Properties of a Mutant of *Pseudomonas aeruginosa* Affected in Aerobic Growth

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SUMMARY

A mutant of *Pseudomonas aeruginosa* affected in aerobic growth (*aer*) was partially characterized. When shifted from anaerobic to aerobic conditions mutant bacteria continued growth. During this aerobic growth after the shift the mutant bacteria differed from the wild type in some respects. First, the concentration of intracellular haem decreased gradually, whereas coproporphyrin was released into the culture fluid. Apparently haem synthesis is blocked under aerobic conditions, so oxygen uptake by the mutant culture did not increase as much as by the wild type after a shift. Second, the anaerobic nitrate-respiratory system of the mutant exhibited an altered sensitivity to oxygen after a shift to aerobiosis. Nitrate and nitrite reduction were not inhibited and nitrite reductase synthesis was not repressed by oxygen. P/O values determined in intact bacteria showed that respiration with oxygen was still coupled to ATP formation in the mutant.

INTRODUCTION

Pseudomonas aeruginosa can grow anaerobically only by anaerobic respiration, in which nitrate is used as terminal electron acceptor (Yamanaka, Ota & Okunuki, 1961). Van Hartingsveldt & Stouthamer (1973) isolated a mutant (*aer*) affected in aerobic growth and which can grow only under anaerobic conditions, or at low oxygen tensions, in the presence of nitrate. After anaerobic cultivation no differences in respiration rate, cytochrome spectra, or nitrate and nitrite reduction were observed between *aer* mutant and wild-type bacteria. However, differences might be expected only when mutant and wild type are incubated aerobically. We report the effects of a shift from anaerobic to aerobic conditions in growing cultures of mutant and wild-type bacteria.

METHODS

Bacterial strains. Wild-type strain s838 and mutant strain s1276 of *Pseudomonas aeruginosa* were used (van Hartingsveldt, Marinus & Stouthamer, 1971; van Hartingsveldt & Stouthamer, 1973).

Media and general culture procedures. Bacteria were grown in a Microferm laboratory fermentor (New Brunswick Scientific Co.) in brain-heart infusion (Oxoid) supplemented with 0.4 % glucose (BHI). Aerobic growth was obtained by stirring at 400 rev./min with sparging of air at 2 l/min/l medium. Anaerobic growth was achieved by adding 0.5 % (w/v) KNO₃ to the medium and sparging with pure nitrogen. Growth at fixed dissolved oxygen concentrations was carried out by means of a dissolved oxygen controller (New Brunswick Scientific Co., Model DO-60). Growth was followed by measuring the extinction at 660 nm (E_{660}).

Shift experiments. At an extinction of about 0.25, bacteria growing anaerobically were shifted to aerobic conditions and cultured to the stationary phase. To follow aerobic growth after a shift in the absence of nitrate or nitrite, a smaller amount of nitrate (0.02 %, w/v, instead of 0.5 % KNO_3) was added to the medium in some experiments, so that nitrate and nitrite were already spent at the moment of the shift.

Oxygen consumption. The oxygen consumption during growth was measured by means of a Servomex oxygen analyser OA 184 (Servomex Controls Ltd, Crowborough, Sussex).

Enzyme assays. In cell-free extracts nitrate reductase was determined by measuring the production of nitrite from nitrate with reduced benzylviologen as hydrogen donor in open tubes (van 'T Riet, Stouthamer & Planta, 1968). Specific activity is expressed as μmol nitrite produced/min/mg protein, total activity as μmol nitrite produced/min/100 ml culture. Nitrite reductase activity was measured anaerobically in resting bacteria as described by Pichinoty, Bigliardi-Rouvier & Rimassa (1969). Specific activity is expressed as μmol nitrite converted/min/100 ml culture. Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Determination of haem, coproporphyrin, nitrite and nitrate. Intracellular haem was determined as pyridine-haemochromogen according to the procedure of Wittenberg & Siström (1971). The amount was calculated from $\epsilon(550-530 \text{ nm}) = 22.1 \text{ l/mol/cm}$. Coproporphyrin was extracted from the culture fluid and determined as described by Falk (1964). The amount was calculated from $\epsilon(401-430 \text{ nm}) = 489 \text{ l/mol/cm}$.

