

## Effect of Growth Conditions on the Involvement of Cytochrome *c* in Electron Transport, Proton Translocation and ATP Synthesis in the Facultative Methylotroph *Pseudomonas* AM1

By C. William KEEVIL\* and Christopher ANTHONY

Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

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The stoichiometry of proton translocation, the amounts of cytochromes firmly bound to membranes, and cell yields with respect to succinate and O<sub>2</sub> have been measured in the facultative methylotroph *Pseudomonas* AM1 and in the mutant lacking cytochrome *c* (mutant PCT76) during carbon-limited growth and carbon-excess growth. →H<sup>+</sup>/O ratios during endogenous respiration of about 4 were measured in wild-type bacteria grown in carbon-excess conditions, and in the mutant in all growth conditions. During methanol- or succinate-limited growth of wild-type bacteria the →H<sup>+</sup>/O ratio increased to about 6. Cell yields with respect to succinate and O<sub>2</sub> were higher in wild-type than in the mutant lacking cytochrome *c* by an amount suggesting loss in the mutant of 30% of the ATP-generating capacity of wild-type bacteria. During carbon-limited growth on methanol or succinate some cytochrome *c* was tightly bound to bacterial membranes, whereas none was tightly bound in bacteria grown in batch-culture or in NH<sub>4</sub><sup>+</sup>-limited conditions. It is proposed that the role of cytochrome *c* in *Pseudomonas* AM1 depends on growth conditions and hence on the 'needs' of the bacteria. During growth in carbon-excess conditions it is only required for methanol oxidation, mediating between methanol dehydrogenase and cytochrome *a/a*<sub>3</sub>. In these conditions oxidation of NADH and succinate by way of cytochrome *b* and cytochrome *a/a*<sub>3</sub> occurs without the mediation of cytochrome *c*. This is the only route for oxidation of NADH and succinate in the cytochrome *c*-deficient mutant in all growth conditions. During carbon-limited growth the cytochrome *c* becomes bound to the membrane in such a way that it can mediate between cytochromes *b* and *a/a*<sub>3</sub>, hence becoming involved in proton translocation and ATP synthesis during NADH and succinate oxidation. An alternative possibility is that in wild-type bacteria the cytochrome *c* is always involved in electron transport, but that its involvement in measurable proton translocation only occurs in carbon-limited conditions.

*Pseudomonas* AM1 is a facultative methylotroph unable to grow on methane, but able to grow on other C<sub>1</sub> compounds and on a wide range of multicarbon compounds such as succinate. Loss of cytochrome *c* by mutation (in mutant PCT76) prevents growth on C<sub>1</sub> compounds, whereas growth on succinate is unaffected (Anthony, 1975; Widdowson & Anthony, 1975). Unexpectedly, the stoichiometry of respiration-driven proton translocation in batch-grown bacteria was not affected by the loss of cytochrome *c* (O'Keefe & Anthony, 1978). These observations suggested that cytochrome *c* may never be involved in electron and proton transport from succinate and NADH in *Pseudomonas* AM1 (O'Keefe & Anthony, 1978). Such a suggestion is at variance with the conclusions of a comprehensive survey by Jones *et al.* (1977), who demonstrated that bacteria possessing a high-potential membrane-bound cytochrome *c* were capable of higher →H<sup>+</sup>/O

ratios and higher growth yields than those lacking this cytochrome.

To investigate this apparent discrepancy further *Pseudomonas* AM1 has been grown under a variety of cultural conditions and the cytochromes, →H<sup>+</sup>/O ratios and cell yields with respect to O<sub>2</sub> and succinate measured and compared with those measured in the mutant lacking cytochrome *c*. A preliminary account of the results of this investigation has been published (Keevil & Anthony, 1979a).

### Materials and Methods

#### Chemicals

All chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K., except for carbonyl cyanide *m*-chlorophenylhydrazone (Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.); the column packings for gas chromatography (Phase Separations, Rockferry, Flintshire, U.K.); and Dow Corning Antifoam RD Emulsion (Hopkin and Williams, Chadwell Heath, Essex, U.K.).

\* Present address: Tate and Lyle (Research), Whiteknights Park, Reading, Berks, U.K.

### Organisms and growth media

*Pseudomonas* AM1 (N.C.I.B. 9133) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K. The cytochrome *c*-deficient mutant (PCT76) and the carotenoid-deficient mutant (PCT7) were isolated by Dr. Patricia Dunstan in this laboratory and have been described previously (Anthony, 1975). The defined liquid growth medium was that of Maclellan *et al.* (1971), with the exception that instead of  $\text{KH}_2\text{PO}_4$ , the medium contained 20 mM-phosphoric acid for batch culture (neutralized with NaOH/KOH) or 6 mM-phosphoric acid neutralized in the culture vessel by addition of NaOH/KOH for continuous culture. The nitrogen source was 27 mM- $(\text{NH}_4)_2\text{SO}_4$  and the carbon and energy source was either methanol (125 mM) or succinate (20 or 40 mM). During nitrogen-limited continuous culture the  $(\text{NH}_4)_2\text{SO}_4$  concentration was either 5.4 mM with excess methanol (250 mM) or 1 mM with excess succinate (40 mM).

