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Three nitrate reductase activities in *Alcaligenes eutrophus*

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Abstract. Three nitrate reductase activities were detected in *Alcaligenes eutrophus* strain H16 by physiological and mutant analysis. The first (NAS) was subject to repression by ammonia and not affected by oxygen indicating a nitrate assimilatory function. The second (NAR) membrane-bound activity was only formed in the absence of oxygen and was insensitive to ammonia repression indicating a nitrate respiratory function. The third (NAP) activity of potential respiratory function occurred in the soluble fraction of cells grown to the stationary phase of growth. In contrast to NAR and NAS, expression of NAP did not require nitrate for induction and was independent of the *rpoN* gene product. Genes for the three reductases map at different loci. NAR and NAS are chromosomally encoded whereas NAP is a megaplasmid-borne activity in *A. eutrophus*.

Key words: Nitrate reductase – Denitrification – *Alcaligenes eutrophus* – Nitrate reductase mutants – Plasmid-encoded nitrate reductase

Alcaligenes eutrophus strain H16 is a facultatively chemolithoautotrophic, obligately respiratory organism which can grow anaerobically with nitrate or nitrite as terminal electron acceptor. The end product of this denitrification pathway of *A. eutrophus* H16 is molecular nitrogen (Pfitzner and Schlegel 1973). Nitrate and nitrite can also serve as nitrogen sources in the presence and absence of oxygen and the end product of this assimilatory pathway is ammonia (Bowien and Schlegel 1981). In a previous paper we described mutants of *A. eutrophus* H16 which had lost simultaneously diverse physiological functions including the ability to denitrify and to use nitrate as the sole source of nitrogen (Römermann et al. 1988). The mutation which conferred this highly pleiotropic phenotype lies in a chromosomal *rpoN*-like gene whose nucleotide-derived amino acid sequence revealed homology to σ^{54} of RNA polymerase (Römermann et al. 1989; Warreimann et al. 1992). This is evidence that the expression of at least some of the genes coding for nitrate-reducing activity is dependent on the minor sigma factor 54.

Curing of the indigenous 450 kilobase pair (kb) plasmid pHG1 of *A. eutrophus* H16 led to an irreversible loss of the ability to denitrify while nitrate assimilatory activity was unaffected (Römermann and Friedrich 1985). Furthermore, it was possible to convert non-denitrifying strains of *A. eutrophus* to denitrifyers by conjugative transfer of plasmid pHG1 (Schneider et al. 1988). These observations assigned an essential denitrification function to the megaplasmid of *A. eutrophus*.

The biochemical pathway of denitrification involves a series of reactions apparently catalyzed by four specific reductases. Starting from nitrate as the electron acceptor, the initial step of the sequence is mediated by nitrate reductase (Zumft et al. 1987). To elucidate nitrate respiration of *A. eutrophus* on the molecular level genetic studies were initiated by the isolation and characterization of mutants impaired in growth on nitrate. In this paper we describe several classes of nitrate negative mutants and give evidence for the presence of three physiologically distinct nitrate reductase activities in *A. eutrophus* strain H16.

Materials and methods

Bacterial strains

Alcaligenes eutrophus H16 and mutants derived therefrom were used in this study (Table 1). Mutants HF312, HF281 and HF231 were obtained by transposon Tn*5* mutagenesis as described (Srivastava et al. 1982). HF313 and HF317 were isolated by screening for chlorate resistance according to Pagan et al. (1977) with the modification that mutants were enriched in chlorate-containing liquid medium instead of directly selecting for chlorate-resistant colonies on agar plates.

Growth conditions

Cells were grown at 30 °C in mineral salts medium (Schlegel et al. 1961) with 0.4% (wt/vol) gluconate as the carbon source and 0.2% (wt/vol) potassium nitrate or ammonium chloride, respectively, as the nitrogen source. Aerated cultures were incubated on a rotary

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Table 1. Strains of *Alcaligenes eutrophus*

Strain[a]	Relevant characteristics[b]	Reference or source
H16	$Nas^+Nar^+Nap^+$	Wild-type (ATCC17699)
HF210	Cured of pHG1	Kortlüke et al. 1992
HF149	$RpoN^-(Hno^-)$	Hogrefe et al. 1984
HF312	$Nas^-Nar^+Nap^+$	This study
HF313	$Nas^+Nar^-Nap^+$	This study
HF317	Mol^-	This study
HF281	$NarC^-$	This study
HF231	$NarF^-$	This study

