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THE RESPIRATORY SYSTEM OF THE MARINE BACTERIUM *BENECKEA NATRIEGENS*

I. CYTOCHROME COMPOSITION

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SUMMARY

1. The cytochrome composition of *Beneckea natriegens* grown under aerobic conditions has been examined.

2. Cell-free extracts obtained by sonication were separated into particulate and supernatant fractions by centrifugation at 150 000 × *g*.

3. The particulate fraction contained cytochromes b_{562}, b_{557}, b or c_{554}, $c_{549.5}$, c_{547} and low concentrations of cytochromes a_1 and a_2. (Subscripts refer to the wavelength optima of the *b* and *c* type cytochrome α-peaks in low temperature (77 °K) difference spectra.) Also present was a second cytochrome $c_{549.5}$ which is capable of binding carbon monoxide (cytochrome $c_{549.5(CO)}$) and which is also found in the supernatant fraction.

4. Reduced plus CO minus reduced difference spectra had spectral peaks corresponding to cytochrome *o* and two *c* type cytochromes, and low concentrations of cytochromes a_1 and a_2.

5. Action spectra for the relief of CO inhibition showed that cytochrome a_2, the CO binding *c* type cytochrome(s) and possibly cytochrome *o*, but not cytochrome a_1, had oxidase activity in intact cells. In cells grown to the late stationary phase, when cytochrome a_2 and particularly cytochrome a_1 were induced, the primary functual oxidase was cytochrome a_1.

INTRODUCTION

The genus of facultatively anaerobic, Gram-negative, marine bacteria *Beneckea* has recently been characterised [1]. These interesting organisms show extreme metabolic versatility combined with an ability to grow uniquely rapidly [2]. In view of the paucity of knowledge concerning their biochemistry, we have decided to investigate the respiratory system of *Beneckea natriegens* (previously classified as *Pseudomonas natriegens* [3]). This work was further prompted by the finding that under certain

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride.

growth conditions (Knowles, C.J., unpublished observations) an exceptionally large ATP pool forms, which should be of great use for studies on oxidative phosphorylation of intact bacteria [4–6].

This paper reports the presence of a complex respiratory system in *B. natriegens* which has a large number of cytochrome components able to combine with carbon monoxide and potentially capable of acting as terminal oxidases.

MATERIALS AND METHODS

B. natriegens strain 111 was grown at 35 °C with high aeration on the DL-lactate, minimal salts medium containing 0.4 M NaCl exactly as described previously [7], with harvesting in the stationary phase (15 h). Cells were disrupted by sonication and the cell free extract, after low-speed centrifugation to remove the cell debris, was fractionated into particulate and supernatant fractions by centrifugation at 150 000 $\times g$ for 90 min. The particulate fraction was washed once in 10 mM $MgCl_2$–50 mM Tris–HCl (pH 7.5) before resuspension in the same buffer.

Cytochrome spectra were measured at room temperature or at liquid N_2 temperature (77 °K) in a Hitachi-Perkin Elmer 356 spectrophotometer. Oxidised minus reduced difference spectra were obtained by reducing one cuvette with a few grains of $Na_2S_2O_4$, 2.5 mM NADH, 30 mM succinate, 30 mM D(−)-lactate, 30 mM L(+)-lactate or 1.5 mM ascorbate plus 4 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) and oxidising the other cuvette by vigorous aeration. Addition of $K_3Fe(CN)_6$ to the cuvette containing the oxidised component resulted in no increase in the peak heights and it was therefore assumed that all the cytochromes were totally oxidised by aeration. Reduced plus CO minus reduced difference spectra were obtained by reducing the cuvettes as described above, and bubbling CO for 30 s through one cuvette. CO combines only slowly with the cytochromes [7]; the spectra were therefore recorded after 10 min incubation with CO, to allow time for the peaks to develop. Difference spectra at 77 °K were measured in cuvettes of 2 mm path length using the single freeze technique [16] and in the presence of 1.0 M sucrose. Room temperature spectra were measured in cuvettes of 1 cm path length. In all cases the slit width was 1 nm. All spectra are compensated to give a linear base line.

