Mapping and Characterization of Mutants of *Pseudomonas aeruginosa* Affected in Nitrate Respiration in Aerobic or Anaerobic Growth

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SUMMARY

Genes which are involved in anaerobic growth with nitrate or nitrite as terminal electron acceptor in *Pseudomonas aeruginosa* were mapped by interrupted mating and linkage analysis after conjugation. Times of entry were mostly consistent with the linkage frequencies. Late markers were not located precisely. A gene order was obtained for the genes involved in dissimilatory nitrate (*nar*) and nitrite reduction (*nir*) and anaerobical growth (*ana*) and for some already known auxotrophic genes. A mutant unable to grow aerobically (*aer*) was found. The *aer* mutation appeared to be an early marker. The cytochrome spectra of *nir* mutants and part of the cytochrome oxidase activities were abnormal in that haem *d* was absent. Most of the *nar* mutants were pleiotropic. *NarA* mutants were affected in assimilatory and dissimilatory nitrate reductase, and *narB*, *narD* and *narE* mutants lacked assimilatory and dissimilatory nitrate reductase and xanthine dehydrogenase. In *narD* mutants high concentrations of molybdate restored xanthine dehydrogenase and assimilatory and dissimilatory nitrate reductase and, consequently, anaerobic growth. Just as in the *aer* mutants, which had a normal respiratory system, the denitrifying system and the cytochrome system in *ana* mutants were normal. It is therefore unlikely that *ana* and *aer* mutants synthesize defective energy generating systems.

INTRODUCTION

Nitrate respiration has been shown to be an important process in *Pseudomonas aeruginosa*. It is the only way in which anaerobic growth occurs (Yamanaka, Ota & Okunuki, 1961). *Pseudomonas aeruginosa* is one of the most important denitrifying organisms in grassland soils (Woldendorp, 1963). Under anaerobic conditions it is responsible for the loss of utilizable nitrogen as it can form free nitrogen from nitrate. Stanier, Palleroni & Douderoff (1966) reported denitrification as a good taxonomic character to differentiate aerobic pseudomonads. Therefore it seemed of interest to know something of the genetical aspects of nitrate dissimilation. Mutants, defective in anaerobic growth in the presence of nitrate, have been isolated. They have been shown to belong to three groups. Mutants of the first (*nar*) lack nitrate reductase A, those of the second (*nir*) lack nitrite reductase, and those of the third (*ana*) are normal in nitrate and nitrite dissimilation but still altered in anaerobic growth (van Hartingsveldt, Marinus & Stouthamer, 1971). Each of these groups appeared to consist of mutants of several genes, some of which have been mapped by recombination and interrupted mating analysis.

Studies of mutants unable to grow aerobically (*aer*) have yielded information on the differences between the aerobic and anaerobic respiration systems.

The *nar* mutants showed some similarities to the *chl* mutants of *Escherichia coli*, in that they are chlorate resistant and most of them are pleiotropic mutants (Piéchaud *et al.* 1967).

Lester & DeMoss (1971) reported a molybdate stimulation of nitrate reductase activity in *E. coli*. High concentrations of molybdate can restore nitrate reductase activity in some *chlD* mutants of *E. coli* (Glaser & DeMoss, 1971) and also in some mutants of *Aspergillus nidulans* (Arst, MacDonald & Cove, 1970). A similar effect has been sought in one of the *nar* mutants of *Pseudomonas aeruginosa*.

As the nitrite reductase of *Pseudomonas aeruginosa* is a haemoprotein with cytochrome oxidase activity, containing a haem *c* and a haem *d* group (Yamanaka & Okunuki, 1963), cytochrome spectra have been recorded and cytochrome oxidase activities determined, to detect possible differences between the *nir* mutants.

METHODS

Bacterial strains and bacteriophage. Most of the bacterial strains of *Pseudomonas aeruginosa* (Table 1) have been described (van Hartingsveldt *et al.* 1971). *NarE* mutants were isolated as chlorate resistant strains from strain S1073 FP⁻ *ilvB112 his-6 leu-1 str* and *aer* mutants by treating strain S840 FP⁻ *met-28 trp-6* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by van Hartingsveldt *et al.* (1971). The transducing bacteriophage was F116 (Holloway, Monk, Hodgins & Fargie, 1962).

