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The Microbial Metabolism of C_1 Compounds

THE ELECTRON-TRANSPORT CHAIN OF *PSEUDOMONAS* AM1

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Pseudomonas AM1, *Hyphomicrobium* X and *Pseudomonas* MS all contain cytochrome a/a_3 and a *b*-type cytochrome able to react with CO. *Pseudomonas* AM1 and *Hyphomicrobium* X also have a CO-binding cytochrome *c*. The purified cytochrome *c* (redox potential 0.26V) of *Pseudomonas* AM1 was not susceptible to oxidation by molecular oxygen. CO reacted slowly with the reduced form giving a CO difference spectrum with a peak at 412nm and troughs at 420nm and 550nm. Similar results were obtained with the cytochrome *c* of *Hyphomicrobium* (aerobically grown or anaerobically grown with nitrate) and with that of *Pseudomonas extorquens*. The results given in the present paper are incompatible with an oxygenase or oxidase function for the soluble cytochrome *c* of methylotrophs. Studies with whole cells of *Pseudomonas* AM1 and a cytochrome *c*-deficient mutant have demonstrated that cytochrome *b* (redox potential 0.009V) is the first cytochrome in the electron-transport chain for oxidation of all substrates except methanol (and ethanol) whose oxidation does not involve this cytochrome. All substrates are usually oxidized by way of cytochrome *c* and cytochrome oxidase (cytochrome a/a_3), but there is an alternative route for the reduction of cytochrome a/a_3 in the mutant lacking cytochrome *c*. Results of experiments on cyanide inhibition of respiration and cytochrome oxidation support the suggestion that the susceptibility of cytochrome *b* to oxidation by molecular oxygen (reflected in its ability to react with CO) is probably irrelevant to the normal physiology of *Pseudomonas* AM1.

Pseudomonas AM1 is a typical facultative methylotroph (Quayle, 1972; Anthony, 1975*a*) able to grow on a wide range of carbon and energy sources, including C_1 compounds. The properties of its cytochrome *c* have been previously described, and mutant, studies have suggested that this soluble cytochrome is necessary for the oxidation of methanol and ethanol but not absolutely essential for the oxidation of other substrates (Anthony, 1975*b*); this work also indicated that some of the cytochromes *b* and *c*, as well as the cytochrome a_3, was able to react with CO and might therefore have an oxidase function. By contrast Tonge *et al.* (1974) failed to observe any CO-binding cytochrome *b* in *Pseudomonas* AM1 and they have suggested that its cytochrome *c* and the 'cytochrome *o*' previously described in two methane-utilizing bacteria may have an oxidase function in methane and/or methanol oxidation (see also Ferenci, 1974).

The present paper describes a further investigation of the three CO-binding cytochromes of *Pseudomonas* AM1 (*a*, *b* and *c*) and elucidates the role of the cytochrome *b* in the growth of this facultative methylotroph.

Preliminary reports of some of this work have been published (Anthony & Widdowson, 1975; Widdowson & Anthony, 1975).

Experimental

Chemicals

All chemicals were obtained from BDH Ltd., Poole, Dorset, U.K., except the following. Horse heart cytochrome *c* (type VI), prepared without the use of trichloroacetic acid, and ox heart cytochrome *c* (Type V) were obtained from the Sigma Chemical Co., St. Louis, Mo. 63178, U.S.A. Toluidine Blue was obtained from George T. Gurr Ltd., London S.W.6, U.K.

Organisms

Pseudomonas AM1 (N.C.I.B. 9133) and *Pseudomonas extorquens* (N.C.I.B. 9399) were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K. *Hyphomicrobium* X and *Pseudomonas* MS were kindly supplied by Dr. M. M. Attwood, Department of Microbiology, University of Sheffield, Sheffield, U.K.

Mutant organisms

Two mutants of *Pseudomonas* AM1 were used in the present work. Mutant PCT7 has no carotenoid pigments; its growth properties and oxidative

properties are identical with those of the parent organism. Mutant PCT761 is a carotenoid-less derivative of a mutant of *Pseudomonas* AM1 containing no cytochrome *c*. These mutants have been fully described previously (Anthony, 1975*b*).

Growth of bacteria

Unless otherwise stated, methods (including those for growth and harvesting bacteria) were as previously described (Anthony, 1975*b*). *Pseudomonas* AM1, mutant PCT7, and *Hyphomicrobium* X were grown aerobically with methanol as substrate, mutant PCT761 was grown on succinate, and *Pseudomonas* MS on methylamine.

Preparation of bacterial extracts

Washed bacterial suspensions (300mg wet wt./ml) were sonicated for 10min in 2min periods in a 100W MSE ultrasonic disintegrator at 20kHz in an ice bath. Whole cells were removed by centrifugation at 3000*g* for 10min. The resulting crude extract was divided into a membrane fraction and a soluble fraction by centrifuging at 380000*g* for 1.5h at 2°C. The pellet (membrane fraction) was washed three times in 20mM-potassium phosphate buffer, pH7.0 (by resuspension, homogenization and centrifugation) and finally resuspended in the same buffer and homogenized with a Tri-R homogenizer. Protein was determined by the method of Lowry *et al.* (1951); crystalline bovine serum albumin (fraction V) was used as standard.

