The membrane-bound respiratory chain of *Pseudomonas pseudoalcaligenes* KF707 cells grown in the presence or absence of potassium tellurite

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The respiratory chain of *Pseudomonas pseudoalcaligenes* KF707 in membranes isolated from cells grown in the presence or absence of the toxic oxyanion tellurite (TeO$_2$\textsuperscript{3-}) was examined. Aerobic growth in the absence of tellurite shows an NADH-dependent respiration which is 80% catalysed by the cytochrome (cyt) bc\textsubscript{1}-containing pathway leading to two terminal membrane-bound cyt c oxidases inhibited by different concentrations of KCN (IC\textsubscript{50} < 2 and 1 µM). A third oxidase, catalysing the remaining 20% of the cyanide-resistant respiration and fully inhibited by 2–3 mM KCN, is also present; this latter pathway accounts for 60–70% of the total NADH-dependent respiration in membranes from cells grown in LB medium supplemented with potassium tellurite (35 µg ml\textsuperscript{-1}). Two high-potential b-type haems ($E_m$ +395 and 318 mV) are redox centres of a membrane-bound cyt c oxidase (possibly of the cbb\textsubscript{3} type) which shows a 50% decrease of its activity in parallel with a similar decrease of the c-type haem content (mostly soluble cyt c) in membranes from tellurite-grown cells; the latter type of cells specifically contain a cyt b type at $E_m$ +203 mV (pH 9–0) which is likely to be involved in cyanide-resistant respiration. Comparison of the growth curve of KF707 cells in parallel with tellurite uptake showed that intracellular accumulation of tellurium (Te\textsuperscript{0}) crystallites starts from the mid-exponential growth phase, whereas tellurite-induced changes of the respiratory chain are already evident during the early stages of growth. These data were interpreted as showing that reduction of tellurite to tellurium and tellurite-dependent modifications of the respiratory chain are unrelated processes in *P. pseudoalcaligenes* KF707.

**Keywords:** cytochromes, tellurite uptake, tellurium crystallites

**INTRODUCTION**

Tellurite (TeO$_2$\textsuperscript{2-}) is a rare-earth oxyanion that can however be found in high concentrations in land and water near sites of waste discharge of industrial manufacturing processes (Taylor, 1999). Tellurite is highly toxic to mammalian cells (Turner *et al.*, 1992; Wagner *et al.*, 1995) and micro-organisms (Turner, 2001) at concentrations as low as 1 µg ml\textsuperscript{-1} (4 μM). Intrinsic low-level resistance to tellurite has been reported for a few Gram-positive bacteria (e.g. *Corynebacterium diphtheriae* and *Streptococcus faecalis*) and micro-organisms (Taylor, 1999) at concentrations as low as 1 µg ml\textsuperscript{-1} (4 µM). Intrinsic low-level resistance to tellurite has been reported for a few Gram-positive bacteria (e.g. *Corynebacterium diphtheriae* and *Streptococcus faecalis*) while a constitutive high-level resistance at concentrations between 1 and 2.5 mg ml\textsuperscript{-1} (0.4–1 mM) has been described for anaerobic (e.g. *Rhodobacter sphaeroides*) and aerobic (e.g. *Erythromicrobium hydrolyticum*) photosynthetic bacteria of the alpha subclass of *Proteobacteria* (Yurkov & Beatty, 1998; Yurkov *et al.*, 1996). Resistance to tellurite (Te\textsuperscript{R}) has been extensively studied in Gram-negative bacteria and there appear to be no simple unifying mechanisms for this resistance. Indeed,
at least five Te\textsuperscript{R} determinants have been identified by genetic studies and DNA sequencing, all apparently unrelated to one another at either DNA or protein level (Taylor, 1999; Turner, 2001). Most bacteria convert tellurite to elemental tellurium (reduction of Te\textsuperscript{IV} to Te\textsuperscript{0}), which is accumulated intracellularly as black crystals (Taylor, 1999). It was proposed that reduction of tellurite into less toxic elemental tellurium might represent a secondary pathway for detoxification; however, Te\textsuperscript{R} without metal accumulation has been observed in several cases, implying that Te\textsuperscript{R} does not strictly depend on formation of crystallites of elemental tellurium (Yurkov et al., 1996).