Media, culture procedures, conjugation, transduction and preparation of cell-free extracts. These were as described by van Hartingsveldt *et al.* (1971).

Incubation under low oxygen tension. Plates were incubated in anaerobic jars, which had been evacuated partially to 10, 7, 4 or 2% of the air pressure and refilled with nitrogen to a pressure of 1 atmosphere.

Mapping by time of entry. Logarithmic-phase donor and stationary-phase acceptor suspensions at about 5×10^8 bacteria/ml in nutrient broth (NB) + 0.5% KNO₃ were mixed at 37 °C and left to pair for 5 min. They were gently diluted 10^{-2} in fresh NB + KNO₃ at 37 °C. Samples were removed at 5 min intervals, shaken on a Vortex Genie Mixer for 1 min to separate the pairs, concentrated tenfold in the presence of streptomycin and plated, undiluted or diluted 10^{-1} , on selective medium. The streptomycin sensitivity and auxotrophy of donor bacteria ensured contra-selection. For late markers dilution and concentration were omitted.

Enzyme assays and cytochrome spectra. In cell-free extracts nitrate reductase activity was measured as described by Van'T Riet, Stouthamer & Planta (1968). Specific activity is expressed as μmol nitrite produced/min/mg protein. Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951). In cell-free extracts cytochrome oxidase activity was measured by a manometric technique (Umbreit, Burris & Stauffer, 1964). Endogenous cytochrome *c*, reduced by sodium ascorbate, was used as substrate. Specific activity is expressed as nmol O₂ consumed/min/mg protein. NADH-oxidase activity was measured in a Unicam SP 800 spectrophotometer with 1 cm light-path quartz cuvettes, by following the rate of oxidation of reduced NADH. The system contained 400 μg NADH and 0.1 or 0.2 ml cell-free extract with a total of 5 to 10 mg protein in 3 ml 0.025 M-potassium phosphate buffer, pH 7.0. Activity is expressed as nmol NADH oxidized/min/mg protein. The molar extinction coefficient of NADH is $6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm. Oxygen uptake was measured for whole resting bacteria, using standard Warburg techniques.

Stationary-phase bacteria semi-anaerobically grown in NB + KNO₃ were washed twice in cold 0.065 M-phosphate buffer, pH 7.2, and resuspended in about 1/40 of the initial volume in the same buffer. In these bacterial suspensions spectra of cytochromes (reduced minus oxidized) were recorded at room temperature in 1 cm light-path glass cuvettes in an Aminco

Table 1. Genetic characteristics of the strains of *Pseudomonas aeruginosa* used in the investigation

Strain number	Characteristics	Origin
S838	FP ⁻ <i>str</i>	.
S840	FP ⁻ <i>met-28 trp-6</i>	.
S841	FP ⁺ <i>trp-6</i>	.
S925	FP ⁻ <i>met-28 trp-6 nirA</i>	S840
S1062	FP ⁻ <i>met-28 trp-6 anaB</i>	S840
S1068	FP ⁻ <i>met-28 trp-6 leu str nirA</i>	S840
S1073	FP ⁻ <i>ilvB112 his-6 leu-1 str</i>	.
S1074	FP ⁻ <i>ilvB112 met-9 leu-1 str</i>	.
S1125	FP ⁻ <i>met-28 trp-6 anaA</i>	S840
S1128	FP ⁻ <i>narB str</i>	S838
S1130	FP ⁻ <i>narA str</i>	S838
S1133	FP ⁻ <i>narC str</i>	S838
S1135	FP ⁻ <i>narB str</i>	S838
S1229	FP ⁻ <i>ilvB112 met-9 leu-1 str narA</i>	S1074
S1230	FP ⁻ <i>ilvB112 met-9 leu-1 str narA</i>	S1074
S1231	FP ⁻ <i>ilvB112 met-9 leu-1 str narB</i>	S1074
S1232	FP ⁻ <i>ilvB112 met-9 leu-1 str narC</i>	S1074
S1233	FP ⁻ <i>ilvB112 met-9 leu-1 str narC</i>	S1074
S1234	FP ⁻ <i>ilvB112 met-9 leu-1 str narD</i>	S1074
S1235	FP ⁻ <i>ilvB112 met-9 leu-1 str narD</i>	S1074
S1239	FP ⁻ <i>ilvB112 met-9 leu-1 str nirD</i>	S1074
S1240	FP ⁻ <i>ilvB112 met-9 leu-1 str nirE</i>	S1074
S1241	FP ⁻ <i>ilvB112 his-6 leu-1 str narE thi</i>	S1073
S1275	FP ⁻ <i>met-28 trp-6 str nic aer</i>	S840
S1276	FP ⁻ <i>met-28 trp-6 str nic aer</i>	S840
S1401	FP ⁻ <i>ilvB112 his-6 leu-1 str narE thi</i>	S1073