Estimation of haems and cytochromes

Haem *a* and haem *b* were estimated by the method of Peterson (1970). Cytochromes were estimated from reduced-minus-oxidized difference spectra or from (reduced+CO)-minus-reduced difference spectra. The molar extinction coefficients used were those of mammalian cytochromes a/a_3 (16mM$\cdot$cm^{-1}), a_3 (91mM$\cdot$cm^{-1}), *b* (22mM$\cdot$cm^{-1}), and *c* (19mM$\cdot$cm^{-1}) (Chance, 1957) or of bacterial cytochrome *o* (91mM$\cdot$cm^{-1}) (Chance, 1961) for estimation of cytochrome *b* reacting with CO. The following wavelength pairs were used; cytochrome a/a_3, 605nm minus 635nm; cytochrome *b*, 560nm minus 575nm; cytochrome *c*, 550nm minus 535nm; cytochrome a_3, 430nm minus 442nm; CO-binding cytochrome *b*, 418nm minus 430nm. Cytochrome a_3 was measured after 15s bubbling with CO (2 bubbles/s). Observation of the spectrum in the α region indicated that no further combination of CO with cytochrome a_3 occurred after this time. In the presence of cytochrome *b* absorption at 418nm increased and absorption at 430nm decreased after a further 1min passage of CO followed by 10min incubation in the dark. Subtraction of the 15s spectrum from the spectrum obtained after longer exposure to CO allowed an estimate of the cytochrome *b* reacting with CO to be made. For complete combination of cytochrome *c* with CO, solutions were saturated by bubbling CO for two 1min periods separated by 10min; spectra were recorded after a total of 15min exposure to CO. For an indication of the rate of binding of CO the rate of increase of $\Delta E_{412-420}$ was measured.

Measurement of absorption spectra

All spectra were recorded in a Cary 118C double-beam spectrophotometer (Varian Associates Ltd., Walton-on-Thames, U.K.). Cuvettes were placed as close as possible to the photomultiplier to detect the maximum amount of scattered light. Spectra were recorded at 20–25°C. For recording reduced-minus-oxidized difference spectra, samples were reduced with a small amount of solid sodium dithionite and oxidized with a small amount of solid potassium ferricyanide or with H_2O_2 [0.01ml of a 3% (v/v) solution]. H_2O_2 was used for recording the Soret end (γ) of the absorption spectrum. (Reduced+CO)-minus-reduced difference spectra were recorded after bubbling CO through the dithionite-reduced sample (reduced sample without CO was used in the reference cuvette). Cuvettes were sealed with SubaSeal caps to exclude air during incubations with CO. Before recording difference spectra, base lines were always checked by first recording the spectrum obtained with untreated or reduced preparations in both cuvettes.

Reduction of cytochromes by respiratory substrates in whole cells of Pseudomonas AM1 and its mutants

Whole cells were aerated in 20mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] buffer, pH7.0 until all cytochromes were completely oxidized. They were then transferred to a spectrophotometer cuvette containing substrate, and spectra run once per minute; the reference cuvette contained no added substrate. That endogenous substrate was able to slowly reduce the cytochromes was demonstrated by a similar experiment in which no substrate was added and the cells in the reference cuvette were oxidized by H_2O_2. Spectra produced in such experiments were eventually the same when cells were incubated in cuvettes aerobically or after O_2 was excluded by bubbling N_2 through for 3min before sealing the cuvette and injecting substrate. In anaerobic conditions the cytochromes became reduced more rapidly than in aerobic conditions.

Binding of CO to cytochromes (in whole cells of mutant PCT761 reduced by substrate)

Substrate (added or endogenous) or dithionite was allowed to reduce the cytochromes in previously aerated cells in two spectrophotometer cuvettes. In aerobic conditions this process required at least 15min in the absence of added substrate but less than 1min with added substrate. As soon as complete

cytochrome reduction had occurred CO was bubbled for 1 min through one cuvette and the (reduced+CO)-minus-reduced difference spectrum measured after 3 min.

Inhibition of respiration and cytochrome oxidation by cyanide in whole cells

The effect of cyanide on respiration of whole cells of the mutants PCT7 and PCT761 was measured by using the Rank oxygen electrode as previously described (Anthony, 1975*b*).

To measure the effect of cyanide on the oxidation of cytochromes the spectrum of cells reduced by endogenous substrate was first recorded with aerated cells (all cytochromes oxidized) in the reference cuvette. This spectrum was then compared with that obtained by using cells (in the reference cuvette) in which cytochrome had been reduced by endogenous substrate and which had then been extensively aerated in the presence of various concentrations of KCN (this was added to the anaerobic cells 5 min before submitting them to aeration). The cytochrome observed in this spectrum is that which has become oxidized during the aeration process.

