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Effect of Oxygen Limitation on the Formation of the Electron Transport System of the Phytopathogenic Fluorescent Bacterium *Pseudomonas cichorii*

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Abstract

The composition of the membrane-bound electron transport system of the phytopathogenic bacterium *Pseudomonas cichorii* underwent modification in response to oxygen supply. Growth adaptation to low oxygen concentrations was characterized by repression of cytochromes involved in ubiquinol-cyt. *c* oxidoreductase and cyt. *c* oxidase activities. By contrast, cyto. *o*, i.e., the alternative cyanide-insensitive oxidase of *P. cichorii*, was unaffected by low oxygen tension. No *a*-type cytochromes could be detected at any stage of growth.

Key Words: *Pseudomonas cichorii*; respiratory chain; cytochrome *o*; control of cytochrome formation.

Introduction

Pseudomonas cichorii is a Gram-negative, obligately aerobic fluorescent bacterium isolated from *Chicorium intybus* and *C. endivia* plants, for which it is pathogenic. Recent studies on the membrane-bound cytochromes in cells of *P. cichorii* grown under high oxygen tension have shown that this phytopathogenic species contains a branched respiratory chain leading to two high-potential cytochrome oxidases of *b* type (Zannoni, 1982). Two protohemes with *Em*, 7.0 of +380 and +250 mV (cyt. *b* 380 and cyt. *b* 250) were suggested to be components of the cytochrome *c* and "alternative" oxidases, respectively. Since the alternative oxidase-containing pathway is largely insensitive to KCN (K_i = 0.2 mM) but CO-sensitive, cyt. *b* 250 should correspond to the spectrally detectable cyt. *o* (cyt. *o* is a CO-binding *b* type

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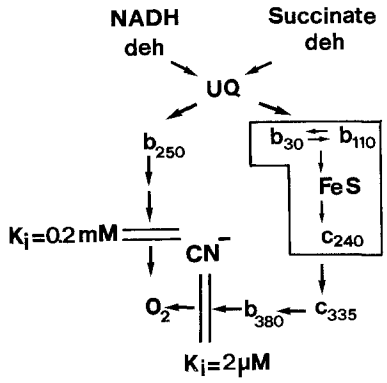

Fig. 1. A schematic model of the electron transport pathways of the phytopathogenous *Pseudomonas cichorii*. The redox interactions occurring within the enclosed box in the diagram await further clarification. Cytochromes are indicated with their midpoint potentials at pH 7.0. Abbreviations: CN^-, cyanide; deh, dehydrogenase. (Modified from Zannoni, 1982; Cocchi and Zannoni, 1985.)

cytochrome). Conversely, cyt. *b* 380 would be the cytochrome *b* component of the cytochrome *c* oxidase which is affected by low KCN concentrations (K_i = 2 μM) and linked to energy transduction through proton translocation (Zannoni, 1984). In addition to these two oxidases, the membrane-bound electron transport system of *P. cichorii* is composed of other cytochromes of *b* (*Em*, 7.0 of +110 and +30 mV) and *c* type (*Em*, 7.0 of +335 and +240 mV), along with several ferredoxin-like centres involved in either dehydrogenase or ubiquinol-cyt. *c* oxidoreductase activities (Zannoni, 1982; Zannoni and Ingledew, 1984). Figure 1 shows a schematic diagram of the branched respiratory chain of *P. cichorii*.

The present work reports on the effects of low oxygen tension on the composition and function of *P. cichorii* respiratory chain. Indeed, it is generally accepted that under oxygen limiting conditions of growth, obligate aerobes and facultative anaerobes accommodate the major part of the respiratory electron flow through an "alternative KCN-insensitive" oxidase (Jones, 1977). A peculiarity of this growth adaptation to low oxygen concentrations is that both a generalized increase of cytochrome concentration and the synthesis of cytochrome *d* (formerly classified as cyt. *a*) have been observed (Jones, 1977).