aer, No aerobic growth; *ana*, affected in anaerobic growth; *his*, histidine requirement; *ilv*, isoleucine + valine requirements; *leu*, leucine requirement; *met*, methionine requirement; *nar*, no dissimilatory nitrate reductase activity; *nic*, nicotinic acid requirements; *nir*, no dissimilatory nitrite reductase activity; *str*, streptomycin resistance; *thi*, thiamin requirement; *trp*, tryptophan requirement.

Chance Duochromator spectrophotometer. Reduction was achieved by adding small amounts of sodium dithionite, and oxidation by adding nitrate (40 mM) or nitrite (40 mM) to the bacterial suspensions or by bubbling through oxygen.

RESULTS

New mutants. Among the strains used in this work (Table 1) two mutants, S1241 and S1401 were isolated which did not utilize nitrate under anaerobic conditions and simultaneously developed a thiamin requirement. These *nar* mutants belonged to the same transduction group which was located away from the *narA*, *B*, *C* and *D* genes; they were designated *narE*. The two *aer* mutants, S1275 and S1276, were auxotrophic for nicotinic acid (*nic*) and they too belonged to one transduction group.

Mapping by linkage analysis. From mating experiments the linkage frequencies of different markers with the *nar* mutants were determined (Table 2). In strain S1230 *narA* was not linked with the early markers *ilvB112* and *met-9* (Loutit, 1969) and only 2 or 3 % linked with the late marker *leu-1*. *NarA* was not linked with *met-28* and *trp-6* (van Hartingsveldt *et al.* 1971). Therefore *narA* must be located distally from *leu-1* (Fig. 1). *NarB*⁺ and *narC*⁺ recombinants (strain S1231 and S1232) on the contrary had a high recovery of *leu-1*⁺; *leu-1*⁺ recombinants showed a lower recovery of *narB*⁺ than of *narC*⁺. Therefore *narB* must be situated more distally from *leu-1* than *narC*. In strain S1234 the *narD* marker was unlinked to any of the

Table 2. *Percentage linkage of unselected and selected markers in the nar mutants of Pseudomonas aeruginosa after mating with strain S1141 or S841*

Two hundred colonies were screened in each cross.

Recipient	Selected markers					Unselected markers
	<i>ilvB112</i> ⁺	<i>met-9</i> ⁺	<i>his-6</i> ⁺	<i>leu-1</i> ⁺	<i>nar</i> ⁺	
S1230 <i>narA</i>	n.d. 0	n.d. 0	. .	. 2	3 .	<i>leu-1</i> ⁺ <i>narA</i> ⁺
S1231 <i>narB</i>	n.d. 0	n.d. 0	. .	. 26	38 .	<i>leu-1</i> ⁺ <i>narB</i> ⁺
S1232 <i>narC</i>	n.d. 0	n.d. 0	. .	. 35	50 .	<i>leu-1</i> ⁺ <i>narC</i> ⁺
S1234 <i>narD</i>	0 .	0 .	. 33	0 n.d.	. .	<i>narD</i> ⁺ <i>narE</i> ⁻
S1241 <i>narE</i>	. n.d.	n.d. n.d.	n.d. .	n.d. n.d.	60 10	<i>ilvB112</i> ⁺ <i>his-6</i> ⁺

n.d., Not determined.

auxotrophic markers used. With the *narE* mutants no *ilv*⁺ recombinants could be selected as isoleucine and valine are not required with addition of thiamin to the minimal medium, as Loutit & Davis (1970) observed for some of their *ilvB* mutants.