Measurement of redox potentials

These were obtained by measuring the amounts of the oxidized and reduced forms of cytochromes and redox indicator dyes at equilibrium in the presence of various amounts of dithionite. Reactions were in anaerobic spectrophotometer cuvettes under an atmosphere of N_2 in 20mM-potassium phosphate buffer, pH 7.0, at 21°C. Cytochrome *c* was purified from *Pseudomonas* AM1 by the method of Anthony (1975*b*) and the dye used with this cytochrome was 2,6-dichlorophenol-indophenol (E'_0 0.217V). For measurements with cytochrome *b*, washed-membrane preparations of mutant PCT7 were used with Toluidine Blue (E'_0 0.034V) and Methylene Blue (E'_0 0.011V). Experiments were done over a range of cytochrome and dye concentrations and the method was checked with cytochromes of known redox potential. Redox potentials were calculated by the method of Clark (1960).

Results

Distribution of cytochromes in washed-membrane and soluble fractions of Pseudomonas AM1 (wild-type and mutants), Pseudomonas MS and Hyphomicrobium X

The two mutants of *Pseudomonas* AM1 used in this work were described in the Experimental section.

The results in Table 1 show that the concentrations of cytochromes in mutant PCT7 (lacking carotenoid pigments) were not markedly different from those in wild-type *Pseudomonas* AM1. Although the concentrations of all cytochromes in mutant PCT761 were less than in wild-type bacteria, the major difference was the complete lack of cytochrome *c*. Succinate- and β-hydroxybutyrate-grown bacteria had higher concentrations of *a*- and *b*-type cytochromes, and cytochrome *c* was more firmly attached to membranes of these bacteria compared with methanol-grown bacteria.

There were relatively higher concentrations of all cytochromes in *Hyphomicrobium* X than in *Pseudomonas* AM1 and *Pseudomonas* MS, and no cytochrome *c* in *Pseudomonas* MS, in contrast with the other two methylotrophs.

Table 1. *Cytochrome concentrations in washed-membrane and soluble fractions of Pseudomonas AM1, Pseudomonas MS and Hyphomicrobium X*

Extracts were prepared and cytochromes were estimated as described in the Experimental section. The washed pellet was resuspended in a volume of buffer equal to that of the crude extract from which it was derived. The concentration of cytochromes varied from one batch of cells to another, giving a range of values within ±30% of the values given below. This variation may be only a reflection of the difficulty of estimating the contributions of cytochromes *a*, *b* and *c* to the observed peaks in spectra of mixtures. Cytochrome *c* was the only cytochrome present in the soluble fractions; n.d., not detected.

Organism	Growth substrate	Fraction ...	Cytochrome concentration (pmol/mg of protein)					Protein in extracts (mg/ml)	
			Membrane				Soluble	Membrane	Soluble
			a/a_3	*b*	*c*	a_3	*c*		
Pseudomonas AM1	Methanol		97	157	n.d.	30	1390	21.9	10.9
Mutant PCT7	Methanol		132	196	8	53	1200	8.0	11.0
Mutant PCT7	Succinate		215	442	510	190	810	17.4	31.6
Mutant PCT7	β-Hydroxybutyrate		191	231	372	151	840	12.4	41.2
Mutant PCT761	Succinate		85	148	n.d.	74	n.d.	16.0	26.0
Pseudomonas MS	Methylamine		52	179	n.d.	78	81	24.0	16.0
Hyphomicrobium X	Methanol (aerobic)		420	660	590	140	1160	25.0	11.0

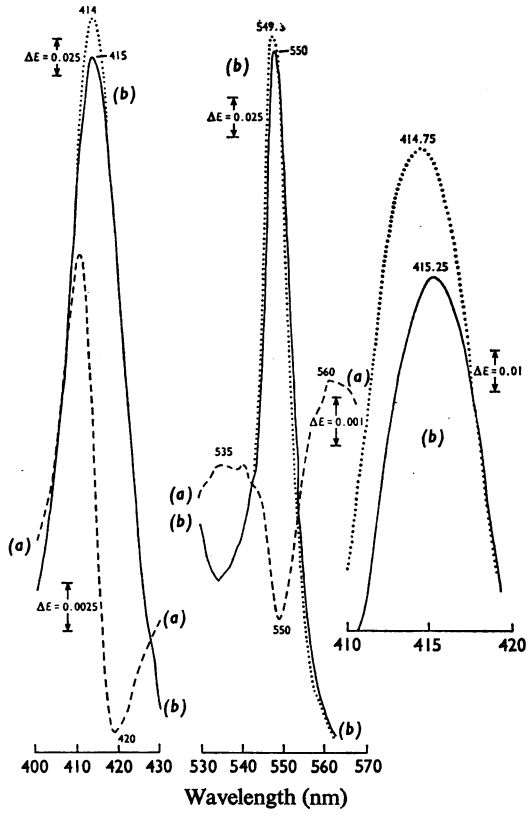


Fig. 1. *Reaction of CO with purified cytochrome c of Pseudomonas AM1*

The absorption spectrum of reduced cytochrome *c* (0.076 mg/ml) was recorded, CO was passed through a sample of the reduced cytochrome for 2 min and the absorption spectrum of the CO-treated sample recorded after 15 min incubation as described in the Experimental section. The difference spectrum was then recorded of the CO-treated sample with the untreated material in the reference cuvette. (*a*) ----, (Reduced+CO)-minus-reduced difference spectrum; (*b*) ——, absorption spectrum of reduced cytochrome *c*; ····, absorption spectrum of the same material after CO treatment.

