Journal of General Microbiology (1980), **118**, 229–234. *Printed in Great Britain*

The Assimilatory and Dissimilatory Nitrate Reductases of *Pseudomonas aeruginosa* are Encoded by Different Genes

By S. R. SIAS,[1] A. H. STOUTHAMER[2] AND J. L. INGRAHAM[1]*

[1] *Department of Bacteriology, University of California, Davis, California* 95616, *U.S.A.*
[2] *Microbiology Department, Biological Laboratory, Free University, De Boelelaan* 1087, *Amsterdam, The Netherlands*

(*Received* 21 *September* 1979; *revised* 30 *November* 1979)

The phenotypes of certain mutant strains of *Pseudomonas aeruginosa* were reported to be pleiotropic for nitrate reduction; these strains were selected for their inability to dissimilate nitrate and were found also to have lost the ability to assimilate nitrate. We now report that the isolation procedure selected two mutations, one in genes encoding the synthesis of dissimilatory nitrate reductase (*narA*, *narB* or *narE*) and another in one of the genes (*nas*) encoding the synthesis of assimilatory nitrate reductase. Thus in *P. aeruginosa* dissimilatory and assimilatory nitrate reductases are genetically distinct. However, a loss of both enzymes is necessary to prevent slow dissimilatory growth on nitrate. Assimilatory nitrate reductase requires molybdenum to function, as does dissimilatory nitrate reductase. Lesions in *narD* affect incorporation of molybdenum into both enzymes, and hence exert a pleiotropic effect.

INTRODUCTION

Pseudomonas aeruginosa is capable of assimilatory and dissimilatory reduction of nitrate. By the assimilatory pathway, nitrate is reduced to nitrite, which is then further reduced by assimilatory nitrite reductase to ammonia, which serves as a source of nitrogen for biosynthesis. Nitrate is also reduced to nitrite by the dissimilatory pathway. However, in this case, nitrite is reduced by several enzymic steps to nitrogen gas; ammonia is not an intermediate of the dissimilatory pathway. The overall dissimilatory reduction of nitrate to nitrogen gas is coupled to ATP generation, thus allowing this non-fermentative organism to grow in the absence of oxygen.

Mutant strains of *P. aeruginosa* have been isolated by selecting for chlorate-resistance and for inability to utilize nitrate as a terminal electron acceptor for anaerobic growth. The mutations were mapped in a series of genes designated *narA*, *narB*, *narC*, *narD* and *narE* (van Hartingsveldt *et al.*, 1971). The *nar* mutants, with the exception of *narC* mutants, simultaneously lost the ability to assimilate nitrate (van Hartingsveldt *et al.*, 1971). The genes *narA*, *narB*, *narC* and *narD* were located between 45 and 55 min on the *P. aeruginosa* strain PAO chromosome; *narE* was located at 9 min (van Hartingsveldt & Stouthamer, 1973).

Mutants of *P. aeruginosa* have also been isolated by selecting for their inability to assimilate nitrate while growing aerobically. These fell into three classes: those that lacked assimilatory nitrate reductase, designated *nasA*, *nasB* and *nasC*; those that lacked assimilatory nitrite reductase, designated *nis*; and those that lacked assimilatory nitrate reductase and assimilatory nitrite reductase, designated *nasD*. Strains with mutations in the *nas* genes share the defect in assimilatory nitrate reductase with the *nar* mutants but they are unaffected in the dissimilatory reduction of nitrate (Sias & Ingraham, 1979). The *nas* genes are located between 18 and 28 min on the *P. aeruginosa* chromosome, quite distant from any *nar* genes (Sias & Ingraham, 1980).

0022-1287/80/0000-8961 $02.00 © 1980 SGM

Table 1. *Pseudomonas aeruginosa strains used*

Our strain designation	Genotype	Source	Original designation	Parent
JM41	wild-type	T. C. Hollocher	PAO1	
JM54	*nasB5*	Sias & Ingraham (1979)		JM41
JM62	*nasC6*	Sias & Ingraham (1979)		JM41
JM74	*nasD12*	Sias & Ingraham (1979)		JM41
JM100	*nasA17*	Sias & Ingraham (1979)		JM41
JM114	*narA nasB met-9 leu-1 str*^{r} *ilvB112*	A. Stouthamer	S1228	
JM116	*narC met-9 leu-1 str*^{r} *ilvB112*	A. Stouthamer	S1232	
JM117	*narD met-9 leu-1 str*^{r} *ilvB112*	A. Stouthamer	S1234	
JM126	*narC str*^{r}	This paper		JM116
JM129	*narA nasB str*^{r}	This paper		JM114
JM130	*narB nasB str*^{r}	A. Stouthamer	S1128	
JM132	*narE nasA str*^{r}	A. Stouthamer	S1401	
JM136	*narD str*^{r}	This paper		JM117