CO action spectra were measured at the Johnson Research Foundation, University of Pennsylvania, in the apparatus described by Castor and Chance [8, 9]. Pyridine haemochromes of acid–acetone extracts and residues were measured as described previously [7].

Protein was assayed by a modified Biuret method [10] using bovine serum albumin as standard.

Carbon monoxide was obtained from British Oxygen Company. NADH, succinate, D(−)-lactate, L(+)-lactate and TMPD were obtained from Sigma Chemical Company. All other reagents were the finest grade available and distilled water was used throughout.

RESULTS

Room temperature $Na_2S_2O_4$ reduced minus oxidised difference spectra of the cell free extract and particulate fraction of *B. natriegens* grown to the stationary phase

(16 h growth) show the presence of *c* type (peaks at 552 and 523 nm) and *b* type cytochromes (shoulder at 560 nm, peak at 529 nm). Peaks at 615–628 and 588–595 nm, which are usually hardly distinguishable and sometimes not resolved, indicate the presence of very low concentrations of cytochromes a_2 and a_1 respectively. The presence of cytochrome a_1 is confirmed by a shoulder at 432–436 nm on the Soret peak at 426 nm. The supernatant fraction contains only *c* type cytochrome (α-peak at 552 nm); the properties of this high potential, CO-binding cytochrome have been described previously [7].

Difference spectra of the acid–acetone extract and residue of the particulate fraction have α-peaks at 557 nm (protohaem) and 551 nm (covalently linked protohaem) respectively (not shown). Peaks at 580–590 nm and 610 nm in the extract due to haem *a* and haem *d* respectively were barely visible. The relative peak heights at 557 nm in the extract and at 551 nm in the residue indicate that the total cytochrome *b* is about 70 % of the total cytochrome *c* concentration in the particulate fraction.

Fig. 1 shows reduced minus oxidised difference spectra of the particulate fraction measured at 77 °K. Chemical reduction (by $Na_2S_2O_4$) gave α- and β-peaks similar to those found in the room temperature spectra but shifted 1–3 nm towards the ultraviolet region. NADH reduced 80–90 % of the *c* type (549.5 nm) and *b*-type (556.5 nm) cytochromes, whilst succinate, D(−)-lactate or L(+)-lactate caused only an apparent 50–60 % reduction of these cytochromes. D(−)- and L(+)-lactate are oxidised

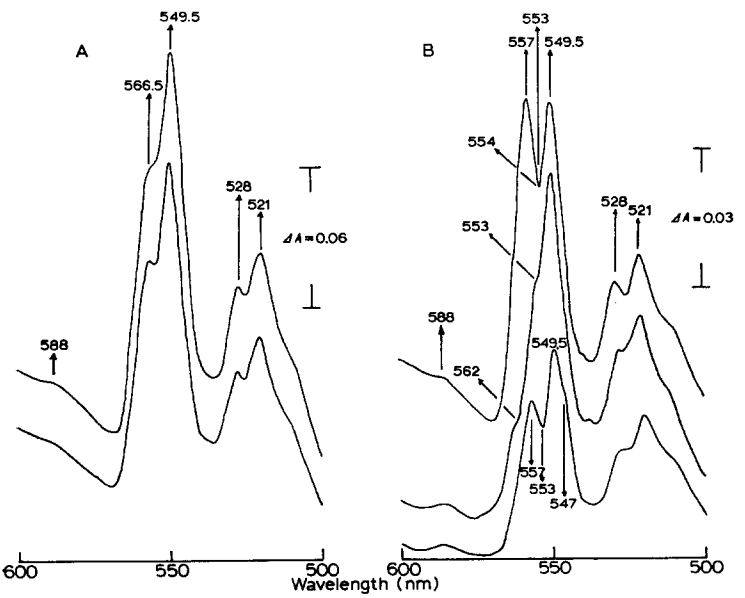

Fig. 1. Low temperature spectra (77 °K) reduced minus oxidised difference spectra of the particulate fraction. The particles (3.4 mg protein per ml) were suspended in 10 mM $MgCl_2$–50 mM Tris–HCl (pH 7.8) in 1.0 M sucrose: the cuvette path length was 2 mm. A, upper curve, $Na_2S_2O_4$ reduced minus oxidised; lower curve, NADH reduced minus oxidised. B, upper curve, succinate reduced minus oxidised; middle curve, NADH reduced minus succinate reduced; lower curve, ascorbate–TMPD reduced minus oxidised. The cuvettes were left 10 min after substrate addition before recording the spectra, in order to attain anaerobic conditions. Note the greater expansion of the absorbance scale in B than in A.