Reaction of CO with purified cytochrome c of Pseudomonas AM1

No haem *a* or *b* was present in the purified cytochrome *c*.

That the ferrocytochrome *c* of *Pseudomonas* AM1 reacted with CO is shown by the (reduced+CO)-minus-reduced difference spectrum shown in Fig. 1; there was no reaction with ferricytochrome *c*. [This spectrum is very similar to that obtained by using a crude extract prepared from *Pseudomonas extorquens* (Tonge *et al.*, 1974).] The CO difference spectra obtained by using cytochrome *c* purified by either of the two methods previously described (Anthony, 1975*b*) were identical (Fig. 1*a*) and had absorption maxima at 412, 535 and 560 nm and troughs at 420 and 550 nm. The extinction coefficient measured between 412 and 420 nm in this spectrum was 8% of that measured between 415 and 390 nm in the absolute absorption spectrum of the reduced cytochrome *c*. This percentage was the same at all stages of the purification procedure and was similar to that observed in whole cells.

The CO-binding cytochrome *c* is also clearly seen in whole cells of *Pseudomonas* AM1. After complete reaction the (reduced+CO)-minus-reduced difference spectrum had peaks at 412, 535, 565 and 590 nm, with troughs at 442, 550 and 603 nm. [When this spectrum was first recorded (Anthony, 1975*b*) insufficient time of incubation with CO was allowed for development of the 412 nm Soret peak of the spectrum.] The 412 nm peak in CO difference spectra was also observed in those membrane fractions that retained some bound cytochrome *c*. The rate of binding of CO to ferrocytochrome *c* was the same in whole cells grown on methanol, methylamine, succinate, malate or β-hydroxybutyrate, and for cytochrome at all stages of purification. Maximum binding occurred at about 15 min after saturation with CO, 60% occurring at 4 min after initial exposure. Removal of CO from solution (by passing O_2-free N_2 for 5 min) led to complete dissociation of the CO–cytochrome *c* complex.

The position of the Soret (*γ*) band in the absolute absorption spectrum changed by only 1 nm on reaction of purified ferrocytochrome *c* with CO (415 nm to 414 nm) and there was a 6% increase in the extinction coefficient measured between 390 nm and the peak wavelength; the α peak shifted from 550 nm to 549.3 nm (Fig. 1*b*). When the spectra in Fig. 1(*b*) were subtracted, the difference spectrum showed a peak at 412 nm and was the same shape as that shown in Fig. 1(*a*).

Mutant PCT761 (derived from *Pseudomonas* AM1) was previously shown to have no cytochrome *c* (Anthony, 1975*b*) and this is consequently a valuable mutant for the study of CO-binding cytochromes. In this mutant no soluble cytochromes were present and hence no spectrum corresponding to the CO-binding cytochrome *c* occurred in whole cells or extracts (Fig. 3*c*).

Reaction of CO with the soluble cytochrome c of Hyphomicrobium X and Pseudomonas extorquens

By using the soluble fraction from *Hyphomicrobium* X and from *Pseudomonas extorquens* (methanol-grown or succinate-grown) it was shown that binding of CO to the cytochrome *c* of these bacteria was identical with that observed with *Pseudomonas* AM1 and horse heart or bovine cytochromes *c*; reaction with CO shifted Soret maxima in the absolute absorption spectrum from 415 nm to 414 nm, with a 5–15%

increase in extinction coefficient. Identical results were obtained with cytochrome *c* from *Hyphomicrobium* when grown aerobically or when grown anaerobically with nitrate as terminal oxidant. The CO difference spectra of cytochrome *c* were essentially the same in extracts and whole cells of *Hyphomicrobium X*, *Pseudomonas extorquens* and *Pseudomonas* AM1. Similar difference spectra are also observed with commercial preparations of horse heart and bovine cytochromes *c*.