The pleiotropic phenotype of *nar* mutants implies that dissimilatory and assimilatory nitrate reduction are determined by common genes. If this is the case, one would expect that mutants selected for loss of nitrate assimilation (*nas*) should simultaneously lose the ability to dissimilate it, but this is not so. The inconsistency between the phenotype of mutations in *nas* and *nar* genes could be resolved by assuming that chlorate-resistance and inability to grow anaerobically require loss of assimilatory nitrate reductase as well as dissimilatory nitrate reduction, i.e. that *nar* strains were actually double mutants. Indeed, we have found that certain *nar* strains also carry mutations in *nas* genes. It now seems clear that assimilation and dissimilation of nitrate by *P. aeruginosa* are genetically and physiologically distinct except for their common requirement for molybdenum (Fewson & Nicholas, 1961), the incorporation of which is facilitated by a factor encoded by *narD*.

METHODS

Strains. Strains used are all derivatives of *Pseudomonas aeruginosa* PAO1 (Holloway, 1969) and are listed in Table 1.

Media and culture conditions. The media employed in these studies were described previously (Sias & Ingraham, 1979). In experiments on the effect of tungstate on growth, the procedure described by Guerrero *et al.* (1973) was used. In other experiments, the conditions described previously were used (Sias & Ingraham, 1979).

Resistance to chlorate. Chlorate-resistance was determined by the method of van Hartingsveldt *et al.* (1971).

Transductions. Transductional crosses and growth of phage G101 were done as previously described (Sias & Ingraham, 1979).

RESULTS

Most mutant strains of *P. aeruginosa* selected for their inability to dissimilate nitrate (DNR^-) were found to be unable to assimilate nitrate (ANR^-) (van Hartingsveldt *et al.*, 1971). Although the lesions conferring the DNR^- phenotype (*narA*, *narB*, *narD* and *narE*) have been mapped (van Hartingsveldt & Stouthamer, 1973), it was not possible to score the ANR phenotype of the recombinants because the strains used for mapping also carried several amino acid auxotrophic markers. Thus, the ANR phenotype was masked.

On the basis of the finding that mutants selected as ANR^- (*nasA*, *nasB*, *nasC* and *nasD*) remain DNR^+ (Sias & Ingraham, 1979), we suspected that the ANR^- phenotype of the strains selected as being DNR^- (*narA*, *narB*, *narD* and *narE*) might be a consequence of a second mutation in one of the *nas* genes. To test this possibility it was first necessary to render prototrophic certain strains carrying *nar* mutations, because the mutations were

Table 2. *Transductional crosses between wild-type P. aeruginosa* PAO1 *and strains with mutations in various nar genes*

In all cases the donor strain was PAO1 wild-type. Crosses were made by phage G101-mediated transductions. Ability to assimilate nitrate (ANR^+) was determined by aerobic growth on minimal medium plates containing nitrate as the sole source of nitrogen. To select ANR^+ transductants, plates were incubated for 3 d at 37 °C. Ability to dissimilate nitrate (DNR^+) was scored by anaerobic growth on complex medium plates containing nitrate (TYN-5) (Sias & Ingraham, 1979). To select DNR^+ transductants, plates were incubated for 4 to 5 d at 37 °C. To score the DNR phenotype plates were incubated for 3 d at 37 °C. To score the ANR phenotype plates were incubated for 1 d at 37 °C.

	Cross 1 ANR^+ selected, DNR scored		Cross 2 DNR^+ selected, ANR scored	
Recipient	No. of ANR^+ transductants tested	No. of ANR^+DNR^+ transductants	No. of DNR^+ transductants tested	No. of ANR^+ DNR^+ transductants
JM129 (*narA*)	50	0	50	0
JM130 (*narB*)	50	0	50	0
JM136 (*narD*)	50	50	50	50
JM132 (*narE*)	50	0	50	0

Table 3. *Transductional crosses between strains carrying mutations in nar genes and strains carrying mutations in nas genes*

Crosses were mediated by phage G101. Transductants were selected for NAS^+ on minimal nitrate plates incubated aerobically at 37 °C for 3 to 4 d.