Since all *narE*⁺ recombinants lost the requirement for thiamin, however, close linkage of *narE* and *ilvB112* could be demonstrated by using minimal medium without thiamin. Linkage of *narE* and *his-6* was much lower. *NarE* must be proximal to *his-6* (Fig. 1).

The linkage data for the *aer* mutants, S1275 and S1276 (Table 3), demonstrated that *aer* is proximal to *met-28*. This observation and the relatively great number of recombinants in conjugation (about 10⁻⁵/acceptor bacterium) suggest that *aer* is an early gene. No *met*⁺ and *trp*⁺ recombinants could be obtained to confirm this. Selection of *nic*⁺ recombinants did not result in recovery of *aer*⁺, *met-28*⁺ or *trp-6*⁺, and therefore the nicotinic acid requirement must be due to a secondary mutation located away from these three genes.

Mapping by interrupted mating. In contrast to the distal *narA* and *narD* genes, *leu-1*, *narB*, *C* and *E* could be mapped easily by interrupted mating (Table 4). The distal *leu-1* marker (Loutit, 1969) and *narB* and *narC* entered at about 45 min and *narE* at 9 min. This is in accordance with the close linkage of *narB* and *narC* to *leu-1* and of *narE* to *ilvB112*. The times of entry of *narA* and *narD* were very late and consequently not very precise. The results obtained with the reference markers *met-28*, *trp-6* and *ilvB112* differed at most by 2 min from the data of Loutit (1969) and Stanisich & Holloway (1969).

For some unknown reason strain S925 did not give *nir*⁺ recombinants in interrupted mating so that no entry time could be determined. At any time from the moment of entry of *met-28*⁺, about 55% of the *met*⁺ recombinants were *nirA*⁺. Evidently, *nirA*⁺ entered prior to *met-28*⁺, as was proposed in our previous paper (van Hartingsveldt *et al.* 1971). *NirD* and *nirE* appeared to be late markers.

Strains S1125 and S1062 (formerly *nirB* and *nirC*) did not lack nitrite reductase activity, but were still affected in anaerobic growth. They were thus designated *anaA* and *anaB*. Mapping of the *ana* and *aer* genes by interrupted mating was impossible. Transduction revealed that *anaA* (formerly *nirB*) was located between *met-28* and *trp-6* (van Hartingsveldt *et al.* 1971).

Transduction. *NarB* and *leu-1*, *narC* and *leu-1* and *narE* and *ilvB112* were not co-transducible by phage F116.

Table 3. *Percentage linkage of selected and unselected markers of Pseudomonas aeruginosa after mating of aer mutants with strain S1141*

Two hundred recombinants were screened in each cross.

Recipient	Markers		Double recombinants (%)
	Selected	Unselected	
S1275	<i>aer</i> ⁺	<i>met-28</i> ⁺	11
		<i>trp-6</i> ⁺	2
		<i>nic</i> ⁺	0
	<i>nic</i> ⁺	<i>met-28</i> ⁺	0
		<i>trp-6</i> ⁺	0
		<i>aer</i> ⁺	0
S1276	<i>aer</i> ⁺	<i>met-28</i> ⁺	10
		<i>trp-6</i> ⁺	3
		<i>nic</i> ⁺	0

Table 4. *Time of entry of some nar and nir genes and some reference markers in interrupted mating of Pseudomonas aeruginosa*

Recipient	Marker	Time of entry (min)
S840	<i>met-28</i>	29
S840	<i>trp-6</i>	31
S1068	<i>nirA</i>	< 29
S1073	<i>leu-1</i>	44
S1128	<i>narB</i>	44
S1230	<i>narA</i>	65
S1232	<i>ilvB112</i>	7
S1232	<i>narC</i>	45
S1234	<i>narD</i>	65
S1239	<i>nirD</i>	70
S1240	<i>nirE</i>	70
S1241	<i>narE</i>	9

Linkage map. From these data and from those of Loutit (1969), Stanisich & Holloway (1969) and van Hartingsveldt *et al.* (1971) a linkage map was constructed (Fig. 1). Loutit proposed two linkage groups (I and II) in *Pseudomonas aeruginosa*. Linkage between markers of these groups has not been demonstrated. The late genes *narA*, *narD*, *nirD* and *nirE* might belong either to group I or II. The positions of *aer*, *nic* and *anaB* are not given in this map. *Aer* is most probably in linkage group II and *nic* in group I.