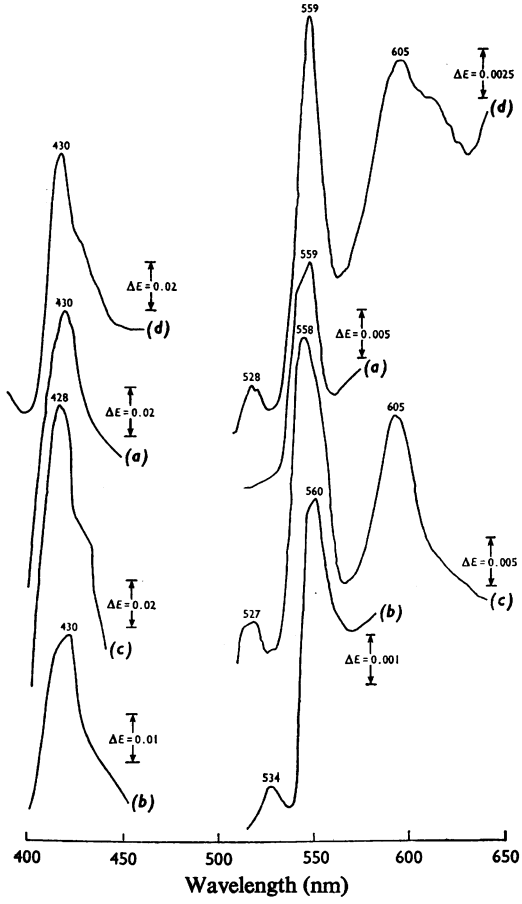


Fig. 2. *Cytochrome b in whole cells and extracts of mutant PCT7 and PCT761*

Extracts were prepared and spectra recorded as described in the Experimental section. (*a*) Mutant PCT7, whole cells (80mg dry wt./ml); reduced-minus-untreated difference spectrum. (*b*) Mutant PCT7, crude extract (38.8mg of protein/ml); reduced-minus-untreated difference spectrum. (*c*) Mutant PCT7, washed-membrane fraction (27mg of protein/ml); reduced-minus-oxidized difference spectrum. (*d*) Mutant PCT761, washed-membrane fraction (25.6mg of protein/ml); reduced-minus-oxidized difference spectrum.

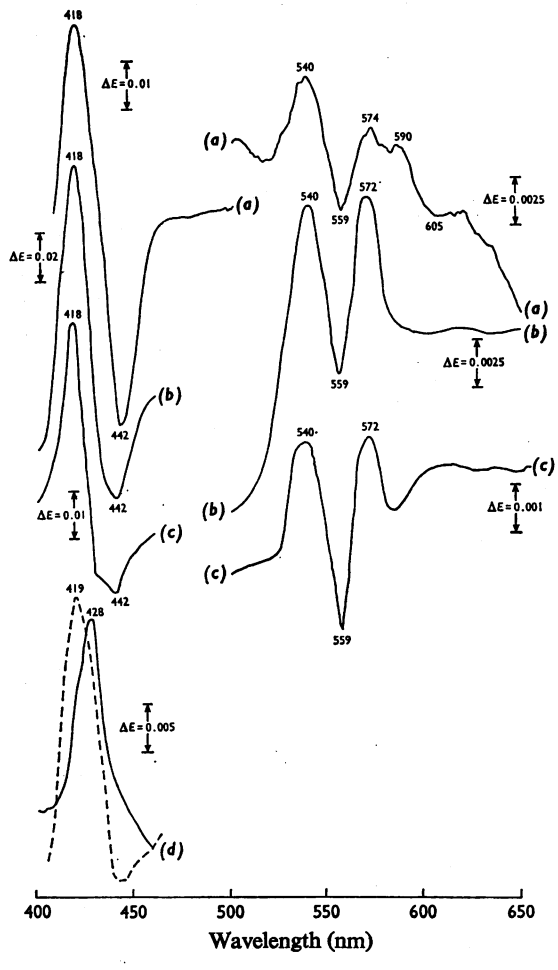


Fig. 3. *Reaction of cytochrome b with CO in membrane fraction of mutant PCT7 and PCT761 and in whole cells of mutant PCT761*

(*a*) Mutant PCT7, washed membrane fraction (27mg of protein/ml); (reduced+CO)-minus-reduced difference spectrum. (*b*) Mutant PCT761, washed-membrane fraction (25.6mg of protein/ml); (reduced+CO)-minus-reduced difference spectrum. (*c*) Mutant PCT761, whole cells (372mg dry wt./ml); (reduced+CO)-minus-reduced difference spectrum. (*d*) Mutant PCT7, washed membrane (27mg of protein/ml); ——, reduced-minus-oxidized difference spectrum; ----, (reduced+CO)-minus-oxidized difference spectrum.

Reaction of CO with cytochrome b in Pseudomonas AM1

Characterization of the cytochrome *b* of methylotrophs has been hindered by the presence of relatively large amounts of cytochrome *c* in most preparations. The binding of CO to the cytochrome *c* (shown above) has likewise hindered demonstration of a *b*-type

cytochrome able to react with CO. Such cytochromes are often designated cytochrome *o*, but, as this tends to automatically imply an oxidase function by analogy with the cytochromes *o* of *Micrococcus pyogenes*, *Acetobacter suboxydans*, *Escherichia coli* and *Azotobacter vinelandii* (Castor & Chance, 1959), we have not used this terminology.