### Growth and harvesting of cultures

For batch culture bacteria were grown in volumes of 1 litre in 2 litre baffled flasks at 30°C on a rotary shaker; they were harvested at the end of the logarithmic phase by centrifugation. For continuous culture the bacteria were grown in 5 litre fermenters (working volume, 1.7 litres; L. H. Engineering Co., Stoke Poges, Bucks., U.K.). The interiors of the glass fermenters were silicon-treated with Repelcote (Hopkin and Williams) to decrease wall growth. Fermenters were stirred at 750 rev./min and were controlled at 30°C and pH 7.0 ( $\pm 0.05$ ). Foaming was controlled by regular addition of anti-foaming agent. Peristaltic pumps for supply of growth medium were MHRE-200 pumps (Watson-Marlow, Wembley, Middx., U.K.) for high flow rates and Pharmacia P3 pumps (Pharmacia, Uppsala, Sweden) for low rates. The concentration of dissolved  $\text{O}_2$  was monitored by using a lead/silver electrode (L.H. Engineering Co.) and maintained at 40–80% of the air saturation value by varying the flow rate of air into the fermenter.

Bacteria were harvested for measurement of cytochromes or of  $\rightarrow\text{H}^+/\text{O}$  ratios directly from the culture vessel or from the overflow line (collected overnight at 2°C). The measured  $\rightarrow\text{H}^+/\text{O}$  ratios were found to be the same size when bacteria were harvested from the overflow line after overnight storage as when collected directly from the culture vessel.

### Analysis of cultures

All analyses were of material taken directly from the fermenter vessels. Purity of cultures was tested by streaking on to methanol-, succinate- and nutrient-agar plates and incubating at 30°C for 96 h. The concentration of bacteria in the fermenter was measured by rapidly filtering 20 ml samples through

preweighed Millipore cellulose filters (pore size, 0.22  $\mu\text{m}$ ; Millipore, Wembley, Middx., U.K.); the filters were dried at 105°C to constant weight. The filtered supernatants were stored at -15°C for subsequent analysis.

The concentrations of methanol and succinate in the filtered culture media were measured by gas chromatography. Methanol was measured by comparison with standard solutions by using a Perkin-Elmer F11 chromatograph (Perkin-Elmer, Beaconsfield, Bucks., U.K.) with a flame-ionization detector at 60°C. The sample size was 2  $\mu\text{l}$ , the carrier gas was  $\text{N}_2$  flowing at 8 ml/min and the column packing (in a stainless-steel column, 2.1 m  $\times$  3 mm) was 10% Carbowax 1540 on Teflon (30–60 mesh).

Succinate was measured as the more volatile trimethylsilyl derivative. Sodium malonate was used as internal standard; 10  $\mu\text{l}$  of 500 mM solution was added to 1 ml of culture filtrate followed by 0.5 ml of IR-120H<sup>+</sup> ion-exchange resin. The well-mixed suspension was allowed to stand for 5 min and 40  $\mu\text{l}$  of supernatant injected into a 0.8 ml Autojector vial (Pye-Unicam, Cambridge, U.K.). The contents were freeze-dried and 10  $\mu\text{l}$  of pyridine was added followed by 5  $\mu\text{l}$  of hexamethyldisilazane and 5  $\mu\text{l}$  of trimethylchlorosilane. After 30 min 2  $\mu\text{l}$  was injected on to a glass column (2 m  $\times$  6 mm) packed with 3% OV-1 on diatomite CQ (100–200 mesh). A Pye-Unicam 104 chromatograph was used with a flame-ionization detector, the injection port and oven temperatures being 170 and 120°C respectively. The carrier gas was  $\text{N}_2$  at a flow rate of 40 ml/min. The succinate was measured by electronic integration of the peak areas and comparison with known standards, data being normalized to the peak areas of the internal malonate standard for decreasing the error. The concentration of  $\text{NH}_3$  in deproteinated culture supernatants was determined by the method of Mulramatsu (1967).

The effluent gases from continuous cultures were passed through a sterile filter by way of Butyl XX rubber tubing (Esco Rubber Co., London, U.K.), which has a low permeability to  $\text{O}_2$ . The gases were dried over anhydrous  $\text{CaCl}_2$  and the concentrations of  $\text{O}_2$  and  $\text{CO}_2$  were measured;  $\text{O}_2$  was measured with a Taylor Servomex type OA 272 paramagnetic gas analyser and  $\text{CO}_2$  was measured with a Beckman type L81 i.r. gas analyser. Analyses were calibrated before each determination with a similar flow rate of white spot  $\text{N}_2$ , a mixture of  $\text{CO}_2$ ,  $\text{O}_2$  and  $\text{N}_2$  (5:12:83, by vol.; British Oxygen Co., London S.W.19, U.K.) and air. The flow rates of gases were controlled with GAP flow meters and calibrated with a vertical bubble-flow column.