	No. of NAS^+ transductants			
	Donor			
Recipient	JM100 (*nasA*)	JM54 (*nasB*)	JM62 (*nasC*)	JM74 (*nasD*)
JM129 (*narA*)	> 250	21	> 300	> 250
JM130 (*narB*)	> 200	9	> 300	> 200
JM136 (*narD*)	> 250	> 250	> 300	> 200
JM132 (*narE*)	6	> 250	> 300	> 200

only available in an auxotrophic background. This was done stepwise, by selecting for spontaneous reversion to amino acid prototrophy. In this manner prototrophic strains (JM129, JM126 and JM136) which carry the lesions *narA*, *narC* and *narD*, respectively, were generated. Strains carrying *narB* and *narE* (JM130 and JM132, respectively) were available from the original collection. The phenotypes of all these prototrophic strains agreed with their original description, namely, strains carrying mutations in *narA*, *narB*, *narD* and *narE* were DNR^- and ANR^-; the strain carrying a mutation in *narC* was only DNR^-.

Those prototrophic strains exhibiting the double defective phenotype (ANR^-DNR^-) were used as recipients in transductional crosses with the wild-type (JM41) to determine whether or not the defects ANR^- and DNR^- could be separated genetically (Table 2). In each cross, following phage adsorption, half the transduction mixture was plated on minimal nitrate medium and incubated aerobically at 37 °C to select ANR^+ transductants. The remaining half was plated on a complex medium with nitrate, TYN-5 (Sias & Ingraham, 1979), and incubated anaerobically at 37 °C to select DNR^+ transductants. When colonies appeared (after 2 to 5 d) 50 transductants were picked from each cross and each selection plate and cloned twice. The purified clones were scored for their unselected phenotype. In

Table 4. *Effect of tungstate on nitrate assimilation of strain* JM41

Cultures were grown aerobically at 37 °C in minimal media with the additions listed. The inoculum was grown aerobically in minimal nitrate medium without molybdenum.

Additions to nitrogen-free minimal medium	Doubling time (min)
1. 0·1% $NaNO_3$	64
2. 0·1% $NaNO_3$ + 1 μM-Na_2WO_4	85
3. 0·1% $NaNO_3$ + 10 μM-Na_2WO_4	80
4. 0·1% $NaNO_3$ + 100 μM-Na_2WO_4	100
5. 0·1% $NaNO_3$ + 1 mM-Na_2WO_4	180
6. 0·1% $NaNO_3$ + 1 mM-Na_2WO_4 + 2 mM-MoO_3	90
7. 0·1% $NaNO_3$ + 1 mM-Na_2WO_4 + 0·025% $NaNO_2$	90
8. 0·1% $NaNO_3$ + 1 mM-Na_2WO_4 + 0·1% NH_4Cl	51

the case of JM136 (*narD*) the ANR$^-$ and DNR$^-$ phenotypes were always jointly cured, suggesting that defects in a single gene (*narD*) cause both ANR$^-$ and DNR$^-$. However, in the cases of JM129 (*narA*), JM130 (*narB*) and JM132 (*narE*) the unselected phenotype of the parent remained unchanged, i.e. strains transduced to DNR$^+$ remained ANR$^-$ and strains transduced to ANR$^+$ remained DNR$^-$. Thus, these strains must carry two lesions, one in a gene encoding ANR and the other in a gene encoding DNR. In order to determine if the ANR$^-$ phenotype was a consequence of a mutation in one of the *nas* genes, phage grown on *nasA*, *nasB*, *nasC* or *nasD* strains was used to transduce *narA*, *narB*, *narD* and *narE* strains to ANR$^+$ (Table 3). Taken together, Tables 2 and 3 show that the phenotypes of JM129, JM130 and JM132 are due to two mutations; a lesion in *narA*, *narB* and *narE*, respectively, causing the DNR$^-$ phenotype and a lesion in *nasB*, *nasB* and *nasA*, respectively, causing the ANR$^-$ phenotype.

All transductants selected as ANR$^+$ were examined for their ability to grow anaerobically. With the exception of those from JM136 (*narD*) (Table 2), all transductants were incapable of significant growth anaerobically. However, a variety of slow growth responses was observed. After 3 d anaerobic incubation on plates of complex nitrate medium some NAS$^+$ transductants showed significantly more growth than their NAS$^-$ parents. Others reverted to wild-type anaerobic growth at a significantly greater rate than their parents.