Nitrate was measured with a nitrate ion electrode, Model 92-07 (Orion Research Inc., Cambridge, Massachusetts, U.S.A.). Concentrations were calculated from a standard curve. Nitrite was measured as described by van 'T Riet *et al.* (1968).

Oxidative phosphorylation. Stationary phase bacteria were washed twice with cold 25 mM-potassium phosphate buffer containing 0.4 M- MgSO_4 (pH 7.2) and resuspended in this buffer to a concentration of 40 to 80 mg dry wt/ml. Oxidative phosphorylation in these bacteria was determined as described by van der Beek & Stouthamer (1973).

Growth yields. Bacteria were grown in the defined medium of MacKee & Dawes (1969), with sodium succinate as sole carbon and energy source. Molar growth yields were calculated from the dry weight of bacteria and the consumption of succinate, both determined several times during the exponential phase of growth. Dry weights were determined by filtration (de Vries & Stouthamer, 1968). Succinate was assayed by gas-liquid chromatography (de Vries, van Wijck-Kaptein & Stouthamer, 1972). It was measured as the methyl derivative prepared as described by Cato *et al.* (1970).

RESULTS

Shift experiments

Growth. The *aer* mutant bacteria were able to continue growth after a shift from anaerobic to aerobic conditions (Fig. 1). Even in the absence of nitrate or nitrite growth did not stop after the shift. The growth rate did not alter in the mutant, whereas it increased in the wild type. The maximum extinction of the culture was lower for the mutant than for the wild type.

Nitrate and nitrite reductase activities. Immediately after a shift from anaerobic to aerobic conditions the total nitrate reductase activity in the wild type decreased. In the *aer* mutant it hardly changed for about 30 min and then decreased to the same level as in the wild type (Fig. 2). However, the total nitrite reductase activity increased in the mutant and decreased in the wild type (Fig. 2). Specific activities of nitrate and nitrite reductase decreased both in the *aer* mutant and the wild type after the shift.

Fig. 1

Fig. 2

Fig. 1. Growth of *Pseudomonas aeruginosa* s838 wild type and s1276 *aer* in BHI with 0.5 % KNO_3 before and after a shift from anaerobic to aerobic conditions, given as the extinction at 660 nm. $\circ$, s838; $\triangle$, s1276.

Fig. 2. Total activity of nitrate reductase ($\mu\text{mol nitrite produced/min/100 ml culture fluid}$) and nitrite reductase ($\mu\text{mol nitrite converted/min/100 ml culture fluid}$) in cultures of *P. aeruginosa* s838 wild type and s1276 *aer* before and after a shift from anaerobic to aerobic conditions. Growth conditions as in Fig. 1. $\circ$, Nitrate reductase in s838; $\triangle$, nitrate reductase in s1276; $\square$, nitrite reductase in s838; +, nitrite reductase in s1276.

Nitrate and nitrite concentrations in the culture fluid. The concentrations of nitrate and nitrite in the culture fluids of wild-type and mutant bacteria were measured before and after a shift from anaerobic to aerobic conditions (Fig. 3). In the wild type both nitrate and nitrite reduction stopped within about 30 min of the shift, whereas they continued in the mutant. Since nitrite accumulated, the rate of nitrate reduction was greater than of nitrite reduction.

Haem and porphyrin determinations. When *aer* mutant bacteria were shifted from anaerobic to aerobic conditions the pink colour of the bacteria, caused by haem compounds, gradually disappeared. Therefore intracellular haem was determined before and after a shift from anaerobic to aerobic conditions (Fig. 4). The total haem concentration in the mutant culture decreased after the shift, whereas a strong increase was observed in the wild-type culture. Subsequently porphyrin concentrations in the culture fluid were determined (Fig. 5). In the mutant culture considerable amounts of coproporphyrin accumulated under aerobiosis. No protoporphyrin could be detected. The wild type hardly released porphyrins. In the *aer* mutant a defect in the synthesis of haem under aerobic conditions is probably present.