The role assumed by the respiratory chain of bacterial cells resistant to potassium tellurite remains elusive, although several reports suggest that plasma-membrane redox enzymes might be involved in at least one of the two enzymic steps required to generate tellurium from tellurite (Moore & Kaplan, 1992). The extent of tellurite reduction in R. sphaeroides was inversely related to the oxidation state of the carbon source and it has also been shown to be dependent on FADH\textsubscript{4} oxidation activity (Moore & Kaplan, 1992, 1994). Chiong et al. (1988) purified a protein fraction from Thermus thermophilus which contained an NADH/NADPH-dependent tellurite-reducing activity, while Terai et al. (1958) demonstrated tellurite reduction in cell extracts of Mycobacterium avium. Tellurite reductase activity encoded by a large conjugative plasmid of the IncHI-2 incompatibility group has also been observed in Alcaligenes sp. (Jobling & Ritchie, 1988). The membrane-bound nitrate reductases (NarG and NarZ) have been found to reduce tellurite and contribute to the basal level of resistance in Escherichia coli (Avazeri et al., 1997). Additionally, the periplasmically located nitrate reductase (Nap) also exhibits tellurite reductase activity (R. J. Turner, personal communication). Recently, the electron-transport activity catalysed by the branched respiratory chain of Pseudomonas aeruginosa (Matsushita et al., 1980; Zannoni, 1989; Cunningham & Williams, 1995) has been correlated with reduction of potassium tellurite, although at a rate which is three orders of magnitude lower than the rate of oxygen reduction (Trutko et al., 2000).

Strain KF707 of Pseudomonas pseudoalcaligenes has been extensively described in the past for its capacity to cometabolize polychlorinated biphenyls (PCBs) under aerobic conditions (Taira et al., 1992; Fedi et al., 2001). Notably, during a selection procedure for the isolation of Te\textsuperscript{R} strains from a PCB-contaminated soil, we have recently observed that P. pseudoalcaligenes KF707 cells have a branched respiratory chain which is functionally and structurally affected by tellurite; we also conclude that tellurite-dependent modifications of the respiratory chain and tellurite reduction to elemental tellurium are unrelated phenomena.

**METHODS**

**Bacterial strain.** Pseudomonas pseudoalcaligenes KF707 (kindly provided by K. Furukawa, Fukuoka, Japan) was grown aerobically in 1 litre flasks containing LB (Luria–Bertani) broth in either the presence or the absence of 35 µg potassium tellurite (K\textsubscript{2}TeO\textsubscript{4}) ml\textsuperscript{−1}.

**Preparation of membranes.** P. pseudoalcaligenes KF707 membrane fragments from washed cells grown aerobically in LB medium in the presence or absence of potassium tellurite were prepared using a French pressure cell and ultracentrifugation as described previously (Zannoni et al., 1978) either in 50 mM MOPS buffer (pH 7.0) containing 1 mM KCl, EDTA and PMSF for PAGE analyses or in 50 mM Tricine buffer (pH 7.5) containing 5 mM MgCl\textsubscript{2} for spectroscopic studies. Membrane fragments were suspended at a known protein concentration in the same buffer, and used immediately for electron-transport measurements or stored frozen at −80 °C for PAGE analyses.

**Protein determination.** Protein content of samples was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

**Measurement of oxygen uptake and inhibitor titrations.** Respiratory activities in membrane fragments were determined by monitoring oxygen consumption with a Clark-type oxygen electrode YSI 53 (Yellow Springs Instruments) as detailed elsewhere (Daldal et al., 2001).