via NAD^+-independent dehydrogenases (Weston, J. A. and Knowles, C. J., unpublished).

NADH reduced minus succinate reduced spectra have a peak at 549.5 nm indicating that NADH causes a greater reduction of this component than succinate, whilst the shoulder at 557 nm is not seen. Additional distinct shoulders at 554 and 562 nm are resolved. Ascorbate–TMPD reduced minus oxidised spectra show only a small reduction of the 557 and 549.5 nm components, but a further clear shoulder at 547 nm is now visible. Reduction by $Na_2S_2O_4$ or any of the substrates gave a Soret peak at 426 nm, with a shoulder at 435 nm and very small peaks at 588–594 nm and 620–630 nm (not shown).

With reference to the peak positions observed at 77 °K (since some of the components are not resolved at room temperature) we will designate the *b* and *c* type cytochromes as b_{562}, b_{557}, *c* or b_{554}, $c_{549.5}$, and c_{547}. From the relative peak heights the major components are cytochromes b_{557} and $c_{549.5}$: in each case they represent about 75 % of the total *b* and *c* type cytochromes, respectively. CO spectra (see below) suggest that the $c_{549.5}$ peak is due to two cytochromes: a minor component capable of binding CO (cytochrome $c_{549.5(CO)}$) and a major component that does not interact with CO ($c_{549.5}$). Cytochrome $c_{549.5(CO)}$ presumably corresponds to a small fraction of the soluble *c* type cytochrome (α-peak at 550–549.5 nm at 77 °K) [7] which remains bound to the membrane.

Room temperature $Na_2S_2O_4$ reduced plus CO minus reduced difference spectra of the cell free extract, particulate fraction and supernatant fraction each have a Soret peak at 413 nm, a trough at 423 nm and a trough at 553 nm due to a CO-binding *c* type cytochrome, corresponding to the α-peak at 552 nm in room temperature or at 549.5 nm in 77 °K reduced minus oxidised difference spectra. In addition all three fractions have a shoulder at 435 nm on the Soret peak due to small quantities of cytochrome a_2; though no peaks were seen in the 580–640 nm region. The cell free extract and particulate fraction, but not the supernatant fraction, exhibit a shoulder at 560–562 nm on the trough in the visible region of the spectrum. This is due to cytochrome *o*, whose Soret peak is obscured by that of the CO-binding *c* type cytochrome.

The improved resolution of low temperature spectra (Fig. 2) over room temperature spectra shows that there is a shoulder on the Soret peak at 417–419 nm of the particulate fraction. This shoulder is due to cytochrome *o* and the peak at 410–412 nm corresponds to the CO-binding *c* type cytochrome. In addition, the trough in the visible region splits into three components, with minima at 558 nm (due to cytochrome *o*) and 552 nm (due to a *c* type cytochrome), and a shoulder at 547 nm (due to second *c* type cytochrome, or to splitting of the α-peak of the 552 nm component). CO spectra of the supernatant fraction at 77 °K have a Soret peak at 412–413 nm and a trough at 552–553 nm in the visible region, with no shoulders in either region [7]. This strongly suggests that the *c* type cytochrome with the trough at 552 nm in the particulate fraction corresponds to some of the soluble CO-binding *c* type cytochrome ($c_{549.5(CO)}$) which is membrane-bound, and that the shoulder at 547 nm in CO spectra of the particulate fraction is due to a second CO-binding *c* type cytochrome present in lower concentrations (as no such shoulder is seen in spectra of the supernatant fraction). This latter cytochrome could correspond to cytochrome c_{554} or cytochrome c_{547}.