A clear demonstration of the cytochrome *b* and its reaction with CO in *Pseudomonas* AM1 has now been effected by the removal of cytochrome *c* from membrane fractions by thorough washing and by using mutants lacking carotenoid pigments (mutant PCT7) or carotenoids and cytochrome *c* (mutant PCT761). From spectra presented in Fig. 2 the cytochrome *b* is shown to have absorption peaks at 558–560nm and 430nm. The spectra presented in Fig. 3 show that the cytochrome *b* is able to react with CO, the proportion binding being between 50 and 100% (calculated by using the extinction coefficients given in the Experimental section for cytochromes *b* and *o*). Fig. 3(*d*) shows that, on reaction of CO with ferrocytochrome *b*, its characteristic γ peak was shifted from 428nm to 419nm; such a shift is typical of the CO-binding *b*-type cytochromes which may function as cytochrome oxidases and which are often designated cytochrome *o* (Lemberg & Barrett, 1973). The reaction of CO with cytochrome a_3 was extremely rapid, complete binding occurring during a 15s period of exposure to CO. By contrast, passage of CO for 1min was required for 50% of maximum binding to cytochrome *b* (maximum binding occurred during a further 10min incubation). The complexes with CO of both cytochrome a_3 and cytochrome *b* were reversed by bubbling O_2-free N_2 through suspensions containing these cytochromes; 1min was sufficient for complete dissociation of the cytochrome a_3 complex, whereas a 5min passage of N_2 was required for 85% dissociation of the cytochrome *b* complex.

In membrane preparations all of the cytochrome *b* and cytochrome a_3 was present in the oxidized state as would be expected if they function as oxidases. However, because these cytochromes were always present together it is not possible to conclude unequivocally from this that both are able to be directly oxidized by molecular oxygen; it is possible that the cytochrome a_3 oxidizes all other cytochromes bound to membranes (membrane-bound cytochrome *c* is also found in the oxidized state).

Results shown in Fig. 4 show that *Hyphomicrobium* X and *Pseudomonas* MS also have a cytochrome *b* which is able to react with CO and which may therefore possibly function as an oxidase (cytochrome *o*).

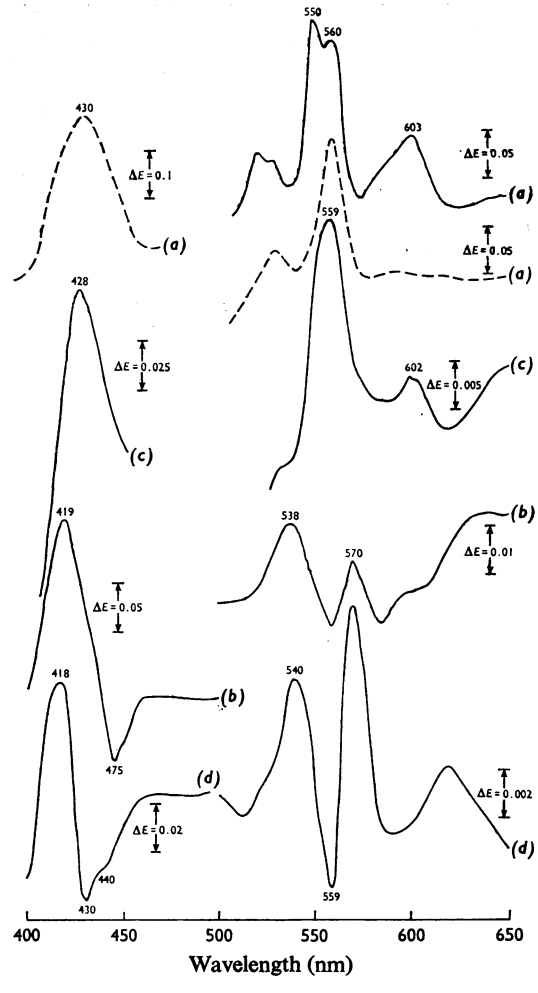


Fig. 4. *Cytochrome b and its reaction with CO in washed membrane fractions of Hyphomicrobium X and Pseudomonas MS*

(*a*) *Hyphomicrobium* X (43.2mg of protein/ml); ——, reduced-minus-oxidized difference spectrum; – – – –, reduced-minus-untreated difference spectrum. (*b*) *Hyphomicrobium* X (43.2mg of protein/ml); (reduced+CO)-minus-reduced difference spectrum. (*c*) *Pseudomonas* MS (24mg of protein/ml); reduced-minus-oxidized difference spectrum. (*d*) *Pseudomonas* MS (24mg of protein/ml); (reduced+CO)-minus-reduced difference spectrum.