The ANR$^-$DNR$^-$ phenotype of JM136 (*narD*) is not attributable to the presence of two mutations. The *narD* mutation causes a requirement for high levels of molybdenum to become ANR$^+$DNR$^+$ (van Hartingsveldt & Stouthamer, 1973). A common requirement of the two nitrate reductases for molybdenum could explain the pleiotropic phenotype. Fewson & Nicholas (1961) demonstrated that DNR of *P. aeruginosa* does, indeed, require molybdenum. The technique of Guerrero *et al.* (1973) was used to test for a molybdenum requirement of ANR. Tungsten is a specific competitive inhibitor of molybdenum (for review, see Stouthamer, 1976). To examine the effect of tungstate on nitrate assimilation, molybdenum was omitted from minimal nitrate media and tungstate was added in varying amounts (Table 4). A culture of the wild-type (JM41) was grown overnight in minimal nitrate medium lacking molybdenum to dilute cellular molybdenum. This culture was used to inoculate a series of subcultures containing increasing amounts of sodium tungstate and nitrate as the sole source of nitrogen. Incubation of these cultures aerobically at 37 °C (Table 4) showed that the growth rate was inversely proportional to the concentration of tungstate (lines 1 to 5, Table 4). Added molybdenum, nitrite or ammonium reversed the toxicity of tungstate (lines 6, 7 and 8, Table 4). Thus tungstate appears to be a competitive antagonist of molybdenum, which is required for the proper function of ANR. Therefore, it seems likely that ANR as well as DNR is a molybdo-protein.

To compare the molybdenum requirement of JM136 (*narD*) with JM41 (wild-type),

Table 5. *Effect of added molybdenum on the aerobic growth of strains* JM136 (*narD*) *and* JM41 (*wild-type*) *with nitrate as sole source of nitrogen*

MoO_3 was added to minimal nitrate medium to give the concentrations listed. After 48 h shaking at 37 °C, growth was determined by measuring the absorbance at 650 nm.

MoO_3 concn (μM)	Growth (A_{650})	
	JM136	JM41
0	0·004	0·136
1	0·030	0·852
5	0·441	0·796
10	0·835	0·911
50	0·777	0·841
100	0·739	0·893
500	0·028	0·010
1000	0·001	0·000

these strains were grown anaerobically at 37 °C on minimal medium containing excess ammonium and nitrate with varying amounts of molybdenum trioxide (MoO_3). After 3 d the plates were examined and growth was scored. The wild-type required at least 5 μM-MoO_3 while JM136 required at least 100 μM-MoO_3 for anaerobic growth. Concentrations of MoO_3 of 500 μM or greater inhibited the growth of both strains. Contaminating molybdenum in the agar was sufficient for both JM136 and JM41 to assimilate nitrate aerobically without added MoO_3. In order to measure the assimilatory molybdenum requirement the strains were grown in liquid minimal nitrate media with varying amounts of MoO_3 (Table 5). Each inoculum used was grown in minimal nitrate with 5 μM-MoO_3. After 48 h incubation at 37 °C with shaking, the absorbance at 650 nm was measured. Aerobically JM136 required 10 μM-MoO_3 for ANR function. The 10-fold difference in the molybdenum requirement of DNR and ANR could be due in part to the different activities of enzyme required for wild-type anaerobic nitrate dissimilation and aerobic nitrate assimilation.

Certain *nar* mutants were isolated anaerobically by selection for resistance to chlorate (van Hartingsveldt *et al.*, 1971). Chlorate can be reduced by DNR to chlorite, which is toxic. Presumably chlorate-resistant mutants are blocked in dissimilatory reduction of nitrate (Pichinoty *et al.*, 1969; Piéchaud *et al.*, 1967; Stouthamer, 1967). This procedure selects for double mutants, as the previous experiments showed. However, when scored for sensitivity to chlorate, the DNR^+ transductants of JM129 (*narA*) remained resistant to chlorate although they had regained DNR activity. This implies that JM129 carries at least three distinct mutations. In contrast, the DNR^+ transductants of JM130 (*narB*) were all sensitive to chlorate. In the case of JM130, a single mutation causes loss of DNR function and resistance to chlorate.

DISCUSSION

Genetic analysis by transduction of the assimilatory and dissimilatory defects of JM129, JM130 and JM132 showed that the pleiotropic phenotypes of these strains are due to two distinct mutations: JM129 carries mutations in *narA* and in *nasB*; JM130 carries mutations in *narB* and in *nasB*; and JM132 carries mutations in *narE* and in *nasA*. The *narA*, *narB* and *narE* genes which are located at approximately 55, 45 and 9 min, respectively, on the chromosome of *P. aeruginosa* (van Hartingsveldt & Stouthamer, 1973) cause loss of the ability to synthesize active DNR, hence, loss of the ability to grow anaerobically. The lesions in *nasA* and *nasB* are located at 20 and 27 min, respectively, (Sias & Ingraham, 1980) and cause loss of the ability to synthesize active ANR and, hence, loss of the ability to assimilate nitrate aerobically. Thus assimilatory nitrate reduction is quite distinct from dissimilatory nitrate reduction.