The CO spectra of the particulate fraction at 77 °K are shown in Fig. 2 with

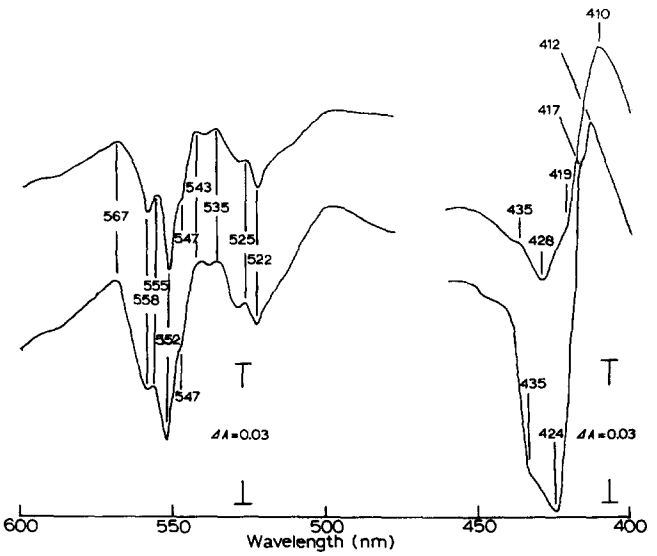

Fig. 2. Low temperature (77 °K) reduced plus CO minus reduced difference spectra of the particulate fraction. Upper curve, succinate plus CO minus succinate; lower curve, NADH plus CO minus NADH. The particles (6.5 mg protein per ml) were suspended in 10 mM $MgCl_2$–50 mM Tris–HCl (pH 7.8) in 1.0 M sucrose, and the cuvette path length was 2 mm.

reduction of the cytochromes by NADH and succinate. The shoulder at 417–419 nm on the Soret peak and the trough at 558 nm, both due to cytochrome *o*, are routinely found to be much larger for reduction by NADH than for reduction by succinate. Reduction by $Na_2S_2O_4$ and ascorbate–TMPD gave CO spectra similar to that for NADH reduction, whilst D(−)-lactate and L(+)-lactate gave spectra similar to that for succinate reduction.

Reference to published values for the extinction coefficients of similar cytochromes [11] enables an approximate measure to be made of the concentrations of the *b* and *c* type cytochromes of the particulate fraction (Table I). The high degree of overlap in the CO spectra Soret peaks of cytochrome *o* and the CO-binding *c* type cytochromes means that their individual concentrations cannot be determined. Castor and Chance [8, 9] have shown "peak to plateau" millimolar extinction coefficients of about 80 for various cytochromes *o* in the Soret region, whilst for the 3 haem binding cytochrome c_{553} from *Chromatium* $\varepsilon_{mM} = 165$ (i.e. 165/3 = 55 per haem) [12]. If an average of these values ($\varepsilon_{mM} = 68$) is taken as a guide, a rough measure of the combined concentration of cytochrome *o* plus CO-binding *c* type cytochrome can be obtained. The supernatant fraction contains only cytochrome $c_{549.5(CO)}$ and a value of $\varepsilon_{mM} = 55$ can be used [12].

It can be seen from Table I that fractionation of the cell-free extract into particulate and supernatant fractions results in a 3-fold increase in the total concentration of the *b* and *c* type cytochromes in the particulate fraction, but with some cytochrome $c_{549.5(CO)}$ also present in the supernatant fraction. The distribution of the CO-binding components is, however, markedly different. The supernatant fraction probably contains only one *c* type cytochrome, which is capable of CO binding (i.e.

TABLE I

DISTRIBUTION OF CYTOCHROME CONCENTRATIONS

The concentrations are given as nmoles/mg protein. The concentrations of the total *b* and *c* type cytochromes were determined from the heights of their α-peaks in room temperature $Na_2S_2O_4$ reduced minus oxidised difference spectra (Fig. 1) using the extinction coefficients given by Jones and Redfearn [15]. The total concentration of the CO-binding *c*-type cytochrome plus cytochrome *o* was determined from their overlapping Soret peaks in room temperature $Na_2S_2O_4$ reduced plus CO minus reduced difference spectra (Fig. 3). The extinction coefficients used are discussed in the text.