[a] Strains carrying the initials HF are derivatives of the wild-type H16

[b] Phenotype designations for: NAS, assimilatory nitrate reductase; NAR, respiratory membrane-bound nitrate reductase; NAP, nitrate reductase formed in the stationary phase of growth. Hno^- is the former designation of $RpoN^-$ mutants (Römermann et al. 1988); Mol^-, molybdenum cofactor deficiency; $NarC^-$ and $NarF^-$ are regulatory phenotype designations explained in the text

Table 2. Nitrate reductase activities in cell extracts of *A. eutrophus*

Growth conditions[a]			Nitrate reductase (U/mg of protein)[b]		Enzyme
NO_3^-	NH_4^+	O_2	Soluble extract[c]	Membrane extract	
−	−	+	0.8	0	NAS
−	+	+	0	0	nr
+	+	+	0	0	nr
+	+	−	0.3	2.1	NAR
−	(+)	(+)	2.8	0.3	NAP

[a] Growth conditions are defined in 'Materials and methods'. +, present; −, absent (under exponential growth conditions); (+) present (in stationary phase)

[b] Nitrate reductase activity was determined by measuring the nitrite formed from nitrate by the standard colorimetric method (Lowe and Evans 1964) as described in 'Materials and methods'. Specific activity is defined as 1 µmol nitrite formed per min and mg of protein

[c] Cell-free extracts were prepared and fractionated as described in 'Materials and methods'; nr, not relevant

shaker at 115 rpm. Incubation under anaerobic conditions was performed in 10 ml screw-cap tubes filled to the top with mineral medium containing 0.2% (wt/vol) potassium nitrate as terminal electron acceptor and 0.2% ammonium chloride, unless otherwise stated. Growth was monitored with a Klett-Summerson colorimeter at 520–540 nm.

Preparation of extracts

Extracts were prepared from 100 ml of cells that had been washed twice with 250 mM phosphate buffer, pH 6.5. The cells were concentrated 30-fold and disrupted by sonication. The cell-free suspension was fractionated by centrifugation at 90000 × g for 45 min yielding the soluble and the membrane-containing extracts.

Enzyme assays

Nitrate reductase activity was determined by measuring the nitrite formed from nitrate by the standard colorimetric method (Lowe and Evans 1964). The reaction mixture contained 1 ml 50 mM 2-(*n*-morpholino)-ethane sulfonic acid, 1 ml 30 mM potassium nitrate, 1 ml 6 mM benzylviologen equilibrated to an atmosphere of N_2; pH and temperature were adjusted to 7.5 and 30 °C (for NAS) and pH 5.5 and 50 °C (for NAR and NAP), respectively. Prior to starting the reaction with 5 to 100 µl extract or intact cells, the benzylviologen was reduced by the addition of 50 µl of 23 mM sodium dithionite dissolved in 48 mM bicarbonate.

Results

Nitrate reductase activities under various conditions of growth

Cells grown exponentially under heterotrophic conditions with aeration and nitrate as the only nitrogen source contained moderate levels of nitrate reductase activity in the soluble fraction of the extract (Table 2, line 1). No nitrate reductase activity was found when nitrate was omitted from the medium or ammonium chloride was supplied as additional source of nitrogen (Table 2, lines 2 and 3). These results indicate that the nitrate assimilatory enzyme (NAS) is only formed in the presence of nitrate and in the absence of a preferentially used nitrogen source such as ammonium.

When the cells were grown without aeration in the presence of both nitrate and ammonium chloride, high nitrate reductase activity was measured in the membrane fraction of the extract and approximately only ten percent of this activity was detected in the soluble extract (Table 2, line 4). Both anaerobiosis and the presence of nitrate were required for induction of the nitrate respiratory pathway (NAR) and the presence of ammonium assured that the assimilatory nitrate reductase was repressed.

When cells were grown with aeration in the presence of ammonium and without nitrate, the ammonium should repress the assimilatory pathway and both the lack of anaerobiosis and the lack of nitrate should prevent expression of the nitrate reductase activity. Nevertheless a substantial amount of nitrate reductase activity (NAP) was found under these conditions (Table 2, line 5). However, this activity was seen only during stationary phase (Fig. 1) and was localized in the soluble fraction of the extract (Table 2). Indeed, this NAP activity could