**Spectral analysis of cytochrome content of membranes.** The amounts of cytochromes in membrane fragments and soluble fractions were estimated by recording reduced (with sodium ascorbate or dithionite)-minus-oxidized (with potassium ferrocyanide) optical difference spectra at room temperature with a Jasco 7800 spectrophotometer. Absorption coefficients ε\textsubscript{404-430} of 23 mM\textsuperscript{−1} cm\textsuperscript{−1}, ε\textsubscript{451-557} of 22 mM\textsuperscript{−1} cm\textsuperscript{−1} and ε\textsubscript{551-560} of 19 mM\textsuperscript{−1} cm\textsuperscript{−1} were used for a-, b- and c-type cytochromes, respectively.

**Equilibrium redox titrations.** Dark equilibrium redox titrations were performed in a self-made glass cuvette kept anoxic by means of a stream of argon. A platinum electrode was fitted into the cuvette and the redox potentials were measured against an external calomel electrode connected via a salt bridge. The following redox mediators, at a concentration of 2.5 µM, were used: N-ethylidibenzopyrazine ethyl sulphate, N-methylidibenzopyrazine methyl sulfate, 2,3,5,6-tetramethyl-1,4-benzoquinone, p-benzoquinone, 1,2-naphthoquinone and 1,4-naphthoquinone. Sodium ascorbate and sodium dithionite were used as reductants; potassium ferricyanide was the oxidant. Membranes equivalent to 3 mg ml\textsuperscript{−1} were suspended in MES/TE/Ticine (30 mM each) buffer with 50 mM KCl (pH 7.0–9.0).

**Determination of potassium tellurite in liquid media.** The quantitative determination of tellurite was done using the
reagent diethylthiocarbamate (DDTC) as described by Turner et al. (1992).

Electron micrographs of bacterial cells. To determine the presence of tellurium (Te⁰) in bacterial cells, potassium tellurite (35 µg ml⁻¹) was added to the liquid culture during the mid-exponential growth phase. Two hours later, when the bacterial cultures became black, they were harvested and processed for electron-microscopy analysis as follows. Cell pellets were fixed for 2 h in 0.05 M cacodylate, 1.5% (w/v) glutaraldehyde (pH 7.2); the same buffer was used for overnight washing of the samples followed by 2 h fixation with 2% (w/v) osmium tetroxide and dehydration with ethanol. Samples were finally embedded in Durcupan. Thin sections obtained by an LKB Ultratome Nova were double-stained with uranyl acetate and lead citrate (Reynolds, 1963). Specimens were examined with a Philips CM-100 transmission electron microscope.

TMBZ-SDS-PAGE gels. SDS-PAGE was performed using 16.5% (w/v) acrylamide Tris-Tricine gels as described by Shägger & von Jagow (1987). Samples were denatured for 5 min at 37 °C in SDS loading buffer prior to electrophoresis, and gels were stained with Coomassie brilliant blue to visualize the polypeptides. The c-type cytochromes were revealed via intrinsic peroxidase activity of their haem group, using 3,3',5,5'-tetramethyldibenzidine (TMBZ) and H₂O₂ (Thomas et al., 1976).

RESULTS

Growth curve of P. pseudoalcaligenes KF707 in medium supplemented with K₂TeO₃ and its sequestration as tellurium crystallites

