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The Requirement of RpoN (Sigma Factor σ^{54}) in Denitrification by *Pseudomonas stutzeri* Is Indirect and Restricted to the Reduction of Nitrite and Nitric Oxide

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The *rpoN* region of *Pseudomonas stutzeri* was cloned, and an *rpoN* null mutant was constructed. RpoN was not essential for denitrification in this bacterium but affected the expression levels and enzymatic activities of cytochrome cd_1 nitrite reductase and nitric oxide reductase, whereas those of respiratory nitrate reductase and nitrous oxide reductase were comparable to wild-type levels. Since the transcription of the structural genes *nirS* and *norCB*, coding for nitrite reductase and the nitric oxide reductase complex, respectively, proceeded unabated, our data indicate a posttranslational process for the two key enzymes of denitrification depending on RpoN.

Denitrification is an alternative way of energy conservation for many facultative anaerobic bacteria. The process is thought to be organized in a tripartite modular way, in which the respiratory systems utilizing nitrate, nitrite and nitric oxide (NO), and nitrous oxide (N_2O) have to be induced by environmental signals to form a functional unit for the complete denitrification pathway (for a review, see reference 24). To achieve this, the expression of the genes encoding the four reductases and accessory proteins have to be regulated in a concerted way, which may be controlled by a distinct sigma factor. Sigma factors have been found as coordinating elements for the transcription of specific sets of genes in response to environmental stimuli.

Sigma factor σ^{54} (encoded by the *rpoN* gene) was originally described as a factor involved in the expression of nitrogen-regulated genes; since then multiple and diverse physiological functions have been found to depend on this factor (12). The role of σ^{54} in denitrification is still insufficiently clarified. The factor is required in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*), a denitrifying hydrogen bacterium, for anaerobic growth on nitrate (18). However, it is not clear whether critical genes for denitrification depend on RpoN or whether the requirement is indirect in nature. In *Pseudomonas aeruginosa* RpoN controls diverse sets of genes, such as those for glutamine synthetase, urease, and flagellin, but an *rpoN* mutant grows anaerobically on nitrate (20). In this case it is not known whether the expression of genes for the entire denitrification pathway is independent of RpoN. A comparison of promoters of several denitrification genes of *Pseudomonas stutzeri* did not provide sequence-specific clues with respect to a dependence on RpoN (4). In *Bradyrhizobium japonicum* the expression of genes for nitrate respiration again is independent of *rpoN* (for a review, see reference 6), but as for *P. aeruginosa* the role of σ^{54} in the proper denitrification system remains to be investigated. An *rpoN* mutant of the diazotrophic denitrifier *Azospirillum brasilense* is defective in nitrate assimilation, yet a possible effect on denitrification has not been explored (17).

Here we describe the isolation of the *rpoN* gene region from *P. stutzeri*. To show which step of denitrification is regulated by RpoN we constructed an *rpoN* mutation by gene replacement and analyzed the mutant for the expression of the four terminal reductases of denitrification at the transcriptional and translational levels.

Isolation and cloning of *rpoN*. The experimental strain was *P. stutzeri* MK21, a spontaneously streptomycin-resistant derivative of strain ATCC 14405. The presence of an *rpoN* gene was demonstrated by Southern hybridization of genomic DNA with a probe from *rpoN* of *Pseudomonas putida*. A genomic cosmid library of MK21 was screened with a 1.4-kb *Sac*I-*Hin*dIII fragment derived from the *rpoN*-carrying plasmid pNTR1 (10). Cosmid DNA of 264 clones was isolated (5) and digested with *Sma*I. The DNA was separated on 0.75% agarose gels and blotted onto nitrocellulose membranes. Hybridization was performed at 65°C as described elsewhere (19). The *rpoN* gene of *P. stutzeri* was found on a 3.4-kb *Hin*dIII fragment on cosmid c167. It was cloned as two *Eco*RI-*Hin*dIII fragments of 1.6 and 1.8 kb into the vector pUC18. The 1.8-kb clone, pRpoN1.8, carried the complete *rpoN* gene. Occasionally a bacterium harbors two gene copies of *rpoN* (11). Since hybridization of genomic DNA with the homologous 1-kb *Xho*I probe (see below) at low stringency (45°C) gave a single signal, we assume that *P. stutzeri* possesses only one copy of *rpoN*.