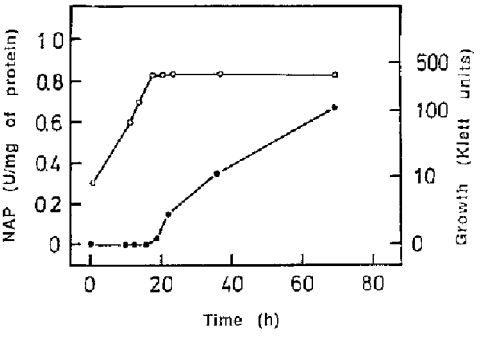

Fig. 1. Activity of the nitrate reductases of *A. eutrophus* H16 during heterotrophic growth in gluconate-containing mineral medium with 0.2% (wt/vol) ammonium chloride as the nitrogen source. —○—, growth; —●—, nitrate reductase activity. The assay conditions are the same as described in the footnote of Table 4

Table 3. Biochemical properties of nitrate reductase activities of *A. eutrophus*[a]

Enzyme	Cell fraction	Optima		Apparent molecular weight
		pH	Temperature	
NAS	Soluble	7–8	30 °C	nd
NAR	Membrane	5.5	50 °C	300000
NAP	Soluble	5.5	50 °C	100000

[a] The experimental conditions were the same as described in the legend to Table 2; nd, not determined due to instability

account for the increase of nitrate reductase activity observed in the stationary phase of cells grown under conditions of nitrate assimilation (data not shown).

Biochemical properties of the three nitrate reductases

The three nitrate reductase activities (NAS, NAR and NAP) differed from each other in several biochemical parameters as summarized in Table 3. Both NAS and NAP were found in the soluble fraction but NAR was in the membrane-containing extract. NAR and NAP revealed a temperature optimum of 50 °C and a pH optimum of 5.5 but NAS had a temperature optimum of 30 °C and a pH optimum of 7 to 8. The electrophoretic mobilities of NAR and NAP, detected with an activity stain (Lund and DeMoss 1976) in non-denaturing PAGE were distinct. NAR showed an apparent molecular weight of 300000 and NAP of 100000 (data not shown). NAS was unstable and thus not characterized with respect to molecular weight.

Mutants defective in nitrate reduction

Further evidence for three distinct nitrate reductases in *Alcaligenes eutrophus* comes from mutant analysis. Seven classes of mutants defective in nitrate metabolism were characterized. Table 4 summarizes the phenotypes of the respective strains. Mutants with the phenotype Nas^- (HF312), were unable to grow on nitrate as the sole nitrogen source, but retained normal NAR and NAP activity. Nar^- mutants (HF313) were defective in denitrification, but were unaffected with respect to NAS and NAP activities. The plasmid-cured strain (HF210) was devoid of NAP activity but showed wild-type specific level of NAS and low but detectable NAR activity. Thus NAP must be a megaplasmid encoded activity. Indeed, transconjugants which have recovered plasmid pHG1 were restored in NAP activity (data not shown).

Mol^- (HF317) and $NarC^-$ (HF281) represent two mutant classes in which the expression of all three nitrate reductases was affected simultaneously. Mol^- mutants are probably impaired in molybdenum metabolism since they could not grow on substrates like formate (Table 4) or xanthine (data not shown), which are degraded by molybdenum-containing enzymes (Johnson et al. 1980). This indicates that all three nitrate reductases in *A. eutrophus* are molybdo-enzymes. In contrast to the Mol^- mutants the $NarC^-$ mutants were exclusively impaired in the expression of the three nitrate reductases. The nature of this mutation is not known yet. The $NarF^-$ (HF231) mutants appeared to be defective in several steps of the denitrification pathway since they lacked the membrane-bound nitrate reductase and, in contrast to the Nar^- mutant HF313, could not grow anaerobically on nitrite (data not shown). NAS and NAP activities were not affected in the $NarF^-$ group suggesting that the $NarF^-$ phenotype may result from mutation in a regulatory gene that controls denitrification in *A. eutrophus*.

Denitrification is dependent on a chromosomally encoded RpoN-like sigma factor (σ^{54}) of RNA polymerase in *A. eutrophus* (Römermann et al. 1988). In this study the expression of NAR, NAS and NAP was

Table 4. Nitrate reductase deficient mutants of *A. eutrophus*

Strain[a]	Growth on[b]			Enzyme activity (U/mg of protein)[c]			Phenotype
	NO_3^- ($+O_2$)	NO_3^- ($-O_2$)	Formate	NAS	NAR	NAP	
H16	+	+	+	0.1	0.2	0.4	Wild-type
HF312	−	+	+	0	0.2	0.4	Nas^-
HF313	+	−	+	0.1	0	0.3	Nar^-
HF210	−	(+)	+	0.14	0.03	0	Nap^-
HF317	−	−	−	0	0	0	Mol^-
HF281	−	−	+	0	0	0	$NarC^-$
HF231	+	−	+	0.1	0	0.5	$NarF^-$
HF149	−	−	nd	0	0.03	0.7	$RpoN^-$