We have recently observed that cells of P. pseudoalcaligenes KF707 are resistant to potassium tellurite concentrations up to 100 µg ml⁻¹ (minimal inhibitory concentration, MIC, approx. 150 µg ml⁻¹; unpublished results). Notably, growth of strain KF707 in the presence of tellurite caused the bacteria to turn black, indicative of the presence of fine tellurium crystallites inside the cells. Fig. 1 shows the growth curves (based on OD₆₆₀) of P. pseudoalcaligenes KF707 in the presence and absence of 35 µg K₂TeO₃ ml⁻¹; in parallel, the decrease of the concentration of potassium tellurite in the growth medium (Fig. 1a) and the influx of K₂TeO₃, expressed as variation of the amount of tellurite in the medium as a function of the cell protein concentration (Fig. 1b), were measured. In the presence of tellurite the initial lag phase of growth varied from 4–6 to 20–22 h in control and tellurite cultures, respectively (not shown). As shown in Fig. 1(a), both control and tellurite growth curves had similar slopes, indicative of quite similar cell doubling times (1.3–1.4 h), although the final optical densities of the cultures were slightly different. However, this latter phenomenon was due to overestimation of the OD₆₆₀ of tellurite-grown cultures from their late exponential growth phase onwards as a consequence of the accumulation of black tellurium crystals in the cells. Further analyses indicated that both control and tellurite-grown cultures had similar numbers of viable cells per ml (≈10⁹). The concentration of potassium tellurite in the medium was roughly constant during the first period of the exponential phase of growth, starting to decrease only in the middle of the exponential growth phase; notably, within the next 4–5 h the concentration of tellurite dropped to less than 5% of the initial value. To test whether or not this latter decrease was linked to reduction of potassium tellurite along with its intracellular accumulation in the form of elemental tellurium crystallites, a control culture (OD₆₆₀ ≈ 2; viable cells ml⁻¹ ≈ 10⁸) was harvested, autoclaved (120 °C, 10 min) and incubated for 10 h in the presence of 35 µg potassium tellurite ml⁻¹. At the end of this treatment, only about 30% of the initial amount of tellurite was nonspecifically sequestered by cell proteins, suggesting that the tellurite uptake shown in Fig. 1 was actually due to cell accumulation of elemental tellurium. To further strengthen this conclusion, electron micrographs of KF707 cells incubated with potassium tellurite were made. As shown in Fig. 2(b), several electron-dense tellurium crystallite granules were present in the cytoplasm of KF707 cells which were harvested at the end of the exponential phase of growth (OD₆₆₀ ≈ 1.5) and incubated for 2 h with 35 µg potassium tellurite ml⁻¹;

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**Fig. 1.** P. pseudoalcaligenes KF707 growth curve (expressed as OD₆₆₀) and variation of the tellurite concentration in the growth medium. ○ (in a), control growth curve (absence of tellurite); ● (in a and b), growth curves in medium supplemented with tellurite; ▲ (dotted traces), tellurite variation, expressed as µg ml⁻¹ and µg h⁻¹ (mg protein)⁻¹, in (a) and (b), respectively.
control cells (Fig. 2a) did not contain electron-dense tellurium granules.

Respiratory activities in membrane fragments from KF707 cells grown in the presence or absence of tellurite

Preliminary experiments were carried out to determine the respiratory activities of membrane fragments isolated from *P. pseudoalcaligenes* KF707 cells grown aerobically in LB broth and harvested at the stationary growth phase (OD₆₆₀ ≥ 2). Table 1 shows that several activities were present with different sensitivities to KCN, rotenone and antimycin A, which are inhibitors of various redox complexes of mitochondrial and bacterial electron-transport chains (Cramer & Knaff, 1990). Interestingly, oxidation of NADH was partially resistant (24–29%) to cyanide and antimycin A, suggesting the presence of a branched respiratory pathway as previously seen in other species of the genus (Zannoni, 1989). To further analyse the presence of multiple membrane-bound oxidases in cells of strain KF707, we investigated the effect of cyanide on the NADH-dependent respiratory activity. The titres reported in Fig. 3(a), indicate that membranes from stationary-phase control cells (grown without tellurite) contain at

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**Table 1. Oxidative activities of membranes from aerobically grown cells of *P. pseudoalcaligenes* KF707**