The present study suggests that the membrane-bound cytochrome complement of *P. cichorii* is drastically repressed by low oxygen tension growth mode. This variation seems to be related prevalently with those redox components functionally involved in the cyt.(s) *c*-containing pathway. Furthermore, in contrast to previous observations in saprophytic species, e.g., *Pseudomonas putida* (Sweet and Peterson, 1978), no significant peaks

were seen in the differential spectra of cytochromes in the 600–650 nm region. This excludes the synthesis of *a* type oxidases in response to restricted oxygen availability.

Materials and Methods

Pseudomonas cichorii (NCPPB 907) was grown in a medium of the following composition: D-glucose, 20 g; yeast extract DIFCO, 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.6 g; K_2HPO_4, 1 g; and adjusted to pH 7.2 in 1 liter of distilled water. Highly aerated cultures were obtained in a Microferm Fermentor (New Brunswick Sci. Co.) at 30°C and then stirred at 250 rpm under a stream of 4 liters/min of humidified air. Less aerated cells were obtained in Feunbach bottles occasionally stirred for 1 min to prevent cell clumping. Respiratory particles were prepared by differential centrifugation after cell disruption by French-pressure cell, as previously described (Zannoni, 1982).

Oxygen concentration during the growth of the culture was determined polarographically through anaerobic transfer of 2 ml of the culture into a Clark oxygen electrode reaction chamber. In the case of less aerated cells, the 3-liter Feunbach flasks contained 500 ml of the culture (1 cm thick with a diffusion surface of approximately 500 cm^2). In the bottle the sample was removed 0.5 cm under the surface of the culture. Cell endogenous respiration was blocked by addition of 0.1 ml anaerobic trichloroacetic acid (30% w/v).

Difference spectra were obtained with a Jasco Mod. UV-VIS spectrophotometer at 25°C. Reduced plus CO minus reduced spectra were obtained by bubbling a stream of CO for 1 min through the test cuvette. Estimations of the amounts of cytochromes present were made by using the following extinction coefficients and wavelength pairs: cyt. *c*, $\varepsilon = 19\,mM^{-1}\,cm^{-1}$, at 552–540 nm; cyt. *b*, $\varepsilon = 22\,mM^{-1}\,cm^{-1}$, at 560–575 nm (Chance, 1957); cyt. *o*, $\varepsilon = 170\,mM^{-1}\,cm^{-1}$, at 417–432 nm (Daniel, 1970).

The determinations of the oxidation–reduction potentials of cytochromes were performed at pH 7.0 in a medium containing 50 mM KCl and 50 mM 2(*n*-morpholino)ethanesulfonic acid (MES) using a dual-wavelength spectrophotometer (SIGMA-ZWSII) according to the technique introduced by Dutton *et al.* (1970) as previously described (Zannoni, 1982). E_o' values were assigned on the basis of a computer-assisted analysis, as in Dutton and Jackson (1972).

Oxidations of NADH, succinate, and ascorbate-*N*,*N*,*N*′*N*′-tetramethyl-*p*-phenylenediamine (TMPD) were measured polarographically. Horse heart cytochrome *c* and dichlorophenol indophenol (DCIP) reductions were

followed spectrophotometrically in the presence of 5 mM KCN. Proteins were measured by using the method of Lowry *et al.* (1951).

Results

Figure 2 shows the growth curves of *P. cichorii* with different oxygen provisions. Traces A and B indicate the cultures grown with high aeration and low aeration, respectively (details under Materials and Methods). Points 1 and 2 on the curves indicate examples of harvest times: when cells were harvested at point 1, hereafter termed stationary highly aerated cells (SHA cells), they were in stationary growth; cells harvested at point 2 are termed stationary less aerated cells (SLA cells). In Fig. 2, the oxygen concentration of the medium on a parallel time scale with growth of the cells is also shown (traces C and D). It is apparent that the time at which the oxygen concentration reached the lowest value corresponded well with the end of logarithmic growth only for highly aerated cells (doubling time of approximately 120 min). At this point, which corresponds to the harvest time for SHA cells, a slight increase of the oxygen concentration was observed. In contrast, less aerated cells, after a lag period of 2–4 h, started growing at