Sequence analysis of *rpoN* and its flanking regions. The nucleotide sequence of *rpoN* was determined by sequencing plasmid pRpoN1.8, and the *rpoN*-flanking regions were obtained by direct sequencing of cosmid c167. For this purpose the dideoxy chain termination method with universal and sequence-specific primers was used together with a Thermo-Sequenase kit (United States Biochemical Corp.) and [^{35}S] dATP (Amersham). The sequence revealed four open reading frames (ORFs), whose derived products were similar to those derived from the *rpoN* region of other denitrifiers (Fig. 1). The *rpoN* sequence extends over 1,503 bp and encodes a protein of 502 amino acids with a M_r of 56,843. The derived amino acid sequence exhibits the three regions which have been determined to be typical of σ^{54} factors (for a review, see reference 15). The N-terminal region has the domain of 50 amino acids, rich in glutamine and leucine, followed by 110 residues with a prevalence of acidic amino acids. The carboxy-terminal region exhibits the helix-turn-helix structure (amino acid positions 387 to 412, *P. stutzeri* count) and the invariant sequence ARRTV AKYR (positions 479 to 487), known as the RpoN box (21).

ORF241, upstream of *rpoN*, is transcribed in the same di-

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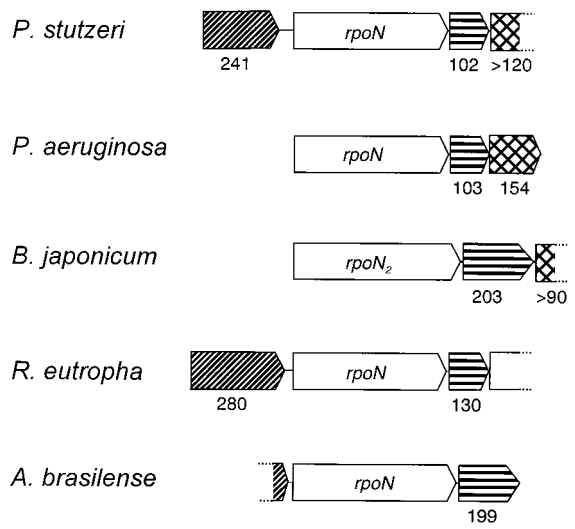

FIG. 1. Organizational conservation of *rpoN* regions in denitrifying bacteria. ORFs are shown as arrow boxes to indicate the direction of transcription. Numbers of amino acids of the derived gene products are shown. Homologous components are indicated by identical patterns: ATP-binding proteins of ABC transporters (▨), modulators of σ^{54} function (▤), and PTS proteins, E_{IIA} (▩). Information about the following organisms was taken from the following references: *P. aeruginosa* (9), *B. japonicum* (11), *R. eutropha* (22), *A. brasilense* (17). Incompletely sequenced ORFs are shown as open boxes.

rection and potentially encodes a 241-amino-acid protein with a M_r of 26,420. Downstream of *rpoN*, ORF102 may encode a 102-amino-acid polypeptide with a M_r of 11,752. The next ORF was sequenced only partially to cover 120 amino acids. The product of this ORF is a homolog of the protein E_{IIA} of the phosphotransferase system. Mutations in the corresponding ORFs 95 and 154 downstream of *rpoN* of *Klebsiella pneumoniae* increase transcription from RpoN-dependent promoters, suggesting that the gene products may act as modulators of σ^{54} activity (16).

Primer extension analysis was used to locate the promoter of *rpoN*. Total RNA was prepared from MK21 cells grown in asparagine-citrate (AC) medium under oxygen-limited conditions and supplemented with 1 g of $NaNO_3$ per liter (3). The RNA was extracted by a method described elsewhere (1), and primer extension was done by a standard protocol (2). We found two transcript initiation sites spaced by only two nucleotides. Upstream of those sites the sequences TATAAT and TAGGCA are thought to be −10 and −35 binding motifs, respectively. The −10 sequence is identical to the σ^{70} consensus sequence of *Escherichia coli* (8), whereas the −35 sequence varies somewhat from the TTGACA consensus sequence. The presence of these motifs suggests that *rpoN* of *P. stutzeri* is under the control of the principal sigma factor σ^{70}, encoded by *rpoD*. Interestingly, the *rpoN* promoter of *P. stutzeri* is identical from positions +2 to −21 to the promoter sequence of *rpoN* of *P. aeruginosa* and extends further to an identical −35 motif (Fig. 2). Overall the derived *P. stutzeri* and *P. aeruginosa* RpoN proteins are 84.5% identical.