<table>
<thead>
<tr>
<th>Electron donor*</th>
<th>Inhibitor (concn)</th>
<th>Activity [µmol O₂ h⁻¹ (mg protein)⁻¹]†</th>
</tr>
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<tbody>
<tr>
<td>NADH</td>
<td>–</td>
<td>41·1</td>
</tr>
<tr>
<td></td>
<td>Rotenone (1 µM)</td>
<td>8·2 (20%)</td>
</tr>
<tr>
<td></td>
<td>Antimycin A (5 µM)</td>
<td>12·1 (29%)</td>
</tr>
<tr>
<td></td>
<td>KCN (100 µM)</td>
<td>9·8 (24%)</td>
</tr>
<tr>
<td>Succinate</td>
<td>–</td>
<td>5·5</td>
</tr>
<tr>
<td></td>
<td>KCN (100 µM)</td>
<td>1·2 (21%)</td>
</tr>
<tr>
<td>Asc/cyt c</td>
<td>–</td>
<td>10·9</td>
</tr>
<tr>
<td></td>
<td>KCN (100 µM)</td>
<td>0·3 (3%)</td>
</tr>
</tbody>
</table>

*Concentrations: NADH, 0·2 mM; succinate, 2 mM; sodium ascorbate (Asc)/horse heart cyt c, 5 mM/50 µM.
†Percentage of corresponding control (no inhibitor) shown in parentheses.
least three terminal oxidases (triphasic curve) with different sensitivities to cyanide (IC$_{50}$ of 0.15, 2 µM, and 1 mM). Apparently, the most cyanide-sensitive oxidase accounts for less than 20% of the total NADH oxidase activity, while the most cyanide-resistant oxidase (IC$_{50}$ 1 mM) might represent the so-called ‘cyanide resistant’ oxidase as also suggested by the data of Table 1. Fig. 3(a) also shows that membranes isolated from cells grown in the presence of tellurite contain a cyanide-resistant oxidase contributing about 60–65% of the total NADH oxidase activity; this result was also confirmed by the use of antimycin A, a specific inhibitor of the cytochrome (cyt) $bc_1$ complex (not shown). The diversion of most of the electron-transport flow through the cyanide-resistant oxidase did not significantly affect the total NADH-dependent respiratory rate [32 µmol O$_2$ (mg protein$^{-1}$)], which was only 22% less than the control [41·1 µmol O$_2$ h$^{-1}$ (mg protein$^{-1}$)].

To better discriminate between the three oxidases present in membranes from cells of KF707 grown in the presence or absence of tellurite, we examined the effect of cyanide on the cyt $c$ oxidase activity catalysed by reduced horse-heart cyt $c$ (30 µM) with sodium ascorbate (5 mM) as reducing agent. As shown in Fig. 3(b), the KCN-titration curve is essentially biphasic (IC$_{50}$ of 0.1 and 1 µM), suggesting the presence of two oxidases catalysing the oxidation of reduced cyt $c$ in membranes from cells grown either in the absence or in the presence of tellurite. However, although the cyt $c$ oxidase inhibition pattern by KCN in membranes from tellurite-grown cells was similar to that seen in control membranes, the cyt $c$ oxidase rate was 50% less than that of the control [5·2 and 10·9 µmol O$_2$ h$^{-1}$ (mg protein$^{-1}$), respectively]. These findings suggest that tellurite strongly affects the functional arrangement of the KF707 respiratory chain, inducing a restriction of the electron flux going through the cyt $c$ oxidase pathway with a parallel enhancement of the activity catalysed by the cyanide-resistant branch (Fig. 3a).

Further, the most cyanide-sensitive oxidase (IC$_{50}$ 0·1–0·2 µM) seems to play a minor role in respiratory electron transport in both types of membranes (catalysing 20–25% of the total activity) and it presumably has an affinity for oxygen different from that exhibited by the oxidase which is totally sensitive to 10 µM cyanide.