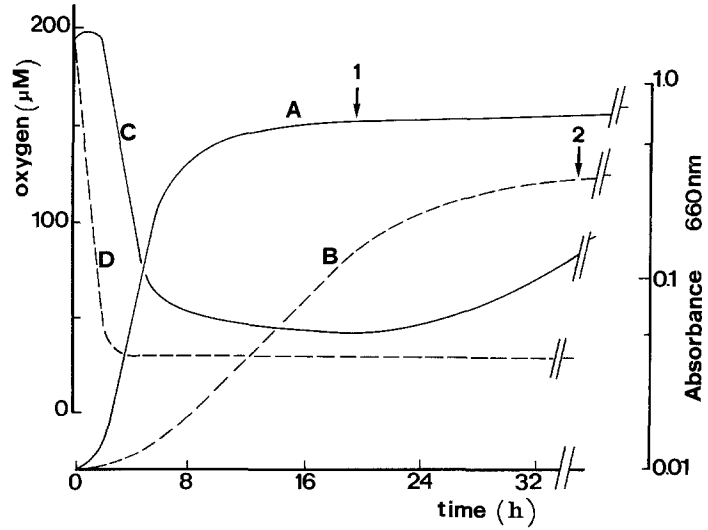

Fig. 2. Growth curves for *P. cichorii* under different aeration conditions. Curves A and B are related to high-aerated and low-aerated cells, respectively (for details see Materials and Methods). Points 1 and 2 on the curves indicate examples of harvest times discussed in the text. Curves C and D show the oxygen concentration of the medium corresponding to growth curves A and B, respectively, on a parallel time scale.

Table I. Respiratory Activities[a] by Plasma Membranes from SHA and SLA Cells of *Pseudomonas cichorii*

Electron donor	Electron acceptor	Membranes	
		SHA	SLA
NADH	Oxygen	28.0	20.0
NADH	Cyt. *c*[b]	4.5	1.0
NADH	DCIP[b]	7.5	10.5
Succinate	Oxygen	4.5	3.0
Succinate	Cyt. *c*[b]	2.8	0.8
Succinate	DCIP[b]	1.0	1.2
Ascorbate/TMPD	Oxygen	65.0	5.5

[a] Expressed as μmol of electron acceptor per hour per mg protein.
[b] Measured in the presence of 5 mM cyanide. Nonstandard abbreviations: TMPD, *N,N,N'N'*-tetramethyl-*p*-phenylenediamine; Cyt. *c*, horse heart cytochrome *c*; DCIP, dichlorophenol indophenol. Additions: NADH, 2 mM; succinate, 5 mM; ascorbate, 5 mM; TMPD, 250 μM; Cyt. *c*, 50 μM; DCIP, 250 μM. Experimental details under Materials and Methods.

a roughly constant oxygen concentration of 20–30 μM (doubling time of approximately 450 min).

Table I presents some respiratory activities of membranes from highly aerated and less aerated cell cultures of *P. cichorii*. From this it appears that cytochrome *c*-oxidase and cytochrome *c*-reductase activities are 4–10 times higher in membranes from SHA than SLA cells. Conversely, NADH- and succinate-oxidases along with NADH- and succinate-dichlorophenol indophenol (DCIP) reductases show similar values in both SHA and SLA membranes. These results indicate that the cyt. *c*-containing pathway in SLA membranes is not responsible for the majority of respiratory activity. Electron flow by SLA membranes would be channelled through the alternative oxidase (see scheme of Fig. 1).