Construction of an *rpoN* mutation by gene replacement. We constructed a null mutant to analyze the role of RpoN in denitrification of *P. stutzeri*. An internal 1-kb *Xho*I fragment which covers 66% of the *rpoN* gene was deleted from pRpoN1.8. The kanamycin resistance (Km^r) cassette from plasmid pUC4-Kiss (Pharmacia) with the aminoglycoside 3′-phosphotransferase gene of transposon Tn*903* was inserted in the opposite orientation into *rpoN*, yielding plasmid pRpoN::Km^r. The replacement construct was linearized at the *Eco*RI site and electroporated into competent *P. stutzeri* cells. For electroporation the bacterium was grown in 3.2% Bacto Tryptone–2% yeast extract–0.5% NaCl, pH 7.5, for 16 h at 30°C. The cells were washed twice with 15% glycerol in 1 mM MOPS (morpholinepropanesulfonic acid), pH 7.5. Conditions for electroporation in a 0.2-cm cuvette were 2.5 kV, 25 μF, 200 Ω (Bio-Rad Gene Pulser). Disruption of the wild-type gene by homologous recombination in mutant MK516 was selected for on the basis of kanamycin resistance (final concentration, 200 μg ml^{-1}) and ampicillin sensitivity (100 μg ml^{-1}). Genomic DNA from MK516 was cleaved with *Hin*dIII. The digest was separated on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with the 1-kb *Xho*I fragment of *rpoN* and the 1.3-kb Km^r cassette to verify gene inactivation. Because of the effected deletion the *Xho*I probe gave no signal, whereas the Km^r probe yielded two signals of 1.5 and 2.6 kb, due to the internal *Hin*dIII site of the inserted cassette (data not shown).

Effect of the *rpoN* mutation on denitrification enzymes. A striking phenotype of the *rpoN* mutant MK516 was its loss of growth on AC minimal medium. Since the structural gene for glutamine synthetase is under RpoN control in *P. aeruginosa* (20), we supplemented AC medium with glutamine but were unsuccessful in restoring growth with or without nitrate either under aerobic or anaerobic conditions. The mutant was also unable to use histidine or proline as the sole nitrogen source. The lack of growth of the *rpoN* mutant on minimal medium indicates that the absence of σ^{54} must affect amino acid metabolism and other routes of intermediary metabolism of *P. stutzeri*. MK516 grew in Luria-Bertani medium with nitrate, aerobically as well as anaerobically, but under the latter conditions grew more slowly than the wild type, demonstrating that *rpoN* exerts a general effect on denitrification. MK516 also lost its motility on 0.3% swarm agar, which indicates that RpoN may be required for flagellin synthesis, as had been demonstrated for *P. aeruginosa* (20).

To find out which step in the denitrification pathway was affected by *rpoN*, we measured the in vitro activities of the reductases for nitrate, nitrite, and NO of MK516 in comparison to those of MK21, representing wild-type traits (Table 1). Denitrification of MK516 was induced by a shift from aerobic to O_2-limited growth conditions in the presence of nitrate. Luria-Bertani medium (500 ml in a 1-liter flask) was inoculated with an aerobic overnight culture to result in an optical density at 660 nm (OD_{660}) of about 0.2. The culture was incubated for 5 h on a gyratory shaker at 240 rpm, 30°C, until the OD_{660} was ≈0.4. Denitrification was induced by adding $NaNO_3$ (final con-