### Molar-growth yields

The respiratory activities *in situ* of growing cultures were determined at various growth rates by

measuring the concentrations of O<sub>2</sub> and CO<sub>2</sub> in the air entering the fermenter and in the effluent gas, the gas-flow rate, the medium-flow rate and cell concentration. The respiratory activities were expressed as  $q_{O_2}$  values (mol of O<sub>2</sub> consumed/h per g of cells) and  $q_{CO_2}$  values (mol of CO<sub>2</sub> produced/h per g of cells). Rates of succinate utilization *in situ* ( $q_{succinate}$ ; mol of succinate utilized/h per g of cells) were also measured. True molar growth yields ( $Y_{max}$ ) with respect to O<sub>2</sub> and succinate consumed or CO<sub>2</sub> produced (g dry wt. of cells/mol of O<sub>2</sub>, CO<sub>2</sub> or succinate) were determined from the equation:

$$q = \frac{\mu}{Y_{max.}} + m$$

where  $\mu$  is the specific growth rate (equivalent to the dilution rate in continuous culture; h<sup>-1</sup>) and  $m$  is the maintenance rate (mol/h) of utilization of O<sub>2</sub> or succinate or of production of CO<sub>2</sub>. The  $q$  values *in situ* were plotted against dilution rates; each plot consisted of not less than six points spread over a 5-fold range of growth rates. The best straight line was fitted by linear regression analysis with a Hewlett-Packard model 10A (1098) calculator; correlation coefficients ( $r^2$ ) were in the range 0.961–0.995.

The best measure of cellular-energy conservation is given by the true molar growth yield with respect to succinate oxidized ( $Y_{succ. ox.}^{max.}$ ; Jones *et al.*, 1977); this was calculated with the formula:

$$Y_{succ. ox.}^{max.} = \frac{\gamma Y_{succ.}^{max.}}{\gamma - \beta Y_{succ.}^{max.}}$$

where  $\gamma$  is the carbon content of succinate (g/mol) and  $\beta$  is the fraction of cell mass assumed to be carbon (0.5; Pirt, 1975).

#### Measurement of bacterial respiration and respiration-driven proton translocation

Bacteria were harvested by centrifugation and washed twice at 2°C with 140 mM-KCl and resuspended in this medium to a cell density of 60–100 mg dry wt./ml; they were stored in ice and used within 10 h of harvesting. →H<sup>+</sup>/O ratios (ng-ions of H<sup>+</sup> translocated from the bacteria into the external medium/ng-atom of O consumed) were measured by the pulse oxidant technique of Mitchell & Moyle (1967) in the conditions described by O'Keeffe & Anthony (1978). →H<sup>+</sup>/O ratios were constant when measured in the pH range 5.6–7.5, most experiments being done at an initial pH of about 7.1, achieved by prior addition of 0.2 M-KOH to the cell suspension. The concentration of exogenous substrates was 7.5 mM except for ascorbate (1.5 mM) plus NNN'N'-tetramethylphenylenediamine (4 mM); this mixture was adjusted to pH 6.5 before use. Rates of O<sub>2</sub> consumption were

measured with a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) in the same reaction mixture, including thiocyanate, as was used for measurement of proton translocation. When inhibitors were used these were incubated for 15 min with bacteria before measurement of respiration rates. Proton translocation coupled to methanol oxidation could not be measured because thiocyanate inhibited methanol oxidation in cells grown in both carbon-limited and carbon-excess cultures.

#### Disruption of bacteria and determination of cytochrome concentrations

Bacteria were washed twice in 20 mM-KH<sub>2</sub>PO<sub>4</sub> adjusted to pH 7.0 with 1 M-KOH and resuspended in this buffer to a density of about 60 mg dry wt./ml. The bacteria were disrupted by sonication (Widdowson & Anthony, 1975) or by passage through a French pressure cell (Aminco, Silver Spring, MD, U.S.A.) at 20000 lb/in<sup>2</sup> (135 MPa). Whole cells were removed by centrifugation at 300 g for 10 min and the membrane fraction obtained by centrifuging the resulting supernatant at 380000 g for 1.5 h at 2°C. This membrane fraction was washed 3 times, resuspended in the same buffer and the cytochromes measured as described previously (Widdowson & Anthony, 1975). With these procedures no vesicles were observed when membrane preparations were negatively strained and examined with a Hitachi HV12 electron microscope; this contrasts with the results of Netrusov & Anthony (1979), who used a different method for preparation of membranes capable of oxidative phosphorylation.

## Results

### The increased stoichiometry of proton translocation in *Pseudomonas* AM1 during carbon-limited growth

The results in Table 1 show that the →H<sup>+</sup>/O ratios measured during respiration of endogenous substrate in *Pseudomonas* AM1 were between 5.5 and 6.0 during carbon-limited growth. The same result was obtained with either methanol or succinate as carbon source. By contrast, in batch culture or in NH<sub>4</sub><sup>+</sup>-limited continuous culture the ratios were always about 4 as previously observed by O'Keeffe & Anthony (1978). During succinate-limited continuous culture when the dilution rate was increased the expected increase in growth yield occurred (Fig. 1a) and during the transition phase when the culture was not strictly carbon-limited the stoichiometry of proton translocation decreased, returning in the new steady state of carbon-limitation to the original higher value (Table 1 and Fig. 1b).

These results suggest that during carbon-limited continuous culture an extra proton-translocating

Table 1.  $\rightarrow H^+/O$  ratios measured during respiration of endogenous substrate in *Pseudomonas* AM1 and in a mutant lacking cytochrome *c* (PCT 76)

$\rightarrow H^+/O$  ratios were measured as described in the Materials and Methods section; they are presented as means  $\pm$  S.E.M., with the numbers of determinations with separate batches of cells or separate continuous cultures in parentheses. Bacteria were grown in continuous culture at a variety of dilution rates between 0.01 and 0.1 h<sup>-1</sup> and were harvested from the steady state or from the transition state occurring after doubling of dilution rate of a carbon-limited culture.