The difference spectra of cytochromes in membranes from SHA and SLA cells are shown in Fig. 3 (traces a, b, and c). It is apparent that the cytochrome content (on a molar/protein basis) is strongly repressed in SLA compared to SHA membranes with *b*/*c* ratios of 1.32 and 0.64, respectively. The carbon monoxide difference spectrum obtained with SLA membranes (trace c maxima at 570, 535, and 417 nm and minima at 557 and 430 nm) indicates that a typical CO-binding activity due to cyt. *o* is present in this type of membranes. Using a millimolar extinction coefficient of 170 (417 versus 432 nm) as recommended by Daniel (1970), it follows that cyt. *o* concentration in SLA membranes (0.1 nmol/mg protein) is similar to that previously found in log-phase aerated cells of *P. cichorii* (0.13 nmol/mg protein) (Zannoni, 1982). A potentiometric titration of cytochromes was therefore carried out (at 561–575 nm) to provide additional evidence for the presence of a normal amount of cyt. *o* (*Em*, 7.0 = +250 mV) in SLA membranes.

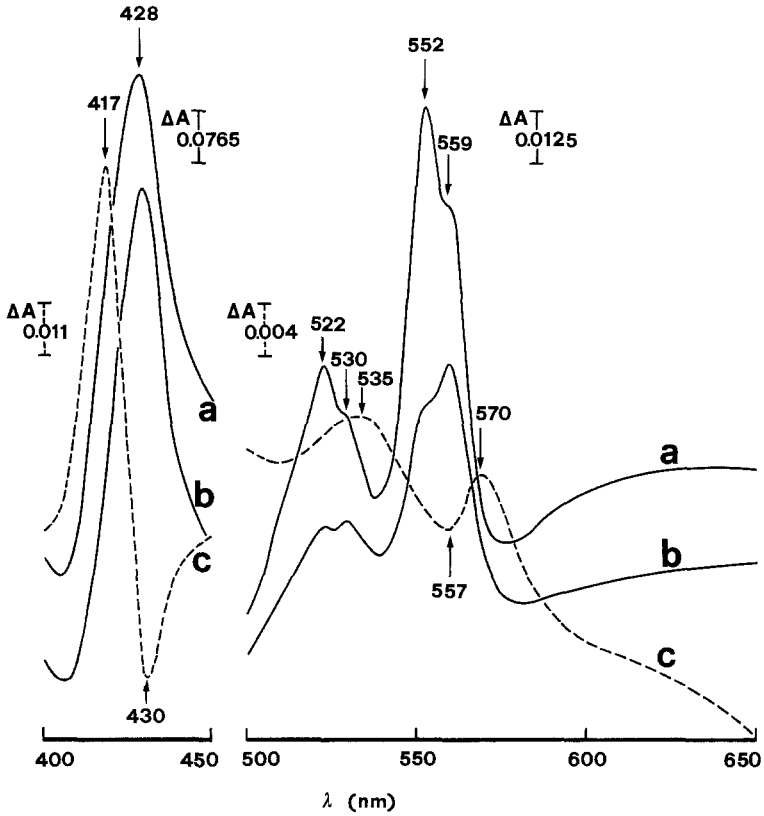

Fig. 3. Room-temperature optical difference spectra of membranes from high- and low-aerated cells of *P. cichorii*. (a, b) Difference spectra (reduced with dithionite minus oxidized with ferricyanide) of SHA and SLA membranes, respectively. Membranes were suspended in 50 mM 2(*n*-morpholino)ethanesulfonic acid (MES) buffer, pH 7.0, at a protein concentration of 4.5 and 9 mg/ml for SHA and SLA cells, respectively. (c) The spectrum (reduced with dithionite plus CO *minus* reduced with dithionite) of SLA membranes at a protein concentration of 9 mg/ml. Experimental details under Materials and Methods.