**Difference spectra and redox potentiometry**

Reduced-minus-oxidized difference spectra recorded under different redox conditions (ascorbate and dithionite as reducing agents) of membranes from KF707 grown in LB broth and harvested at OD$_{660}$ ≥ 2 are in Fig. 4(a). The spectra were recorded from samples with identical protein concentrations (1 mg ml$^{-1}$) and are thus directly comparable. The presence of $c$- and $b$-type haems with $a_2$-bands in their reduced form at 552 nm (peak) and 560 nm (shoulder), respectively, is apparent. In none of the spectra were signals observed which would have indicated the presence of $aa_2$-type haems ($a$-band in their reduced form at 602–605 nm). As shown in Fig. 4(b), the reduced-minus-oxidized difference spectra of membranes isolated from cells grown in the presence of 35 µg potassium tellurite ml$^{-1}$ (OD$_{660}$ ≥ 2·4) were qualitatively different from the controls (Fig. 4a). In particular, the $a_2$-band of $c$-type haems at 552 nm was considerably decreased compared to that of $b$-type haems (560 nm): the cyt $c/b$ ratio [based on nmol haem (mg protein$^{-1}$)] was 0·9, as against a ratio of 1·65 in membranes from control cells (Fig. 4a).

To define the thermodynamic properties of the different cytochromes, dark equilibrium redox titrations of membranes from control cells harvested at OD$_{660}$ ≥ 2 were performed at pH 7·0 and 9·0; series of spectra ranging from fully oxidized to fully reduced conditions were therefore acquired at controlled redox potentials (see Methods). At pH 7·0, analysis of the data at 552–540 nm (indicative of $c$-type species) gave a two-component Nernst curve ($n = 2$) with $E_{m,7}$ of $+379 ± 6$ and $+300 ±...
As expected, analysis of the data obtained at pH 9.0 was qualitatively different from that at pH 7.0. As shown in Fig. 5(b) (open circles) the Nernst curve of the signals at 560–575 nm indicated the presence of four components with $E_{m,5}$ of $+393 \pm 5$, $+293 \pm 2$, $+64 \pm 8$ and $+4 \pm 2$ mV with relative contributions to the absorbance signal of 16, 34, 13 and 37%. Apparently the signal titrated as a single component with $E_{m,5}$ of $+116$ mV at pH 7.0 is resolved at pH 9.0 into two components at +64 and +4 mV (expected $E_{m,5}$ of +124 and +64 mV with a mean slope of 30 mV per pH unit) while the two high-potential haems were slightly affected by pH variation. Similarly to the high-potential $b$-type haems, the mid-point potentials of the $c$-type haems also were not significantly affected by pH (from 7.0 to 9.0) with $E_{m,2}$ of $+360 \pm 5$ and $+293 \pm 4$ mV (Fig. 5a).

Analyses at pH 7.0 (552–540 nm) of the spectra obtained at controlled ambient potentials in membranes from cells grown in the presence of tellurite indicated the presence of components with $E_{m,7}$ of $+380 \pm 4$ and $+297 \pm 2$ mV and relative contributions of 22% and 78%, respectively (data not shown). Notably, the amount of these two high-potential components was 50% lower than the equivalent components of membranes from control cells (see also Fig. 4a, b and Table 2 below). Analogously, the number of $b$-type haems resolved at 560–575 nm (pH 7.0) in membranes from tellurite-grown cells (Fig. 6, filled circles) was similar to the number seen in control membranes ($E_{m,2}$ of $+420 \pm 8$, $+280 \pm 4$ and $+74 \pm 1$, with contributions of 9, 25, 65%, respectively) with some significant quantitative variation, i.e. the amount of the highest-potential $b$-type haems (0.9 nmol (mg protein)$^{-1}$) was lower than the amount of the equivalent components in control membranes (1.2 nmol (mg protein)$^{-1}$). Additionally, redox titrations at pH 9.0 (Fig. 6, open circles) revealed the presence of an extra $b$-type haem along with minor variations of the two high-potential haems ($E_{m,9}$ of $+390 \pm 10$ and $+310 \pm 15$ mV) and a further resolution of the signal titrated at $+74$ mV into two components ($E_{m,9}$ of $+40 \pm 5$ and $-8 \pm 3$ mV); indeed a $b$-type species with $E_{m}$ of $+203 \pm 5$ mV was evident at pH 9.0 due to a shift to lower potentials (30 mV slope per pH unit) of components at $+40 \pm 5$ and $-8 \pm 3$ mV (pH 9.0). Thus, membranes from KF707 cells grown in the presence of potassium tellurite show at least two major specific features: (i) the presence of a new high-potential $b$-type haem (cyt $b_{202}$ at pH 9.0), and (ii) a consistent reduction of the amount of the highest-potential $b$-type haems and of the $c$-type haem complement (see also Table 2 and Fig. 7).
Table 2. Oxidative activities and cytochrome content of membranes from cultures of P. pseudoalcaligenes KF707 grown in the presence or absence of tellurite and harvested in the early exponential phase

