaryotic cells: an overview, p. 3–12. *In* J. G. Belasco and G. Brawerman (ed.), Control of messenger RNA stability. Academic Press, Inc., San Diego, Calif.
10. **Belasco, J. G., and C. F. Higgins.** 1988. Mechanisms of mRNA decay in bacteria: a perspective. Gene **72:**15–23.
11. **Bergmann, D. J., D. M. Arciero, and A. B. Hooper.** 1994. Organization of the *hao* gene cluster of *Nitrosomonas europaea*: genes for two tetraheme *c* cytochromes. J. Bacteriol. **176:**3148–3153.
12. **Braun, C., and W. G. Zumft.** 1992. The structural genes of the nitric oxide reductase complex from *Pseudomonas stutzeri* are part of a 30-kilobase gene cluster for denitrification. J. Bacteriol. **174:**2394–2397.
13. **Camilli, A., and J. J. Mekalanos.** 1995. Use of recombinase fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. **18:**671–683.
14. **Cuypers, H., J. Berghöfer, and W. G. Zumft.** 1995. Multiple *nosZ* promoters and anaerobic expression of *nos* genes necessary for *Pseudomonas stutzeri* nitrous oxide reductase and assembly of its copper centers. Biochim. Biophys. Acta **1264:**183–190.
15. **Cuypers, H., and W. G. Zumft.** 1993. Anaerobic control of denitrification in *Pseudomonas stutzeri* escapes mutagenesis of an *fnr*-like gene. J. Bacteriol. **175:**7236–7246.
16. **Darwin, A. J., and V. Stewart.** 1996. The NAR modulon systems: nitrate and nitrite regulation of anaerobic gene expression, p. 343–359. *In* E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. Chapman & Hall, New York, N.Y.
17. **Denariaz, C. M., M.-Y. Liu, W. J. Payne, J. LeGall, L. Marquez, H. B. Dunford, and J. van Beeumen.** 1989. Cytochrome *c* peroxidase activity of a protease-modified form of cytochrome *c*-552 from the denitrifying bacterium *Pseudomonas perfectomarina*. Arch. Biochem. Biophys. **270:**114–125.
18. **Dolata, M. M., J. J. van Beeumen, R. P. Ambler, T. E. Meyer, and M. A. Cusanovich.** 1993. Nucleotide sequence of the heme subunit of flavocytochrome *c* from the purple phototrophic bacterium, *Chromatium vinosum*. A 2.6-kilobase pair DNA fragment contains two multiheme cytochromes, a flavoprotein, and a homolog of human ankyrin. J. Biol. Chem. **268:**14426–14431.
19. **Gennis, R. B., and V. Stewart.** 1996. Respiration, p. 217–261. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
20. **Guest, J. R., J. Green, A. S. Irvine, and S. Spiro.** 1996. The FNR modulon and FNR-regulated gene expression, p. 317–342. *In* E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. Chapman & Hall, New York, N.Y.
21. **Härtig, E., and W. G. Zumft.** 1996. Nitrate and nitrite response of denitrification genes studied by mRNA analysis. BIOspektrum **2:**88.
22. **Härtig, E. H., and W. G. Zumft.** 1998. Respiratory nitrate reductase of *Pseudomonas stutzeri* is regulated by a two-component system, NarXL, that is ineffective in nitrate control of denitrification sensu stricto. BIOspektrum **4:**51.
23. **Jüngst, A., S. Wakabayashi, H. Matsubara, and W. G. Zumft.** 1991. The *nirSTBM* region coding for cytochrome cd_1-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins. FEBS Lett. **279:**205–209.
24. **Körner, H.** 1993. Anaerobic expression of nitric oxide reductase from denitrifying *Pseudomonas stutzeri*. Arch. Microbiol. **159:**410–416.
25. **Körner, H., and W. G. Zumft.** 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl. Environ. Microbiol. **55:**1670–1676.
26. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74:**4835–4838.
27. **Méjean, V., C. Iobbi-Nivol, M. Lepelletier, G. Giordano, M. Chippaux, and M.-C. Pascal.** 1994. TMAO anaerobic respiration in *Escherichia coli*: involvement of the *tor* operon. Mol. Microbiol. **11:**1169–1179.
28. **Nilson, G., J. G. Belasco, N. S. Cohen, and A. von Gabain.** 1984. Growth-rate dependent regulation of mRNA stability in *Escherichia coli*. Nature **312:**75–77.
29. **Oelmüller, U., H. G. Schlegel, and C. G. Friedrich.** 1990. Differential stability

of mRNA species of *Alcaligenes eutrophus* soluble and particulate hydrogenases. J. Bacteriol. **172:**7057–7064.
30. **Vollack, K.-U., E. Härtig, H. Körner, and W. G. Zumft.** Multiplicity of transcription factors of the FNR family in denitrifying *Pseudomonas stutzeri*: characterization of four *fnr*-like genes, regulatory responses, and cognate metabolic processes. Submitted for publication.
31. **Vollack, K.-U., J. Xie, E. Härtig, U. Römling, and W. G. Zumft.** 1998. Localization of denitrification genes on the chromosomal map of *Pseudomonas aeruginosa*. Microbiology **144:**441–448.
32. **Werner, W.** 1980. Nachweis von Nitrit und Nitrat über die Bildung von Azofarbstoffen. Fresenius Z. Anal. Chem. **304:**117–124.
33. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.
34. **Zumft, W. G., S. Blümle, C. Braun, and H. Körner.** 1992. Chlorate resistant mutants of *Pseudomonas stutzeri* affected in respiratory and assimilatory nitrate utilization and expression of cytochrome cd_1. FEMS Microbiol. Lett. **91:**153–158.
35. **Zumft, W. G., C. Braun, and H. Cuypers.** 1994. Nitric oxide reductase from *Pseudomonas stutzeri*: primary structure and gene organization of a novel bacterial cytochrome *bc* complex. Eur. J. Biochem. **219:**481–490.
36. **Zumft, W. G., K. Döhler, H. Körner, S. Löchelt, A. Viebrock, and K. Frunzke.** 1988. Defects in cytochrome cd_1-dependent nitrite respiration of transposon Tn*5*-induced mutants from *Pseudomonas stutzeri*. Arch. Microbiol. **149:**492–498.