Reduction of cytochromes by respiratory substrates in whole cells of Pseudomonas AM1 and its mutants

The respiratory substrates tested were methanol, ethanol, formaldehyde, formate, succinate, malate and β-hydroxybutyrate. All of cytochromes *c* and a/a_3 were completely reduced in mutant PCT7 by all oxidizable substrates (including endogenous substrate). Because of the masking of the cytochrome *b* spectrum by that of cytochrome *c*, its reduction could only be measured in the mutant lacking cytochrome *c*. In this mutant (PCT761) all oxidizable

substrates reduced both cytochromes *b* and a/a_3. Although methanol dehydrogenase is present, methanol and ethanol were not oxidized and they did not reduce cytochrome *b* or cytochrome a/a_3. Although all of the cytochrome a/a_3 was reduced by oxidizable substrates, only 60–70% of the dithionite-reducible cytochrome *b* was reduced by the substrates. There was no spectral evidence for more than one species of cytochrome *b*. All of the cytochrome *b*, whether reduced by dithionite or by substrate, was able to react with CO.

Inhibition of respiration and of oxidation of cytochromes by cyanide in whole cells

The respiration of all substrates (methanol, ethanol, formaldehyde, formate, succinate, malate and β-hydroxybutyrate) by mutant PCT7 was inhibited by approx. 90% by 200 μM-KCN; (50% occurred between 25 and 75 μM). The aerobic oxidation of cytochromes a/a_3 and *c* was markedly inhibited by 200 μM-KCN, whereas oxidation of cytochrome *b* was unaffected (Fig. 5). A higher concentration of KCN (1 mM) still had relatively little effect on the oxidation of cytochrome *b* whereas oxidation of cytochromes *c* and a/a_3 was completely inhibited.

The relative effects of KCN on the oxidation of cytochromes *b* and a/a_3 were more clearly seen in experiments using the cytochrome *c*-deficient mutant PCT761; 300 μM-KCN completely inhibited oxidation of cytochrome a/a_3 but had little effect on cytochrome *b* oxidation, which required a much higher concentration (4 mM) for complete inhibition.

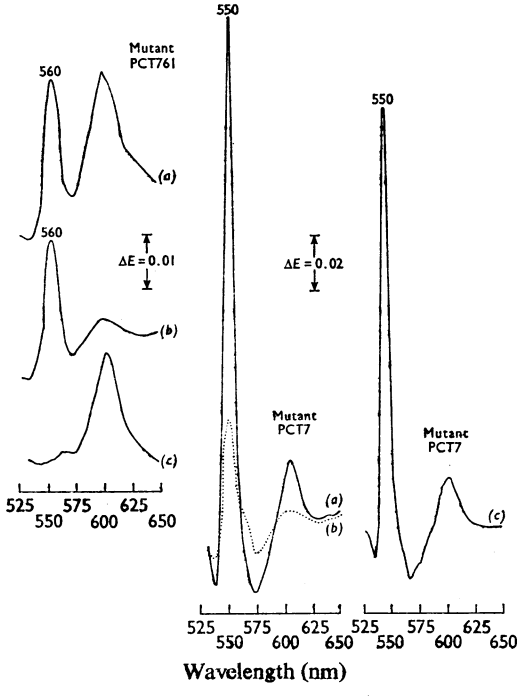


Fig. 5. *Inhibition of cytochrome oxidation by cyanide in whole cells of mutants PCT7 and PCT761*

The effect of cyanide on the oxidation of reduced cytochromes by aeration in whole cell suspensions was determined as described in the Experimental section. (*a*) (Endogenous substrate reduced)-minus-oxidized difference spectrum. (*b*) (Endogenous substrate reduced)-minus-oxidized difference spectrum (oxidation process inhibited with KCN). (*c*) Spectrum (*a*) minus spectrum (*b*) to demonstrate those cytochromes whose oxidation was inhibited by KCN. For mutant PCT7, 200 μM-KCN was used with a suspension of 75 mg dry wt./ml; for mutant PCT761, 300 μM-KCN was used with a suspension of 60 mg dry wt./ml.

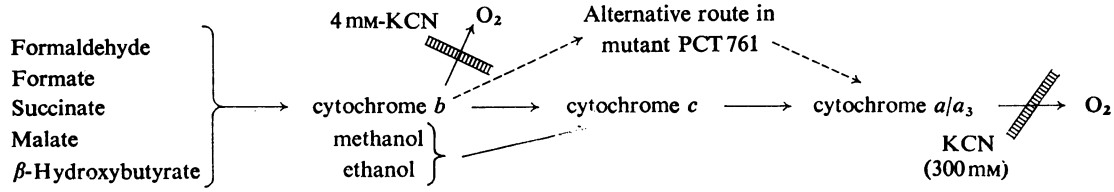
Redox potentials of cytochromes b and c

The redox potential (E'_0) of purified cytochrome *c* was 0.261 V (average of six values between 0.259 and 0.263 V). The redox potential of cytochrome *b* (bound to membrane) was 0.009 V (average of ten values between zero and 0.012 V). This value is typical of cytochromes *b* from other sources and is consistent with our observation that ascorbate+tetramethyl-*p*-phenylenediamine does not reduce cytochrome *b* in whole cells of *Pseudomonas* AM1.