Fraction	*b*-type cytochrome	*c*-type cytochrome	CO-binding *c*-type cytochrome plus cytochrome *o*	$\frac{c_{(CO)}+o}{\text{total } c\text{-type}}$ (%)
Cell-free extract	0.17	0.31	0.076	25
Particulate fraction	0.61	1.02	0.100	9.8
Supernatant fraction	–	0.10	0.086	86

only cytochrome $c_{549.5(CO)}$). In the particulate fraction only about 10 % of the total *c* type cytochrome plus cytochrome *o* is capable of binding CO. Despite the fact that the α-peak at 552 nm (in room temperature reduced minus oxidised difference spectra) is made up of several components it would seem probable that there is insufficient cytochrome $c_{549.5(CO)}$ present to account for more than a small fraction of the total cytochrome $c_{549.5}$. There are therefore two cytochromes with α-peaks at 549.5 nm at 77 °K (cytochromes $c_{549.5}$ and $c_{549.5(CO)}$).

The supernatant fraction derived from cells harvested in the log phase (4 h growth) contained little or no soluble cytochrome $c_{549.5(CO)}$, though in the particulate fraction room temperature CO spectra still had a peak at 413 nm indicating that some CO-binding *c* type cytochrome was present. Growth of the cells for extended periods (30–40 h) resulted in a large increase in synthesis of cytochrome $c_{549.5(CO)}$, present partially in the supernatant fraction and partially membrane bound, which means that in room temperature reduced minus oxidised difference spectra of the cell-free extract and particulate fraction the shoulder at 560 nm is obscured. In addition the concentration of cytochrome a_1 increases sharply, with a distinct peak at 582 nm, whilst the peak at 620 nm due to cytochrome a_2 also becomes somewhat more noticeable.

In order to determine which of the CO-binding cytochromes act as oxidases, action spectra for the relief of CO inhibition by monochromatic light were determined. These experiments were performed with intact cells, measuring the respiration of endogenous substrates. Fig. 3A shows an action spectrum of stationary phase cells (16 h growth). In the visible region there are peaks at 568 and 542 nm, due to either cytochrome *o* or *c* type cytochrome. The Soret peak is at 412 nm and is due to the *c* type cytochrome, since Castor and Chance [8, 9] have reported that the Soret peaks (in action spectra) of cytochrome *o* from a variety of different bacteria are in the range 416–418 nm. The resolution of such spectra is, however, not sufficient to tell whether or not there is a shoulder on the Soret peak due to cytochrome *o*. In addition there is a peak at 637 nm due to cytochrome a_2 (Fig. 3A).

In action spectra of cells grown to the late stationary phase (36 h) (Fig. 3B), where difference spectra show the induction of cytochrome a_1 (see above), the Soret peak shifts to 434 nm and a large peak occurs at 588 nm; both are due to cytochrome