The results of such a titration (Fig. 4) shows that there are at least four components of *b* type present, with $n = 1$ and *Em*, 7.0 of $+380 \mp 5$, $+265 \mp 5$, $+155 \mp 5$, and $+55 \mp 7$ mV. Their relative contributions to the total absorbance signal at 561–575 nm were approximately 10, 45, 20, and 20% for cyt.(s) *b* 380, *b* 265, *b* 155, and *b* 55, respectively (see also Table II). In this respect, the *b*-type complement of SLA membranes is similar to that described in log-phase membranes (Zannoni, 1982). However, in membranes from the latter type of cells, the *b*-type hemes are present in a ratio close to unity, whereas the above reported data with SLA membranes suggest a ratio of approximately 0.2 : 1 : 0.4 : 0.4, due to a decrease of the *b* type species

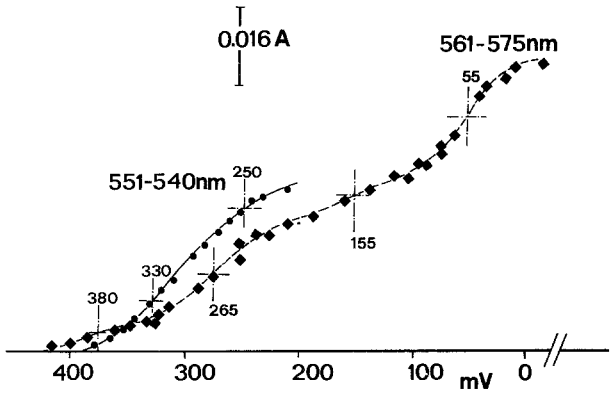

Fig. 4. Dark-equilibrium potentiometric titrations of membrane fragments from SHA and SLA cells of *P. cichorii*. Redox titrations at 561–575 nm (◆) and 551–540 nm (●) are shown. Midpoint potentials (pH 7.0) from best-fit procedures are indicated. Membranes were suspended in 50 mM MES buffer plus 50 mM KCl (pH 7.0) at a protein concentration of 7.5 mg/ml.

except cyt. *b* 265, i.e., cyt. *o*. Conversely, apart from a generalized decrease of the *c*-type hemes (on a molar/protein basis), the relative ratio (1 : 1) of the two cytochromes *c* (*Em*, 7.0 of $+330 \mp 7$ and $+250 \mp 5$ mV) present in SLA membranes was analogous to that previously found in log-phase cells (Zannoni, 1982).

The finding by redox potentiometry that cytochromes of *b* and *c* type, except cyt. *o*, were drastically repressed in SLA membranes, was of special interest because in other facultative and/or obligate aerobes, restriction of the oxygen availability is normally linked to both the synthesis of *a*-type oxidases and increase of the cytochrome content (Jones, 1977).

It is noteworthy that according to previous oxygen affinity determinations of oxidases of *b* type (see Poole, 1983), K_m values for oxygen of the two cytochrome oxidases in both SHA and SLA membranes were of 8–10 μM

Table II. Cytochrome Composition of Respiratory Membranes[a] from SLA Cells of *Pseudomonas cichorii*

Cytochromes type	*Em*, 7.0 (mV)	Concentration (nmol mg^{-1} protein)	Percent of total absorbance signal
b	380	0.025	10
b	265	0.112	45
b	155	0.05	20
b	55	0.05	20
c	330	0.10	50
c	250	0.10	50

[a]Respiratory membranes were prepared as in Materials and Methods.

(not shown). Since it has been shown in Fig. 2 that the dissolved oxygen concentration of the medium in SLA cultures was 20–30 μM, it is possible to conclude that the large changes in cytochromes *c* and *b* seen in less aerated cells of *P. cichorii* is not correlated with the K_m for oxygen of the two respiratory oxidases.