	$\rightarrow H^+/O$ ratio			
	Methanol-grown bacteria		Succinate-grown bacteria	
	Carbon-excess	Carbon-limited	Carbon-excess	Carbon-limited
<i>Pseudomonas</i> AM1				
Batch culture	3.48 $\pm$ 0.18 (15)		4.00 $\pm$ 0.20 (12)	
Continuous culture				
NH <sub>4</sub> <sup>+</sup> -limited	3.90 $\pm$ 0.07 (2)		4.10 $\pm$ 0.08 (6)	
Transition phase	3.10 $\pm$ 0.02 (3)		3.80 $\pm$ 0.35 (2)	
Carbon-limited		5.58 $\pm$ 0.09 (8)		5.90 $\pm$ 0.04 (7)
Mutant PCT76				
Batch culture			3.69 $\pm$ 0.17 (9)	
Continuous culture				
NH <sub>4</sub> <sup>+</sup> -limited			4.35 $\pm$ 0.14 (3)	
Carbon-limited				3.99 $\pm$ 0.08 (3)

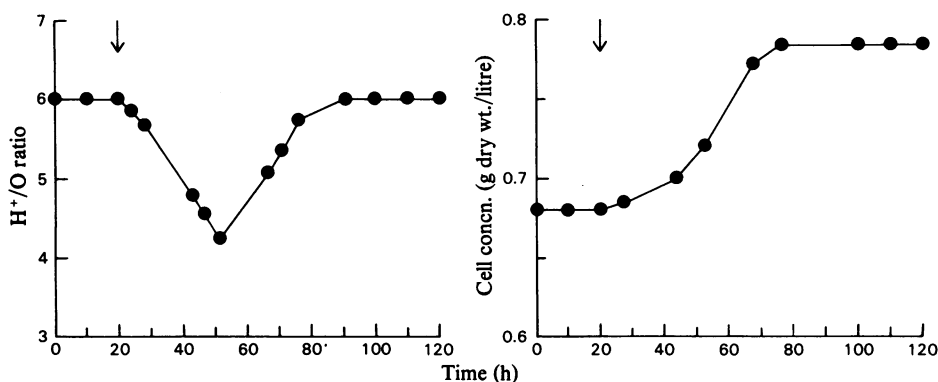


Fig. 1.  $\rightarrow H^+/O$  ratios and cell concentrations during transition from a lower to a higher growth rate

The dilution rate of a succinate-limited continuous culture (40 mM-succinate) of *Pseudomonas* AM1 was increased at the time indicated by the arrow from 0.045 h<sup>-1</sup> to 0.09 h<sup>-1</sup>. Samples were taken directly from the culture vessel for measurement of dry weight and of endogenous respiration-driven  $\rightarrow H^+/O$  ratios.

segment functions during endogenous respiration of *Pseudomonas* AM1. That this extra segment involves cytochrome *c* is indicated by the lower ratios of about 4 always found in mutant PCT76, which lacks cytochrome *c* (Table 1).

It has previously been shown (O'Keeffe & Anthony, 1978) that high concentrations of KCN (4.0 mM) inhibit all respiration and proton translocation in *Pseudomonas* AM1. However, in the presence of a lower concentration of KCN (0.4 mM), although respiration by way of cytochrome *a/a*<sub>3</sub> is prevented, some respiration continues by way of autoxidizable

cytochrome *b* (O'Keeffe & Anthony, 1978). In the present work when *Pseudomonas* AM1 was taken from a succinate-limited continuous culture and the proton translocation driven by endogenous respiration was measured in the presence of 0.4 mM-KCN, the  $\rightarrow H^+/O$  ratio was lowered from about 6 to about 4 [4.35  $\pm$  0.08 (mean  $\pm$  S.E.M.), *n* = 4]. The  $\rightarrow H^+/O$  ratio of about 4 was unaffected by 0.4 mM-KCN in the cytochrome *c*-deficient mutant grown in continuous culture or in wild-type and mutant bacteria when harvested from batch cultures. It thus appears that the extra proton-translocating segment operating

Table 2.  $\rightarrow\text{H}^+/\text{O}$  ratios and respiration rates measured with various added substrates in *Pseudomonas* AM1 and mutant PCT76 grown in batch or continuous culture

For full details see the Materials and Methods section.  $\rightarrow\text{H}^+/\text{O}$  ratios are presented as means  $\pm$  s.e.m., with the number of determinations for separate batch or continuous cultures in parentheses. Respiration rates with added substrate have been corrected for the endogenous rates measured in the same batch of cells. Respiration rates were measured in the same incubation mixture as was used for measurement of  $\rightarrow\text{H}^+/\text{O}$  ratios and these are expressed as nmol of  $\text{O}_2$  consumed/min per mg dry wt. Mutant PCT76 was grown on succinate. Half the cultures of *Pseudomonas* AM1 were grown on methanol and half on succinate. Half of the carbon-excess cultures were batch cultures and half were  $\text{NH}_4^+$ -limited. Abbreviation: TMPD, *NNN'N'*-tetramethylphenylenediamine.