Pleiotropic effects in nar mutants and the influence of molybdate. Nitrate reductase in *Pseudomonas aeruginosa* contains molybdenum (Fewson & Nicholas, 1961) so possibly some of our mutants might form an inactive enzyme complex without molybdenum as Glaser & DeMoss (1971) observed in *chlD* mutants of *Escherichia coli*. The addition of high concentrations of molybdate (5×10^{-4} M) restored nitrate reductase and formate hydrogenlyase activities in these *chlD* mutants. Only the *narD* strains of *P. aeruginosa* regained the ability to grow under anaerobic conditions in the presence of nitrate and 5×10^{-4} M-sodium molybdate. Cell-free extracts of *narD* mutants grown overnight in the presence of molybdate had a nitrate reductase activity of about 70 % of that of the wild-type (Table 5).

In some *Aspergillus nidulans* mutants, which lack both nitrate reductase and xanthine dehydrogenase activity (Pateman, Cove, Rever & Roberts, 1964), these activities could be restored by adding high concentrations of molybdate to the culture medium (Arst *et al.* 1970). It was proposed that nitrate reductase and xanthine dehydrogenase shared a molyb-

Fig. 1. Location of some *nir* and *nar* genes on the maps of the two linkage groups (I and II) of *Pseudomonas aeruginosa*. Gene symbols as in Table 1.

denum-containing co-factor. In *Neurospora crassa* Ketchum, Cambier, Frazier, Madansky & Nason (1970) could restore NADPH-nitrate reductase activity in cell-free extracts of mutants lacking nitrate reductase and xanthine dehydrogenase by adding mammalian xanthine oxidase or chicken-liver xanthine dehydrogenase to the extracts at low pH.

In view of these results it seemed interesting to test our *nar* mutants for the use of hypoxanthine or nitrate as sole nitrogen sources. A number of these mutants appeared to grow with the amino acids, for which they are auxotrophic. In such cases prototrophic recombinants, obtained in conjugation experiments, were used in the growth experiments. *NarB*, *D* and *E* strains were unable to assimilate nitrate or hypoxanthine (Table 6). *NarA* lacks only the ability to assimilate nitrate. As *nar*⁺ recombinants or revertants were also restored in the assimilation of nitrate and hypoxanthine, *narB* and *narD* were likely to be pleiotropic mutations, affected in both assimilatory and dissimilatory nitrate reduction and degradation of hypoxanthine, and *narA* to be a pleiotropic mutation, affected only in assimilatory and dissimilatory nitrate reduction. From *narE* mutants three types of recombinants were isolated in conjugation experiments. In the first the defects in nitrate reductase and degradation of hypoxanthine were restored; in the second the ability to assimilate nitrite was regained; and in the third both defects were overcome. Therefore the *narE* mutants must have two mutations: *narE* causing defects in assimilatory and dissimilatory nitrate reductase and in hypoxanthine degradation, and another resulting in the loss of assimilation of nitrite. Only in *narD* mutants could the use of nitrate and hypoxanthine as sole nitrogen sources be restored by sodium molybdate (Table 6). Molybdate had no effect on the other *nar* mutants.

Cytochrome spectra. In *Pseudomonas aeruginosa* some of the cytochromes are involved in nitrate or nitrite respiration. Purified nitrate reductase has been found to contain a *c* type cytochrome (Fewson & Nicholas, 1961). Yamanaka & Okunuki (1963) demonstrated cytochrome oxidase and nitrite reductase in the same enzyme complex. This complex is a haemoprotein with both haem *c* and *d* groups. Newton (1969) and Lam & Nicholas (1969) observed the same in *Micrococcus denitrificans*. In *P. stutzeri* (Kodama, 1970) and in *Alcaligenes faecalis* (Iwasaki & Matsubara, 1971) too, the *cd* haemoproteins have nitrite reductase activity.