As recently shown, the ubiquinone analog 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) and the antimalarial drug mefloquine drastically inhibit the alternative cyanide-insensitive pathway of *P. cichorii* with a second low-affinity inhibitory site at the *b*/*c* segment of the chain (Cocchi and Zannoni, 1985; Zannoni, 1985). These inhibitors, when used in combination with specific inhibitors of the b/c_1 complex such as antimycin A and myxothiazol (von Jagow and Engel, 1981), have been shown to discriminate between the cytochrome species involved in electron transport of *P. cichorii* (Cocchi and Zannoni, 1985; Zannoni, 1985). In this connection, it is interesting to report that further studies on the patterns of cyt. *b*(s) reduction in membranes from SLA cells have shown that a large fraction (80%) of the substrate-reducible *b*-type complement was insensitive to antimycin A and/or myxothiazol but sensitive to UHDBT and/or mefloquine (not shown). As summarized in Table II, the amount of the *b*-type component of the alternative oxidase, i.e., cyt. *b* 265, in SLA membranes represents 45% of the total protoheme. From this, it is possible to conclude that, apart from cyt. *b* 265, the *b*-type hemes which belong to the *b*/*c* segment of the chain in SLA membranes are inaccessible to reducing equivalents.

Discussion and Conclusions

Previous reports indicated that the electron transport system of *P. cichorii* grown under high oxygen tension is organized as a branched chain containing several cytochromes of *b* and *c* type, plus multiple ferredoxin-like centers (Zannoni, 1982; Zannoni and Ingledew, 1984; Cocchi and Zannoni, 1985). This study shows that, as the supply of oxygen to cells of *P. cichorii* becomes limiting, they begin repressing the content of cytochromes of *b* and *c* type except that of cytochrome *o*. In this respect, *P. cichorii* is quite different from *Pseudomonas putida* in which there is spectral evidence that cytochrome *d* is synthesized and that the total cytochrome amounts relative to protein concentration increased up to severalfold in response to low oxygen concentrations (Sweet and Peterson, 1978). The direct cause of the appearance of cytochrome *d* in many aerobes has been explained in different ways, namely: (i) the synthesis of a new electron transport pathway to oxygen, presumably performing a more efficient oxygen utilization; (ii) a limited

supply of energy to the cell as a general condition triggering the cyt. *d* induction mechanism (Sweet and Peterson, 1978).

At present, the control of cytochrome synthesis by oxygen in *P. cichorii* is unclear. Indeed, a phenotypic control of the respiratory chain components due to the K_m for oxygen of the cytochrome *c* oxidase is unlikely since both respiratory branches of *P. cichorii* have shown quite similar K_m values, severalfold lower than the apparent oxygen concentration under low oxygen conditions of growth. On the other hand, the solubility of oxygen in a salt medium is so low (2.5×10^{-4} M at 25°C) that the lack of stirring in SLA cultures, which greatly decreases the possible diffusion surface through the cell material, must restrict intracellular oxygen provision. This latter consideration, although supporting the hypothesis of the cytochrome synthesis inhibition due to low energy supply, still does not explain differences in concentration of the cytochrome species.

Previous data on electron-transport components of phytopathogenic pseudomonads have shown that the respiratory chain of *P. cichorii* is quite similar to that found in some species of the Rhodospirillaceae family (Zannoni, 1982; Zannoni and Baccarini-Melandri, 1980). In addition, recent comparative analysis of genetic sequence revealed that the genera Pseudomonas and Rhodopseudomonas are closely related (Stackenbrandt and Woese, 1981). In cultures of *R. capsulata* heterotrophically grown with an excess of reducing power, a shift from light-anaerobic to light-aerobic conditions of growth is linked to a bypass of the energy conservation at site I of the redox chain, i.e., NADH-dehydrogenase (Zannoni *et al.*, 1978). This change is achieved by channelling electron flow through the alternative oxidase. By analogy with *R. capsulata* it may be suggested that reducing power dissipation by less aerated cells of *P. cichorii* would be the induction mechanism promoting the restriction of the redox branch leading to the cyt. *c* oxidase with a parallel activation of electron flow through the cyt. *o* containing pathway.

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