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CGAGGGCGACGCCCAAACAATCCTGAGCAACCAACTGGTCAAGGAAGTCTATCTGGGCCA
  E  G  D  A  Q  T  I  L  S  N  Q  L  V  K  E  V  Y  L  G  H
CGAATTCCGTCTGTAGAGCCGTTTTTTTTGCGCACGTCGGCAAATCGGCCCtaggcaaAG
  E  F  R  L end ORF241                                  -35
GCTTCCTCGGcaggcatataatttgctttctgTTGGCGCCTGGCGCGCCACATGTCGGAA
                -10                  +1
                                    RBS
CGGGCGCGGTTGCGCCGGCATAAAAGGTTTGAAGTCCTCTGCCATGAAACCATCGCTAGC
                                          RpoN→ M  K  P  S  L  A
CCTGAAGATGGGCCAGCAGCTGACGATGACACCGCAGCTGCAACAGGCCATACGGCTCCT
  L  K  M  G  Q  Q  L  T  M  T  P  Q  L  Q  Q  A  I  R  L  L
```

FIG. 2. Nucleotide sequence of the *rpoN* promoter. The transcriptional start of *rpoN* was determined by primer extension analysis; the oligonucleotide used for primer extension is underlined. The 5′ ends of transcripts are marked by arrows and +1. Putative −35 and −10 motifs are boxed. RBS, ribosome binding site. The promoter regions identical to that of *P. aeruginosa* are printed in lowercase letters. The C-terminal and N-terminal amino acid sequences of ORF241 and RpoN, respectively, are given below the nucleotide sequence in one-letter code.

TABLE 1. In vitro activities of denitrification enzymes

Strain and genotype	Sp act (nmol · mg of protein^{-1} · min^{-1}), ± SD (n = 4)		
	NO_3^- reduction[a]	NO_2^- reduction[b]	NO reduction[b]
MK21 (wild type)	26 ± 4	118 ± 6	63 ± 9
MK516 (Δ*rpoN*)	24 ± 1	37 ± 6	23 ± 7
MK516c (*rpoN*$^+$)	28 ± 5	68 ± 11	76 ± 7

[a] Assayed with dithionite-reduced methyl viologen (13).
[b] Measured by gas chromatography (7).

centration, 1 mg ml^{-1}) and reducing the shaking speed to 120 rpm. Cells were harvested after 16 h by centrifugation and broken in a French press, and cell extract was obtained as the supernatant from centrifugation (10 min, 20,000 × *g*). MK516 showed reduced activities of cytochrome cd_1 nitrite reductase and NO reductase, whereas the activity of nitrate reductase corresponded to that of the wild type (Table 1). Also, N_2O reductase was not affected in MK516, measured as in vivo activity of whole cells by gas chromatography (data not shown). Next, we complemented the mutation in MK516 with the *rpoN* gene to verify that the lower activity found with nitrite and NO reductases was a direct effect of *rpoN* deletion and not a polar effect of genes located downstream of *rpoN*. The 1.8-kb *Eco*RI-*Hin*dIII fragment, containing the complete copy of *rpoN*, was cloned into vector pUCP24, a broad-host-range vector, that is able to replicate in the genus *Pseudomonas* (23). The construct was transferred to MK516 by electroporation. The complemented cell line MK516c exhibited increased enzyme activities, coming close to the wild-type level and sometimes even surpassing it, indicating that the diminished reductase activities of MK516 were a direct result of the *rpoN* mutation (Table 1).

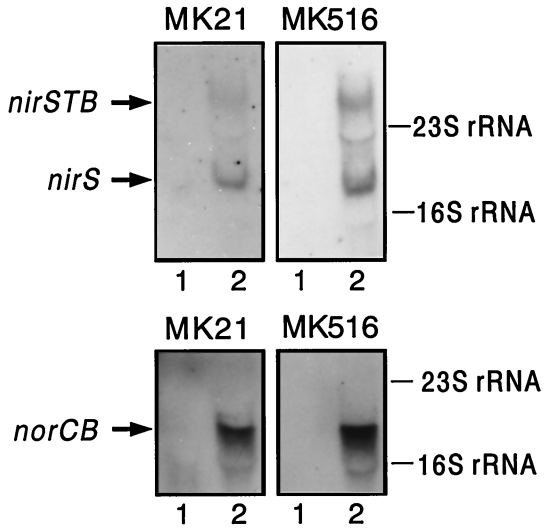

FIG. 3. The *rpoN* null mutant is not affected in the transcription of *nirS* and *norCB*. Total RNA from MK516 and MK21 was prepared according to the method of Aiba et al. (1) from cells grown aerobically (lane 1 of each panel) or under denitrifying conditions (lane 2 of each panel). Induction of denitrification was as described in the text for the activity measurements. Samples (20 μg of RNA) were denatured by glyoxal-dimethyl sulfoxide treatment and separated on a 1.2% agarose gel (14). After transfer to a nylon membrane, the *nirS* and the *norCB* transcripts were detected by hybridization with digoxigenin-labeled probes (labeling kit from Boehringer Mannheim, following the instructions of the manufacturer). A 500-bp *Kpn*I fragment of the *nirS* gene and a 2-kb *Pst*I-*Bgl*II fragment of the *norCB* operon were used as probes. *nirS* exhibited mono- and polycistronic transcripts of 2 and 3.4 kb, respectively; the *norCB* transcript was 2 kb. Equal gel loading was verified by staining with acridine orange. The 16S and 23S rRNA species served as standards.