[a] Reference and sources of strains are given in Table 1 and in 'Materials and methods'

[b] Formate (0.4%, wt/vol) was used as the carbon source instead of gluconate; +, growth; −, no growth; (+), poor growth

[c] Activity of nitrate reductases was determined with cells grown on gluconate-ammonium containing mineral medium. The cells were washed, shifted to nitrate assimilatory conditions (for NAS), nitrate respiratory conditions (for NAR) and to the stationay phase (for NAP) by incubation for 20 h. The assay conditions are described in 'Materials and methods', except intact cells adjusted to a final optical density of 20 (436 nm), were used instead of extracts. Therefore the activities are lower than those listed in Table 2; nd, not determined

examined in RpoN$^-$ mutants (HF149). The activity of NAP was not affected by the *rpoN* mutation whereas activities of the assimilatory and membrane-bound nitrate reductases were almost undetectable in the RpoN$^-$ mutants, suggesting that the expression of *nar* and *nas* requires RpoN whereas NAP synthesis appears to be RpoN independent.

Discussion

It was tentatively concluded that nitrate assimilation and respiration are mediated by the same enzyme in *Alcaligenes eutrophus* (Pfitzner and Schlegel 1973). The results presented in this study, however, show that the two reactions are catalyzed by two distinct proteins, a nitrate respiratory enzyme (NAR) and a nitrate assimilatory enzyme (NAS). Our conclusion is based on the observations that the two activities differ with respect to cellular localization, biochemical and regulatory parameters and genetic origin.

The physiological roles of the two nitrate reductases in *A. eutrophus*, NAS and NAR, can be easily deduced by the pattern of their regulation. NAS is clearly part of the assimilatory pathway responsible for ammonium production from nitrate. As described for other nitrate assimilatory enzymes (Stewart 1988), NAS is a soluble protein whose expression is inhibited by ammonium, but insensitive to aerobiosis. NAR is clearly part of the denitrification pathway responsible for anaerobic respiration with nitrate as terminal electron acceptor. As reported for respiratory nitrate reductases from other sources (Stewart 1988), NAR is almost exclusively located in the particulate fraction and contrary to NAS, expression of NAR is insensitive to ammonium but prevented by aerobiosis.

The role of the third nitrate reductase, NAP, unexpectedly identified in the course of this study, is much less clear. NAP is formed during stationary phase of aerobically grown cells and, in marked contrast to both NAS and NAR, even in the absence of nitrate. The fact that mutants defective in NAR but unimpaired in NAP activity are unable to grow on nitrate anerobically indicates that NAP can not substitute for the physiological function of NAR. Perhaps NAP is formed to prepare the cells for the transition from aerobic respiration with oxygen as terminal electron acceptor to anaerobic respiration with nitrate as terminal electron acceptor. It is also possible that NAP is a reductase for some other compound that accumulates in the medium of heterotrophically grown *A. eutrophus* and that this reductase may have a broad substrate specificity that includes nitrate. This would then mimic the hierarchical induction pattern for terminal electron acceptors seen in other systems when oxygen represses nitrate reduction and nitrate represses other reductases lower in the hierarchy (Stewart 1988). The presence of periplasmic and membrane-bound respiratory nitrate reductases was previously demonstrated for *Thiosphaera pantotropha* (Bell et al. 1990). This organism was reported to reduce nitrate in the presence of oxygen as the initial step of an aerobic denitrification pathway (Robertson and Kuenen 1984). It is unlikely that *A. eutrophus* has the capacity to denitrify under aerobiosis since respiratory reduction of nitrite is strictly inhibited under aerobic conditions (Sann 1990). Thus the precise physiological function of NAP in *A. eutrophus* remains to be explored.

Although the megaplasmid-cured Nap$^-$ strain HF210 was potentially available for a physiological analysis of NAP the strain turned out to be not appropriate for such a study since it accumulates nitrite to a toxic level due to the lack of additional plasmid-encoded functions which are required for the conversion of nitrite to dinitrogen (Römermann and Friedrich 1985). This deficiency may indeed account for the low level of chromosomally encoded NAR activity observed in the megaplasmid-free strain HF210.

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