Respiratory substrate	Carbon-limited cultures		Carbon-excess cultures	
	$\rightarrow\text{H}^+/\text{O}$ ratio	Respiration rate (nmol of $\text{O}_2$ /min per mg dry wt.)	$\rightarrow\text{H}^+/\text{O}$ ratio	Respiration rate (nmol of $\text{O}_2$ /min per mg dry wt.)
<i>Pseudomonas</i> AM1				
Endogenous	5.73 $\pm$ 0.07 (15)	5 – 12	3.75 $\pm$ 0.10 (19)	8 – 16
Malate	5.17 $\pm$ 0.11 (11)	67.1 $\pm$ 11.7 (11)	3.48 $\pm$ 0.07 (12)	22.34 $\pm$ 0.4 (4)
3-Hydroxybutyrate	5.14 $\pm$ 0.12 (5)	21.1 $\pm$ 1.0(4)	3.73 $\pm$ 0.07 (3)	14.1 $\pm$ 2.3 (2)
Ascorbate/TMPD	1.83 $\pm$ 0.15 (7)	50.7 $\pm$ 1.6 (5)		
Mutant PCT 76				
Endogenous	3.99 $\pm$ 0.08 (4)	5 – 9	3.69 $\pm$ 0.17 (9)	8 – 16
Malate	3.40 $\pm$ 0.06 (3)	71.8 $\pm$ 13.3 (3)	3.91 $\pm$ 0.14 (3)	25.2 $\pm$ 1.0 (2)
3-Hydroxybutyrate	3.8 (1)	16.4 (1)	3.86 $\pm$ 0.06 (2)	13.3 $\pm$ 2.7 (2)

during carbon-limited growth of *Pseudomonas* AM1 requires both cytochrome *c* and the usual oxidase, cytochrome *a/a<sub>3</sub>*.

A possibility to be considered if  $\rightarrow\text{H}^+/\text{O}$  ratios vary with growth conditions is that the endogenous respiratory substrate is altering (see Lawford *et al.* 1976). It has previously been suggested that the substrate for endogenous respiration is NADH in *Pseudomonas* AM1 (O'Keeffe & Anthony, 1978). That this remains the endogenous substrate in carbon-limited bacteria is confirmed by the observation (Table 2) that the  $\rightarrow\text{H}^+/\text{O}$  ratios measured during respiration of substrates whose oxidation yields NADH (3-hydroxybutyrate and malate) increased from about 4 to about 6 during carbon-limited growth of wild-type bacteria. The ratios measured during respiration of these substrates was never more than 4 in the mutant lacking cytochrome *c*, even during carbon-limited continuous culture. The  $\rightarrow\text{H}^+/\text{O}$  ratios measured during respiration with ascorbate plus tetramethylphenylenediamine were always about 2 in *Pseudomonas* AM1 grown in batch or continuous (carbon-limited) culture. By contrast the cytochrome *c*-deficient mutant never oxidized ascorbate plus tetramethylphenylenediamine.

#### Lower growth yields resulting from loss of cytochrome *c*

The results described above suggest that the higher  $\rightarrow\text{H}^+/\text{O}$  ratios measured during carbon-limited growth of *Pseudomonas* AM1 depend on the ability of the bacteria to synthesize cytochrome *c*. If the measured  $\rightarrow\text{H}^+/\text{O}$  ratio is reflected in the capacity for synthesis of ATP then the mutant lacking cyto-

chrome *c* should give lower growth yields than wild-type *Pseudomonas* AM1. That this is indeed the case is shown by the results in Fig. 2 and in Table 3. As emphasized by Jones *et al.* (1977) the  $\text{O}_2$  consumed by the culture is used entirely as a terminal electron acceptor for respiration, whereas the carbon substrate is partly oxidized to  $\text{CO}_2$  and partly assimilated into cell material. The best reflections of the efficiency of energy conservation are thus the cell yields with respect to  $\text{O}_2$  ( $Y_{\text{O}_2}$ ) or yields with respect to succinate oxidised ( $Y_{\text{succ. ox.}}$ ). As expected the largest changes resulting from loss of cytochrome *c* in mutant PCT76 were found in these parameters (Table 3). The differences measured between wild-type *Pseudomonas* AM1 and the mutant lacking cytochrome *c* are similar to the differences measured by Jones *et al.* (1977) between those species of bacteria containing cytochrome *c* and those lacking it.

In Table 3 predicted values for the mutant are given and these values are very close to those measured. The predictions were made by using a similar method to that used previously for prediction of factors affecting cell yields in methylotrophs (Anthony, 1978*a*). The assumptions made in the present work are: that the cell composition can be expressed as  $\text{C}_4\text{H}_8\text{O}_2\text{N}$ ; that the  $Y_{\text{ATP}}$  (g. dry wt./mol of ATP available for biosynthesis) for phosphoglycerate assimilation into cell material is 10.5; that the P/O ratios of wild-type bacteria with cytochrome *c* were 3 for NADH oxidation and 2 for oxidation of reduced succinate dehydrogenase; and that the P/O ratios were decreased by 1 for each substrate in the mutant lacking cytochrome *c*.