Discussion

The results obtained in the present study demonstrate that cytochromes *a*, *b* and *c* (where present) all combine with CO in the methylotrophic bacteria *Pseudomonas* AM1, *Pseudomonas* MS and *Hyphomicrobium* X. Short periods of exposure to CO were suitable for demonstration of reaction with cytochromes *b* and a_3, but longer periods were required for maximum combination of cytochrome *c* with CO. Further, when present in relatively large amounts, the CO-binding cytochrome *c* tended to obscure absorbance peaks characteristic of cytochrome *b*. These characteristics suggest why Davey & Mitton (1973) failed to observe the CO-binding cytochrome *c* in *Methylosinus* and *Methylomonas*, which was later reported by Tonge *et al.* (1974), and why these latter workers failed to observe reaction of cytochrome *b* with CO in *Pseudomonas* AM1 and *Hyphomicrobium* X.

The simplest interpretation of the CO difference spectra recorded for cytochrome *c* in the present study is that the cytochrome *c* reacts slowly with CO to form a complex with a high dissociation constant and an absorption maximum at 412 nm. That the CO was reacting with small amounts of irreversibly denatured cytochrome *c* is unlikely because the extent of binding was constant in whole cells and in preparations at all stages of purification.



Scheme 1. *Proposed electron-transport chain in Pseudomonas AM1*

Cytochromes *c* able to react with CO are not uncommon (Lemberg & Barrett, 1973). However, even when these cytochromes are autoxidizable there are very few known to function by activation of O_2, i.e. as an oxygenase or cytochrome oxidase. The soluble ferrocytochrome *c* found in methylotrophs combines slowly with CO; it is not oxidized by molecular oxygen, and is present both in bacteria grown on substrates other than C_1 compounds and in bacteria grown with nitrate in the absence of oxygen. These observations do not support the speculation (Tonge *et al.*, 1974; Ferenci, 1974) that the soluble cytochromes *c* of *Pseudomonas* AM1 and of other methylotrophs have an oxidase or oxygenase function in methanol (or in methane) oxidation.

The results of the present study suggest that electron transport in *Pseudomonas* AM1 is as shown in Scheme 1.

In this scheme the oxidase involved in normal oxidation of all substrates is cytochrome a_3, and cytochrome *c* (redox potential 0.26V) is the usual mediator between cytochrome *b* (redox potential 0.009V) and the oxidase in wild-type bacteria. That methanol does not reduce the cytochrome *b*, and only reduces cytochrome a/a_3 when cytochrome *c* is present, is probably because the redox potential of the methanol dehydrogenase is too high for reaction with the oxidized cytochrome *b*. In the mutant lacking cytochrome *c* (mutant PCT761) there must be an alternative route for reduction of cytochrome a/a_3 by cytochrome *b* (as indicated in Scheme 1) or there must be a route for the reduction of cytochrome a/a_3 by all substrates (except methanol and ethanol) independent of cytochromes *b* and *c*.

That the CO-binding cytochrome *b* of *Pseudomonas* AM1 is not an oxidase (cytochrome *o*) is suggested by the demonstration that its redox potential (0.009V) is much lower than that of typical cytochrome oxidases. Further, cytochrome *b* oxidation was not inhibited by 300 μM-KCN, which inhibited (90–95%) the oxidation of all substrates and also the oxidation of cytochrome *c* and a/a_3. The extent of inhibition by KCN (at various concentrations) of respiration in whole cells was correlated with the inhibition of oxidation of cytochromes a/a_3 and *c* (where present) but not of the oxidation of cytochrome *b*. These results suggest that cytochrome *b* does not function as an oxidase and that the autoxidizability of cytochrome (reflected in its ability to react with CO) is probably irrelevant to the normal physiology of *Pseudomonas* AM1.

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References

Anthony, C. (1975*a*) *Sci. Prog. (Oxford)* **62**, 167–206
Anthony, C. (1975*b*) *Biochem. J.* **146**, 289–298
Anthony, C. & Widdowson, D. (1975) *Proc. Soc. Gen. Microbiol.* **2**, 51–52
Castor, L. N. & Chance, B. (1959) *J. Biol. Chem.* **234**, 1587–1592
Chance, B. (1957) *Methods Enzymol.* **4**, 273–329
Chance, B. (1961) in *Hematin Enzymes* (Falk, J. E., Lemberg, R. & Morton, R. K., eds.), p. 434, Pergamon Press, Oxford
Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Systems*, Bailliere, Tindall and Cox, London
Davey, J. F. & Mitton, J. R. (1973) *FEBS Lett.* **37**, 335–337
Ferenci, T. (1974) *FEBS Lett.* **41**, 94–98
Lemberg, R. & Barrett, J. (1973) *Cytochromes*, Academic Press, London and New York
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Peterson, J. A. (1970) *J. Bacteriol.* **103**, 714–721
Quayle, J. R. (1972) *Adv. Microb. Physiol.* **7**, 119–203
Tonge, G. M., Knowles, C. J., Harrison, D. E. F. & Higgins, I. J. (1974) *FEBS Lett.* **44**, 106–110
Widdowson, D. & Anthony, C. (1975) *Proc. Soc. Gen. Microbiol.* **2**, 52