Selection for DNR mutants resulted in the isolation of double mutants. A strain that lacks only DNR would be expected to be unable to grow anaerobically on complex media containing nitrate. However, under such conditions ANR, normally repressed on complex media, might function at a low level to reduce nitrate and slowly provide a supply of nitrite that would allow poor anaerobic growth. Thus DNR$^-$ strains might appear to be leaky mutants. An additional mutation causing the loss of ANR function would confer on these strains the appearance of being totally defective in DNR. Phenotypically, these would appear as choice mutants for isolation of strains which cannot dissimilate nitrate. However, their inability to assimilate nitrate, because of the loss of ANR, would incorrectly suggest that ANR and DNR are coded for by a common gene or set of genes. Clearly this is not so. We suggest that selection for mutants unable to grow anaerobically on nitrate should be done in a *nas*, rather than wild-type, background.

The existence of at least three lesions in JM129 suggests that selection for chlorate-resistance may not be the best selection for isolation of DNR$^-$ mutants. Using chlorate-resistance to isolate dissimilatory mutants in *Azospirillum*, Magalhaes *et al.* (1978) also found they had isolated pleiotropic mutants unable to dissimilate or assimilate nitrate.

The only component of nitrate reduction common to both the assimilatory and dissimilatory functions involves the gene *narD*. Both ANR and DNR are molybdo-proteins. A mutation in *narD* results in a molybdenum requirement for both ANR and DNR that is higher than that of the wild-type. The *narD* lesion also confers an excessive molybdenum requirement on xanthine dehydrogenase (van Hartingsveldt & Stouthamer, 1973). Perhaps *narD* codes for a molybdenum-incorporating protein.

This research was supported in part by a grant from the National Science Foundation (grant no. PRF 77-07301).

REFERENCES

FEWSON, C. A. & NICHOLAS, D. J. D. (1961). Nitrate reductase from *Pseudomonas aeruginosa*. *Biochimica et biophysica acta* **49**, 335–349.

GUERRERO, M. G., VEGA, J. M., LEADBETTER, E. & LOSADA, M. (1973). Preparation and characterization of a soluble nitrate reductase from *Azotobacter chroococcum*. *Archiv für Mikrobiologie* **91**, 287–304.

HOLLOWAY, B. W. (1969). Genetics of *Pseudomonas*. *Bacteriological Reviews* **33**, 419–443.

MAGALHAES, L. M. S., NEYRA, C. & DOBEREINER, J. (1978). Nitrate and nitrite reductase negative mutants of N_2-fixing *Azospirillum* spp. *Archives of Microbiology* **117**, 247–252.

PICHINOTY, F., PUIG, J., CHIPPAUX, M., BIGLIARDI-ROUVIER, J. & GENDRE, J. (1969). Recherches sur des mutants bactériens ayant perdu les activités catalytiques liées a la nitrate-réductase A. II. Comportement envers le chlorate et le chlorite. *Annales de l'Institut Pasteur* **116**, 409–432.

PIÉCHAUD, M., PUIG, J., PICHINOTY, F., AZOULAY, E. & LEMINOR, L. (1967). Mutations affectant la nitrate-réductase A et d'autres enzymes bactériennes d'oxydoréduction. Etude préliminaire. *Annales de l'Institut Pasteur* **112**, 24–37.

SIAS, S. R. & INGRAHAM, J. L. (1979). Isolation and analysis of mutants of *Pseudomonas aeruginosa* unable to assimilate nitrate. *Archives of Microbiology* (in the Press).

SIAS, S. R. & INGRAHAM, J. L. (1980). Chromosomal location in *Pseudomonas aeruginosa* of genes encoding assimilatory nitrate reductase. *Journal of Bacteriology* (in the Press).

STOUTHAMER, A. H. (1967). Nitrate reduction in *Aerobacter aerogenes*. I. Isolation and properties of mutant strains blocked in nitrate assimilation and resistant to chlorate. *Archiv für Mikrobiologie* **56**, 68–75.

STOUTHAMER, A. H. (1976). Biochemistry and genetics of nitrate reductase in bacteria. *Advances in Microbial Physiology* **14**, 315–375.

VAN HARTINGSVELDT, J., MARINUS, M. G. & STOUTHAMER, A. H. (1971). Mutants of *Pseudomonas aeruginosa* blocked in nitrate or nitrite dissimilation. *Genetics* **67**, 469–482.

VAN HARTINGSVELDT, J. & STOUTHAMER, A. H. (1973). Mapping and characterization of mutants of *Pseudomonas aeruginosa* affected in nitrate respiration in aerobic or anaerobic growth. *Journal of General Microbiology* **74**, 97–106.