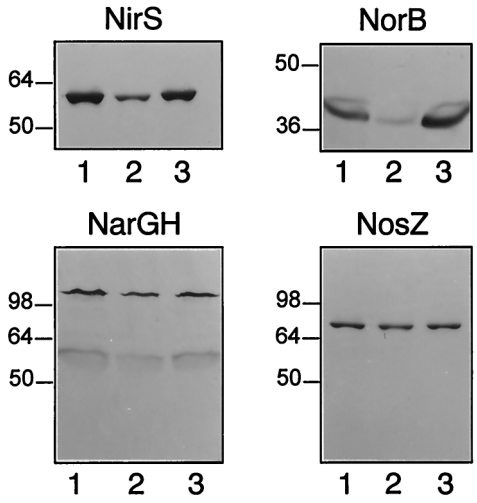

FIG. 4. An *rpoN* mutation reduces the cellular level of nitrite reductase (NirS) and NO reductase (assayed as the NorB subunit). Cell extracts of strains MK21 (lanes 1), MK516 (lanes 2), and the complemented mutant MK516c (lanes 3), all induced for denitrification, were separated electrophoretically on a sodium dodecyl sulfate–12.5% polyacrylamide gel and blotted onto nitrocellulose, and the reductases were detected immunochemically. NarGH, nitrate reductase; NosZ, N_2O reductase. Size markers (in kilodaltons) are indicated to the left of each panel.

Whether the effect on nitrite and NO reductases was caused by a decreased expression of the respective structural genes was investigated by comparing the amount of transcripts of *nirS* (nitrite reductase) and of *norCB* (NO reductase complex) of MK516 with that of the wild type (Fig. 3). Both structural gene sets are organized in independent transcriptional units (24). *nirS* is transcribed both from the *nirSTB* operon and as a monocistronic message and thus yields two signals on Northern blot analysis. Aerobically cultivated cells exhibited no transcripts. In cells grown under denitrifying conditions the *nirS* and *norCB* transcripts were readily detectable. The levels found in denitrification-induced MK516 were not decreased versus that in MK21. This rules out a direct dependence of *nirS* and *norCB* transcription on σ^{54}. Finally, we determined the level of denitrification enzymes, since the lower activities of nitrite and NO reductases could be due to a posttranscriptional mechanism that depends on σ^{54} and affects the concentration of enzymes. Cells were shifted to denitrification, and a cell extract was prepared as described above. The protein pattern was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the reductases were detected with the respective polyclonal antisera by immunoblotting. The *rpoN* mutant showed substantially lower levels of both nitrite reductase and NO reductase, whereas those of nitrate reductase and N_2O reductase corresponded to that of MK21 (Fig. 4). On complementation of MK516 by *rpoN*, wild-type levels of all four reductases were detected.

In conclusion, σ^{54} is not involved in the expression of the denitrification system of *P. stutzeri* by acting as a transcription factor for one or several reductase genes, yet it affected the cellular concentrations of nitrite reductase and NO reductase. Since the transcription of both *nirS* and *norCB* proceeded unabated in the *rpoN* strain, the enzyme levels seem to be affected by a posttranslational mechanism involving one or several products of σ^{54}-dependent gene expression, hence leading to diminished enzyme concentrations and concomi-

tantly decreased denitrification rates. The effect is restricted to the two key enzymes of denitrification, nitrite reductase and NO reductase, and does not affect nitrate and nitrous oxide respiration. Our findings underline once more that the respiratory systems utilizing nitrite and NO are interlaced at several levels of regulation and further support the modular view of the denitrification process that we have detailed elsewhere (24).

Nucleotide sequence accession number. The *rpoN* sequence reported here is available under the accession number AJ223088 in the EMBL/GenBank/DDBJ data banks.

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