Wild-type bacteria, grown semi-anaerobically in the presence of nitrate, contained cytochrome *c* with absorption bands at 522 and 552 nm, cytochrome *b* (530 and 560 nm) and cytochrome *d* (465 and 625 nm). In the cytochrome spectra of *nir* mutants the bands at 465

Table 5. *Specific activity of nitrate reductase in cell-free extracts of a wild-type strain and a narD mutant of Pseudomonas aeruginosa after anaerobic growth in the presence or absence of sodium molybdate*

Strain	Nitrate reductase ($\mu\text{mol nitrite/min/mg protein}$)	
	With molybdate ($5 \times 10^{-4} \text{ M}$)	Without molybdate
s840 wild-type	0.56	0.60
SI234 <i>narD</i>	0.38	0.006

Table 6. *Growth of prototrophic nar mutants of Pseudomonas aeruginosa with nitrate or hypoxanthine as sole nitrogen sources, with or without sodium molybdate*

Strain	Without molybdate		With molybdate ($5 \times 10^{-4} \text{ M}$)	
	Nitrate ($5 \times 10^{-2} \text{ M}$)	Hypoxanthine ($2.2 \times 10^{-3} \text{ M}$)	Nitrate ($5 \times 10^{-2} \text{ M}$)	Hypoxanthine ($2.2 \times 10^{-3} \text{ M}$)
s838 wild-type	+	+	+	+
SI130 <i>narA</i>	—	+	—	+
SI133 <i>narC</i>	+	+	+	+
SI234 <i>narD</i>	—	—	+	+
SI241 <i>narE</i>	—	—	—	—
SI135 <i>narB</i>	—	—	—	—

and 625 nm were absent, possibly due to lack of haem *d*. The cytochrome spectra of the *nar* mutants were normal. Nitrate or nitrite was able to oxidize the cytochrome system in wild-type bacteria but not so well as oxygen; neither compound could oxidize the cytochrome systems of the *nir* mutants. Unexpectedly the *nar* mutants resembled the wild-type, possibly because a little nitrate reductase remained in these mutants.

Cytochrome oxidase activities in aerobically grown wild-type and *nir* mutants were the same (3.5 nmol oxygen consumed/min/mg protein). In wild-type bacteria grown semi-anaerobically in the presence of nitrate, cytochrome oxidase activity increased to 7 nmol oxygen/min/mg protein. *Nir* mutants did not show this increase.

Ana mutants. These possessed both nitrate and nitrite reductase activity (van Hartingsveldt *et al.* 1971). To confirm that their nitrate respiratory system was normal they were cultured with poor aeration and limited amounts of nitrate. The results (Fig. 2) were as for the wild-type (van Hartingsveldt *et al.* 1971). Accumulation of nitrite in the medium started when the oxygen tension had become almost zero. The nitrite concentration in the medium reached a maximum and then gradually decreased to zero. Therefore nitrate and nitrite reduction must be normal in these mutants. The cytochrome spectra and the oxidation of the cytochrome system by nitrate or nitrite were the same as in the wild-type.

Aer mutants. In the *aer* mutants aerobic growth did not occur even in the presence of nitrate. Only oxygen tensions not in excess of about 4 % allowed growth with nitrate on plates. Stationary-phase bacteria obtained after anaerobic growth with nitrate consumed oxygen when incubated with glucose, succinate or pyruvate at about the same rate as the wild-type. With the *aer* mutant SI276 oxygen uptake with glucose amounted to 11 nmol/min/mg protein, with succinate 59 nmol and with pyruvate 37 nmol. NADH oxidase activity in stationary-phase bacteria of these mutants was 59, which exceeds the activity of the wild-type by about 50 %.

Fig. 2. Formation and consumption of nitrite during growth of *Pseudomonas aeruginosa* strain St062 *anaB* in NB with a limited concentration of KNO_3 (0.05 %) and under low oxygen tension. The oxygen tension at the start of the experiment is arbitrarily called 100 %. Growth is given as the extinction at 660 nm. The amount of nitrite is expressed as $\mu\text{mol/ml}$ culture medium. O, Oxygen tension; Δ , growth; $\square$, concentration of nitrite in the medium.