Mutant bacteria cultured under several dissolved-oxygen tensions in the presence of nitrate also released coproporphyrin into the medium (Table 1). An increase in the oxygen tension increased the accumulation of coproporphyrin in the medium. Only at high dissolved-oxygen tensions was a decrease in the intracellular haem observed.

Haemin added to the growth media ($2 \mu\text{g/ml}$) did not restore the ability for normal aerobic growth. Probably this compound is not taken up by *Pseudomonas aeruginosa*.

Respiration rate. Although no differences in respiration rate were found between the *aer* mutant and the wild type after anaerobiosis (van Hartingsveldt & Stouthamer, 1973), a

Fig. 3. Concentrations of nitrate, nitrite and nitrate+nitrite in the culture fluid of *P. aeruginosa* s838 wild type and s1276 *aer* before and after a shift from anaerobic to aerobic conditions. Growth conditions as in Fig. 1. ○, Nitrite concentration for s838; □, nitrite concentration for s1276; +, nitrate concentration for s838; △, nitrate concentration for s1276; ■, concentration of nitrate+nitrite for s838; ●, concentration of nitrate+nitrite for s1276.

difference in oxygen uptake was detected between growing bacteria of both strains after a shift to aerobiosis (Fig. 6). The total oxygen uptake of the wild-type culture increased considerably during growth after the shift, whereas it reached a low level in the mutant culture. Specific oxygen uptake increased in the wild type and decreased in the mutant culture.

Table 1. Intracellular haem and coproporphyrin accumulation in the culture fluid of growing bacteria of the *aer* mutant strain of *P. aeruginosa* cultured at different dissolved oxygen concentrations in the presence of KNO_3 , determined at $E_{660\text{nm}}$ of 0.1 (start of the experiment) and 0.8.

Dissolved oxygen ($\mu\text{M-O}_2$)	Haem ($\mu\text{mol/mg protein}$)		Coproporphyrin (nmol/ml medium)
	$E_{660} = 0.1$	$E_{660} = 0.8$	$E_{660} = 0.8$
12.5	0.68	0.25	75
37.5	0.76	0.20	130
50	0.67	0.14	260
150	0.79	0.02	390

Molar growth yields and oxidative phosphorylation

For wild-type bacteria growing anaerobically a molar growth yield of 23 was found. Raising the dissolved oxygen concentration in the medium resulted in an increase in the molar growth yield and in specific growth rate. At a dissolved-oxygen concentration of

Fig. 4. Intracellular haem in *P. aeruginosa* s838 wild type and s1276 *aer* before and after a shift from anaerobic to aerobic conditions. Growth conditions as in Fig. 1. Haem concentration is expressed as nmol/100 ml culture fluid (—) or as nmol/mg cellular protein (---). ○, △, s838; □, +, s1276.

Fig. 5. Concentration of coproporphyrin in the culture fluid of *P. aeruginosa* s838 wild type and s1276 *aer* before and after a shift from anaerobic to aerobic conditions. Growth conditions as in Fig. 1. ○, s838; □, s1276.

37.5 μM and under strict aerobiosis, molar growth yields of 34 and 40 were found respectively. For the *aer* mutant the anaerobic molar growth yield was also 23, whereas at 37.5 μM dissolved oxygen the value was only 14. This difference might be explained by assuming that mutant bacteria have a defect in phosphorylation coupled to respiration with oxygen but not to respiration with nitrate, in addition to the defect in aerobic haem synthesis.

For the *aer* mutant bacteria cultured anaerobically a P/O ratio of 0.20 was found. For wild-type bacteria grown anaerobically or aerobically P/O ratios of about 0.43 were found, values which agree with those of van der Beek & Stouthamer (1973). After a shift from anaerobic to aerobic conditions a P/O ratio of 0.42 was found for mutant bacteria (Table 2). During the P/O determinations it was observed that the rates of oxygen uptake and ATP production were about five times lower in mutant bacteria grown to the stationary phase

Fig. 6. Total (—) and specific (---) oxygen uptake of cultures of *P. aeruginosa* s838 wild type and s1276 *aer* after a shift from anaerobic to aerobic conditions. Growth conditions as in Fig. 1. Total uptake is expressed as $\mu\text{mol}/\text{min}/100$ ml culture; specific uptake as $\mu\text{mol}/\text{min}/\text{mg}$ cellular protein. $\square$, $\triangle$, s838; $\circ$, $\times$, s1276.