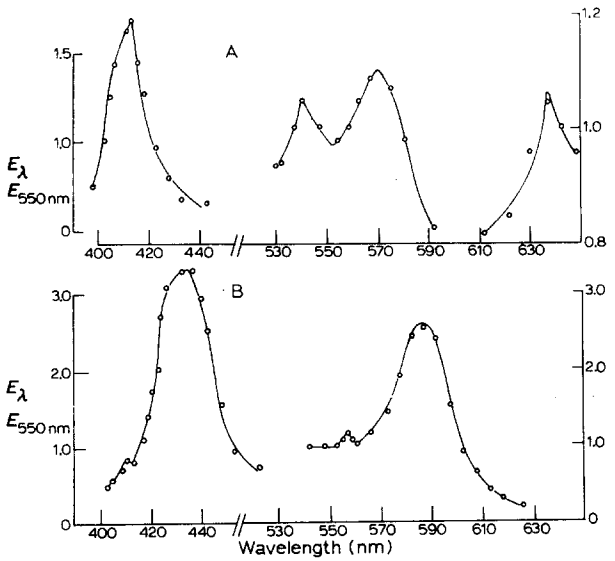

Fig. 3. Action spectra for the relief of CO inhibition by monochromatic light of endogenous activity in intact cells. The oxidase activities measured at each wavelength (ε_λ) are compared to the activity at an arbitrarily chosen wavelength (550 nm). Cells were grown to the early stationary phase (16 h) (upper curve) or the late stationary phase (36 h) (lower curve) and the activities measured in the growth medium. The apparatus and method used was that of Castor and Chance [8, 9].

a_1. The minute shoulder at 410–412 nm shows the relative unimportance of cytochrome *o* and/or CO-binding *c* type cytochrome as terminal oxidases in these cells. Furthermore, there is no peak in the 600–650 nm region due to cytochrome a_2.

DISCUSSION

B. natriegens grown aerobically on DL-lactate minimal salts medium has been shown to form a respiratory system of unusual cytochrome composition. High concentrations of at least six different *b* and *c*-type cytochromes are found in the particulate fraction. The major *b* and *c*-type cytochromes, representing about 70 % of the total, are cytochromes b_{557} and $c_{549.5}$ respectively. Minor components are cytochromes b_{562}, *b* or c_{554}, c_{547}, and a second *c*-type cytochrome absorbing at 549.5 nm, cytochrome $c_{549.5(CO)}$. This latter cytochrome is distinguishable from the dominant cytochrome $c_{549.5}$ in its lower concentration, ability to bind CO, and its additional presence in the supernatant fraction. Cytochromes a_1 and a_2 are present in concentrations less than 5 % of the *b* and *c* type cytochromes in particles derived from cells grown to the routine point of harvesting in the early stationary phase (16 h growth), but present in larger quantities in cells grown for 36–40 h, with cytochrome a_1 particularly increasing in concentration.

Of great interest is the number of cytochromes present that are capable of interaction with CO, and hence possibly acting as terminal oxidases. These are cytochromes a_1, a_2, *o*, $c_{549.5(CO)}$ and possibly a second *c* type cytochrome. It is so far unusual to find *c* type cytochromes capable of binding CO, and cytochrome $c_{549.5(CO)}$

of *B. natriegens* is the first spectrally non-varient, high potential ($E_0' = 0.32$ V), CO-binding *c* type cytochrome to be discovered [7].

The presence of four, or possibly five, different CO-binding cytochromes with possible oxidase function suggests that complex branching of respiration to oxygen may occur in *B. natriegens*. It is not unusual to find more than one possible oxidase in bacterial respiratory systems, with various combinations of cytochromes *a*, a_1, a_2, a_3, and *o* occurring in many bacteria, though never more than a total of three of these cytochromes occurring in any one bacterium [13]. Terminal branching of respiration to oxygen may therefore be typical of many bacterial respiratory systems, though there have been few studies of respiratory branching to oxygen, and the respiratory system of *Azotobacter vinelandii* is the only system to have been investigated in detail [14, 15].

Action spectra of intact cells of *B. natriegens* (Fig. 3) grown for 16 h show that cytochrome a_2, despite being present in very low concentrations, functions as a terminal oxidase, but no peak due to cytochrome a_1 is observed. From the position of the Soret peak at 412 nm it is clear that the CO-binding *c* type cytochromes also function as terminal oxidases; it is not possible to tell whether cytochrome *o* is also acting as a terminal oxidase in these cells. In cells grown for longer periods, when induction of cytochrome a_1 occurs, it has the major function as a terminal oxidase. Under these conditions cytochrome a_2 has little functional oxidase activity and cytochrome *o* plus CO-binding *c* type cytochrome have only minor importance as terminal oxidases.

These results confirm that care must be taken in interpreting CO-binding ability and relative concentration of bacterial cytochromes with actual rather than possible functional importance as terminal oxidases.

Work is in progress to determine whether branching of the respiratory system to oxygen occurs at the terminal oxidases, and, if so, what is the significance of the multiple oxidases.

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