The  $Y_{\text{ATP}}$  is an expression of the ATP requirement

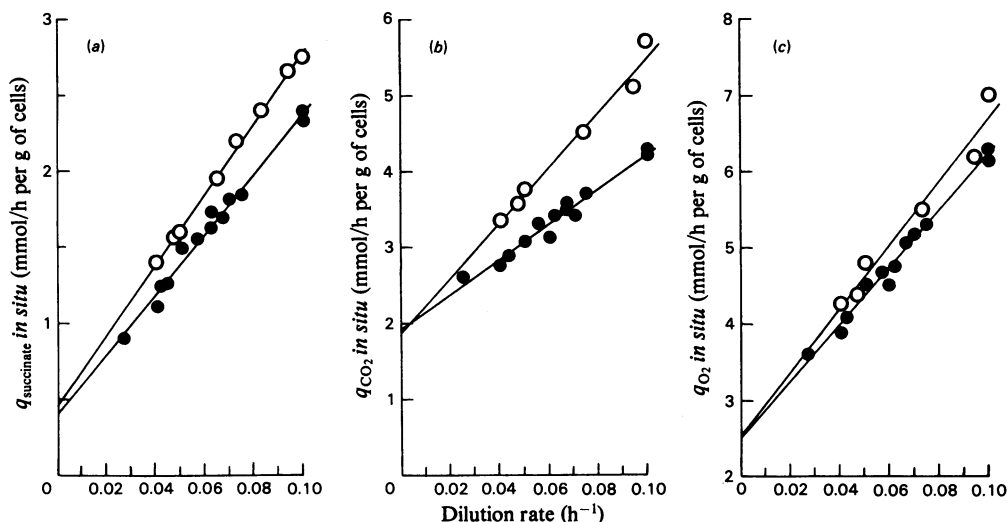


Fig. 2. Effect of loss of cytochrome *c* on rates of utilization of  $O_2$  and succinate and of production of  $CO_2$  *in situ*. Bacteria were grown in succinate-limited continuous culture and cultures analysed as described in the Materials and Methods section. Symbols: ●, *Pseudomonas* AM1; ○, mutant PCT76 lacking cytochrome *c*.

Table 3. Molar growth yields during succinate-limited continuous culture of *Pseudomonas* AM1 and the mutant lacking cytochrome *c* (PCT76)

The rates of  $O_2$  and succinate consumption and of  $CO_2$  production (mmol consumed or produced/h per g of cells) *in situ* were measured and plotted against the specific growth rate (Fig. 2). True molar growth yields were calculated from these plots as described in the Materials and Methods section. The predicted values for the mutant are based on the assumption that loss of cytochrome *c* has diminished the P/O ratios for NADH and succinate from 3 and 2 respectively to 2 and 1 (see the Results section).

	True molar growth yields		
	<i>Pseudomonas</i> AM1	Mutant PCT76	Predicted value for mutant
$Y_{succ.}^{max.}$ (g of cells/mol of succinate consumed)	50.13	42.96	42.6
$Y_{O_2}^{max.}$ (g of cells/mol of $O_2$ consumed)	44.11	27.63	29.1
$Y_{succ. ox.}^{max.}$ (g of cells/mol of succinate oxidized)	104.9	77.76	76.6
$Y_{CO_2}^{max.}$ (g of cells/mol of $CO_2$ produced)	27.12	24.07	21.86

for cell synthesis and it may be related to the  $Y_{O_2}$  by the formula:  $Y_{O_2} = Y_{ATP} \times (P/O \text{ ratio})$ . Thus if it is assumed that the  $Y_{ATP}$  (whatever its actual value) is the same in the mutant and wild-type bacteria, then the  $Y_{O_2}$  value for wild-type divided by that for the mutant will be the ratio of their P/O ratios. The measured values in Table 3 show that this ratio was almost 3:2 (1.6), as expected if the mutant lacking cytochrome *c* has indeed lost one proton-translocating segment, and if this is equivalent to a potential for synthesis of 1 mol of ATP/g-atom of O reduced. It is also noteworthy that the  $Y_{O_2}$  for wild-type divided by that for the mutant (1.6) is almost exactly the same as the ratio of their  $\rightarrow H^+/O$  ratios (1.48).

It can thus be concluded from these growth-yield measurements that during carbon-limited growth on succinate, cytochrome *c* is part of an electron-transport chain from NADH and succinate to  $O_2$  and that this part of the chain containing cytochrome *c* is involved in both proton translocation and ATP synthesis.

#### *Increased binding of cytochrome c to membranes during carbon-limited growth*

Table 4 summarizes the concentrations of cytochromes measured in the bacteria used in the experiments described above. The values were generally

Table 4. The effect of growth conditions on cytochrome concentrations in washed membrane, and soluble fractions of *Pseudomonas* AM1 and the mutant lacking cytochrome *c* (mutant PCT76)

Bacteria were grown, extracts prepared and cytochromes estimated as described in the Materials and Methods section. The concentrations (pmol/mg of protein) are presented as means  $\pm$  s.e.m., with the number of determinations for separate batches or continuous cultures given in parentheses. Half of the carbon-excess cultures were batch cultures and half were  $\text{NH}_4^+$ -limited continuous cultures; there was no significant difference between results from the two types of carbon-excess cultures. A single determination of cytochromes from an  $\text{O}_2$ -limited culture (carbon source, succinate) of *Pseudomonas* AM1 gave similar values to those measured in other carbon-excess cultures. Abbreviation: ND, none detected.