DISCUSSION

The provisional map positions of the genes affecting anaerobic growth with nitrate in *Pseudomonas aeruginosa*, described by van Hartingsveldt *et al.* (1971), have been supplemented and confirmed by interrupted mating studies. In these experiments the time of entry of several genes has been determined. The difficulties of this method, resulting from low frequency of recombination (Loutit, 1969), were partially overcome by using less diluted mixtures of mating bacteria. The number of mating pairs formed on the plate was low and did not disturb the results. The distal genes *narA*, *narD*, *nirD* and *nirE* were not mapped precisely because the conjugation system of *P. aeruginosa* is not as effective as that of *Escherichia coli*. No Hfr strains have yet been found, and donor strains with origins other than of the FP^+ strain used (Loutit, Pearce & Marinus, 1968; Stanisich & Holloway, 1969) are not available. Some of the R-factors able to transfer chromosomal material from different points (Stanisich & Holloway, 1971) might be useful to map late genes more precisely. Both Loutit (1969) and Stanisich & Holloway (1969) proposed two linkage groups in *P. aeruginosa*. As our distal genes did not show linkage with genes of linkage group II, nor with *met-9* of group I (Fig. 1), no conclusion was drawn regarding the group in which they were located. The linkage data for *narB* and *narC* with *leu-1*, all entering at about 45 min, suggested the gene order *leu-1 narC narB*. The location of *anaB* (*nirC*) and of *aer* is not known.

In *Pseudomonas aeruginosa*, as in a number of Enterobacteriaceae, several genes are involved in the reduction of nitrate to nitrite. At least five *nar* genes were found. Four (*narA*, *B*, *D* and *E*) have pleiotropic effects, as have most of the *chl* mutations in *Escherichia coli* (Piéchaud *et al.* 1967), *Salmonella typhimurium* (Stouthamer, 1969) and in *Klebsiella aerogenes* (Stouthamer, 1967*a, b*; Stouthamer *et al.* 1967). In *narB*, *D* and *E* a molybdenum-containing

co-factor, common to dissimilatory and assimilatory nitrate reductase and xanthine dehydrogenase, might be affected (Arst *et al.* 1970). In *narD* mutants molybdenum might not be incorporated in this factor or converted into the proper redox state (Glaser & DeMoss, 1971). There is some evidence that a defect in the uptake of molybdenum in the *narD* mutants can be ruled out (unpublished results). A defect in a part common to both dissimilatory and assimilatory nitrate reductase might account for the *narA* mutation. This agrees with the view that in *K. aerogenes* the assimilatory and dissimilatory nitrate reductase are the same protein, but present in different complexes (Van'T Riet *et al.* 1968). In the *narC* strains, which lacked only dissimilatory nitrate reductase, a defect might exist in the assembly of the membrane and enzymatic components of the enzyme complex, as has been reported for *chlA* and *chlB* mutants of *E. coli* (Azoulay, Puig & Couchoud-Beaumont, 1969). A defect in a membrane component is also possible.

The cytochrome oxidase described by Yamanaka *et al.* (1963), which contains both a haem *c* and a haem *d* group, was only induced in the presence of nitrate in *Pseudomonas aeruginosa*. Furthermore, a second cytochrome oxidase, without nitrite reductase activity, was present under several conditions (Azoulay, 1964; Azoulay & Couchoud-Beaumont, 1965). The high cytochrome oxidase activity of the wild-type strain after semi-anaerobic culturing with nitrate might result from the presence of both cytochrome oxidases under these conditions. The low activity of cytochrome oxidase in the *nir* mutants, grown under the same conditions, must be attributed to the absence of the enzyme described by Yamanaka *et al.* (1963), resulting from lack of haem *d*.

Both in the *aer* and *ana* mutants the cytochrome spectra, nitrate and nitrite reductase activity (van Hartingsveldt *et al.* 1971) and respiration with oxygen were normal. Consequently the absence of aerobic growth in the *aer* mutants and of anaerobic growth in the *ana* mutants are probably not due to a defect in the energy generating system although defective coupling factors cannot be excluded. Further work is required to explain the defects in these mutants.

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