Table 2. Oxidative phosphorylation, expressed as P/O ratios, in intact stationary-phase bacteria of the wild type and the *aer* mutant strain of *P. aeruginosa* after different culture conditions

Strain	Culture conditions		
	Anaerobic	Aerobic	Aerobic after a shift
s838 wild type	0.40	0.46	N.D.
s1276 <i>aer</i>	0.20	N.G.	0.42

N.D., Not determined; N.G., no growth.

after a shift from anaerobic to aerobic conditions than in wild-type bacteria grown aerobically. This is in agreement with the data of Fig. 6. The difference between the P/O ratios of anaerobically grown wild-type and mutant bacteria has previously been unexplained. However, respiration with oxygen is coupled to ATP formation in the mutant, so we conclude that the low molar growth yield of mutant bacteria grown at a dissolved-oxygen concentration of $37.5 \mu\text{M}$ is due to an inefficient coupling between energy production and biomass formation caused by the shortage of haem under these conditions.

DISCUSSION

Under aerobic conditions the *aer* mutant of *Pseudomonas aeruginosa* did not synthesize haem and released coproporphyrin to the medium. After a shift to aerobiosis, *aer* mutant bacteria continued to grow, haem and cytochromes were diluted out, and the respiratory activity decreased. Similar mutants have been isolated from *Rhodopseudomonas spheroides* (Wittenberg & Sistrom, 1971), *Escherichia coli* (Cox & Charles, 1973) and *Saccharomyces cerevisiae* (Miyake & Sugimura, 1968).

Goldfine & Bloch (1963) supposed an aerobic and an anaerobic mechanism to form the vinyl side chains of protoporphyrin, analogous to the aerobic and anaerobic mechanisms for synthesis of mono-unsaturated fatty acids. Conversion of coproporphyrin into protoporphyrin both under aerobic and anaerobic conditions of assay was demonstrated for a *Pseudomonas* species (Ehteshamuddin, 1968), *Rhodopseudomonas spheroides* (Tait, 1969) and *Chromatium* (Tait, 1972). Tait (1972) detected some differences between the systems. Our results with the *aer* mutant of *Pseudomonas aeruginosa* agree with the hypothesis of Goldfine & Bloch (1963). The anaerobic conversion of coproporphyrin into protoporphyrin is unaffected, but the aerobic conversion is blocked in the mutant. Molecular oxygen must repress, inhibit or inactivate the anaerobic route.

Oxygen repressed and inactivated nitrate and nitrite reductase and inhibited these activities in the wild type of *Pseudomonas aeruginosa*, as also found by Fewson & Nicholas (1961). In the mutant, however, nitrate and nitrite reduction and the synthesis of nitrite reductase continued after a shift to aerobic conditions. Probably synthesis of haem compounds is necessary to cause the effects of oxygen on the nitrate respiratory system in the wild type. Both in mutant and wild-type bacteria nitrate reductase is inactivated by oxygen, indicating that haem synthesis is not necessary for this inactivation.

Previously it was considered that the *aer* mutants were most probably not affected in ATP generation under aerobic conditions (van Hartingsveldt & Stouthamer, 1973). However, the possibility could not then be excluded that in the *aer* mutant respiration with oxygen, in contrast to respiration with nitrate, is not coupled to ATP formation. The direct determination of P/O ratios in intact bacteria and the ability to grow after a shift in nitrate-free medium exclude this possibility. None the less, from the present results it must be concluded that the defect in aerobic growth in the *aer* mutant is indeed due to a defect in ATP generation under aerobic conditions, since the block in haem biosynthesis prevents the formation of a functional respiratory chain.

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