	Cytochrome concn. (pmol/mg of protein)							
	Methanol-grown bacteria				Succinate-grown bacteria			
	Membrane			Soluble	Membrane			Soluble
	<i>a/a</i> <sub>3</sub>	<i>b</i>	<i>c</i>	<i>c</i>	<i>a/a</i> <sub>3</sub>	<i>b</i>	<i>c</i>	<i>c</i>
<i>Pseudomonas</i> AM1								
Carbon-limited	(4) 240 $\pm$ 46	260 $\pm$ 45	365 $\pm$ 23	2065 $\pm$ 110	(7) 184 $\pm$ 13	220 $\pm$ 16	405 $\pm$ 381	950 $\pm$ 108
Carbon-excess	(6) 226 $\pm$ 40	245 $\pm$ 33	35 $\pm$ 22	1210 $\pm$ 33	(4) 149 $\pm$ 35	313 $\pm$ 20	ND	399 $\pm$ 56
Mutant PCT76								
Carbon-limited					(2) 200 $\pm$ 20	300 $\pm$ 40	ND	ND
Carbon-excess					(2) 220 $\pm$ 35	273 $\pm$ 80	ND	ND

similar to those previously published (Widdowson & Anthony, 1975; Tonge *et al.*, 1974). The important points to note are: the higher concentrations of cytochrome *c* found in bacteria grown on methanol compared with succinate; the higher concentrations of cytochrome *c* in bacteria grown under carbon limitation; and, most important, the observation that under carbon limitation with either methanol or succinate as substrate there was a much larger amount of cytochrome *c* tightly bound to the membrane fraction than in bacteria grown in carbon-excess conditions. This shows that sonication and washing three times with phosphate buffer was sufficient to remove the cytochrome *c* from membranes prepared from bacteria grown under conditions of carbon abundance, but not from those grown under carbon limitation. It is noteworthy that cytochrome *c* when bound to membranes has the same absorption maximum as the soluble cytochrome *c*. There is no evidence for any membrane-bound cytochrome *c*<sub>1</sub> with a different absorption spectrum or midpoint redox potential as found in mitochondria.

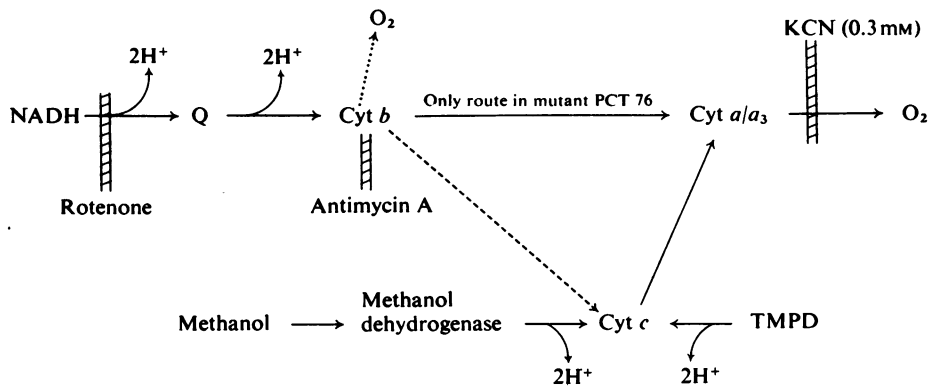
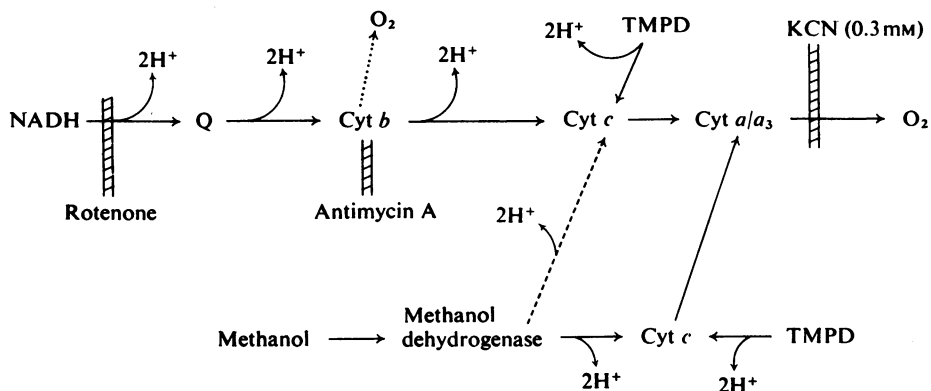
## Discussion

The electron-transport systems proposed in Scheme 1 are consistent with the results in the present paper and they, to some extent, resolve the apparent discrepancies mentioned in the introduction. The Schemes are also consistent with previous measurements in *Pseudomonas* AM1 and mutant PCT76 of reductions of cytochrome *c* and *a/a*<sub>3</sub> in whole cells (Anthony, 1975; Widdowson & Anthony, 1975); of proton translocation in whole

cells (O'Keeffe & Anthony, 1978); and of ATP synthesis in membrane preparations (Netrusov & Anthony, 1979). In these previous papers similar schemes to those presented in the present paper were proposed as alternative explanations for the results. However, the results in the present paper lead to the conclusion that both Schemes are possible and that the growth conditions determine which particular path of electron transport operates.

It is proposed that cytochrome *c* can fulfil two roles in *Pseudomonas* AM1 and that the function of this cytochrome depends on the growth conditions and hence of the 'needs' of the bacteria. We suggest that in carbon-excess growth, cytochrome *c* is not necessarily involved in mediation between cytochromes *b* and *a/a*<sub>3</sub> and that it is only involved in electron transport, proton translocation and ATP synthesis during the first step in methanol oxidation (Scheme 1*a*). This would explain why the stoichiometry of proton translocation is the same during carbon-excess growth in wild-type bacteria and in the mutant lacking cytochrome *c* (mutant PCT76) (Table 1 and O'Keeffe & Anthony, 1978). In mutant PCT76 the only route for oxidation of NADH and succinate even during carbon-limited growth would be that indicated in Scheme 1*a*).

It is further proposed that when growth is carbon-limited, cytochrome *c* becomes incorporated into the electron-transport chain in such a way that it is now involved in proton translocation and ATP synthesis from all substrates (Scheme 1*b*). This proposed mediation by cytochrome *c* between cytochromes *b* and *a/a*<sub>3</sub> during carbon-limited growth appears to be reflected in the experimental difficulty of removal of

(a) *Electron transport in wild-type bacteria (carbon-excess conditions) and mutant PCT 76 (all conditions)*(b) *Electron transport in wild-type bacteria (carbon-limited conditions)*Scheme 1. *Electron transport and proton translocation in Pseudomonas AM1*

The arrows indicate flow of electrons or protons; they do not necessarily imply a direct reaction between components. The hatched bars indicate the site of action of inhibitors. Although only one cytochrome *b* is represented here, at least two cytochromes *b*, differing in wavelength maxima and midpoint redox potentials, have recently been observed in *Pseudomonas AM1* (Keevil & Anthony, 1979*b*). Our results do not preclude the possibility that cytochrome *b* can reduce cytochrome *c* in wild-type *Pseudomonas AM1* in carbon-excess conditions (broken line in *a*); if this occurs, however, it does not appear to be coupled to proton translocation. It is not known if the methanol dehydrogenase reacts, in carbon-limited bacteria, with the same cytochrome *c* that also mediates between cytochromes *b* and *a/a*<sub>3</sub> (broken line in *b*) or if it reacts with a separate cytochrome *c*. The dotted line indicates the flow of electrons to O<sub>2</sub> from cytochrome *b* that occurs when 0.3 mM-KCN inhibits the usual cytochrome oxidase (cytochrome *a/a*<sub>3</sub>; Widdowson & Anthony, 1975). Abbreviations: Q, ubiquinone; Cyt, cytochrome; TMPD, ascorbate-reduced *NNN'N'*-tetramethylphenylenediamine.

cytochrome *c* from membranes of carbon-limited bacteria (Table 4). It is not known if this cytochrome *c* is the same as that accepting electrons from methanol by way of methanol dehydrogenase. That mutant PCT76 has no cytochrome *c* whatsoever suggests that there is only one type of cytochrome *c* in *Pseudomonas AM1* that is capable of reacting with the cytochrome chain in two ways, both involving its oxidation by cytochrome *a/a*<sub>3</sub>. The possibility remains, however, that mutant PCT76 may be a multiple mutant lacking two different cytochromes *c*.

The interpretation described above has assumed

that whenever the cytochrome *c* is involved in electron transport between cytochromes *b* and *a/a*<sub>3</sub> it is also involved in proton translocation. If this is not the case then all our results are consistent with the less conventional possibility that in wild-type *Pseudomonas AM1* cytochrome *c* always mediates electron transport between cytochromes *b* and *a/a*<sub>3</sub>, but that proton translocation is only coupled to this electron transport in carbon-limited bacteria.

If the electron-transport chain during carbon-limited growth is as depicted in Scheme 1(*b*) then it might be expected that *Pseudomonas AM1* would

have two cytochromes *b* as in other bacteria respiring by way of cytochromes *b*, *c* and *a/a*<sub>3</sub> (Haddock & Jones, 1977) and this is now known to be the case. Only one cytochrome *b* had previously been seen in *Pseudomonas* AM1, but recent observations have demonstrated the presence of two cytochromes *b* differing in wavelength absorption maxima and in midpoint redox potential (Keevil & Anthony, 1979b).

The involvement of cytochrome *c* in NADH oxidation and ATP synthesis during conditions of succinate limitation results in higher yields measured with wild-type bacteria compared with those measured with the mutant lacking cytochrome *c* (Table 2). By contrast, incorporation of cytochrome *c* into the electron-transport chain between cytochromes *b* and *a/a*<sub>3</sub> during conditions of methanol limitation will provide relatively little benefit to the organism in terms of yields on methanol. This is because during growth on methanol relatively little NADH is oxidized (Anthony, 1978b), cell yields on methanol being limited to a large extent by the supply of NAD(P)H rather than by ATP supply (Anthony, 1978a).

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