<table>
<thead>
<tr>
<th>Oxidase activity*</th>
<th>Oxidative activity [µmol O₂ h⁻¹ (mg protein)⁻¹]†</th>
<th>Cytochrome content [nmol (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth medium LB</td>
<td>Growth medium LB + TeO₃⁻</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>29·2</td>
<td>19·8</td>
</tr>
<tr>
<td>(+ KCN, 10 µM)</td>
<td>6·5 (22%)</td>
<td>11·7 (59%)</td>
</tr>
<tr>
<td>(+ KCN, 1 mM)</td>
<td>0·5 (17%)</td>
<td>0·3 (1·5%)</td>
</tr>
<tr>
<td>(+ Antimycin A, 5 µM)</td>
<td>4·8 (16%)</td>
<td>12·6 (64%)</td>
</tr>
<tr>
<td>Asc/cyt c oxidase</td>
<td>8·6</td>
<td>2·9</td>
</tr>
<tr>
<td>(+ KCN, 10 µM)</td>
<td>0·1 (1·1%)</td>
<td>0·05 (1·8%)</td>
</tr>
</tbody>
</table>

*Additions and non-standard abbreviations: Asc/cyt c, sodium ascorbate (5 mM) plus horse-heart cyt c (50 µM); NADH, 0.2 mM.
†Percentage of corresponding control (no inhibitor) shown in parentheses.
‡MB-cyt; membrane-bound cytochrome; S-cyt, soluble cytochrome (see text for details).

Fig. 6. Dark-equilibrium potentiometric titrations (●, pH 7·0; ○, pH 9·0) at 560–575 nm of membranes from cells of P. pseudoalcaligenes KF707 grown in the presence of tellurite (OD₆₆₀ 2·4). See text for further details.

Cytochrome c profile

In the preceding paragraphs we have shown that the cyt c-type complement of KF707 is poorly resolved by redox potentiometry. Membrane proteins of strain KF707 were therefore further analysed using TMBZ-SDS-PAGE to determine the profile of the c-type cytochromes. At least six major TMBZ-stained bands of molecular masses ranging from 33 to 10 kDa were discernible (Fig. 7, lane B). Under the separation conditions used here, the c-type profile of KF707 was similar to profiles reported for the facultative phototrophs Rhodobacter capsulatus and R. sphaeroides, which have cbb₃-type oxidases along with a soluble cyt c (c₆) and a membrane-anchored cyt c₅₅ both acting as direct electron donors to the cyt c oxidase (Cox) (Hochkoeppler et al., 1995; Daldal et al., 2001). Using the nomenclature applied to the c-type profile of R. sphaeroides and R. capsulatus the two upper bands of 32 and 33 kDa might be referred to the cyt c₅₅ subunit of the cbb₃-Cox and to the cyt c₅₅ subunit of the cyt bc₁ complex, respectively, which are followed by the cyt c₅₅ subunit of the cbb₃-Cox (27·5 kDa) and cyt c₆ (24 kDa). Soluble cyt c (c₆) of facultative phototrophs ran ahead (10 kDa) of the other c-type cytochromes, while the c₅₅ subunit at 17 kDa does not seem to have a
counterpart in membranes from *R. sphaeroides* and *R. capsulatus*. Further analyses (not shown) indicated that the 17 kDa subunit is not present in the 135 000 g soluble fraction, which contains primarily soluble cyt c of M_r 10000 (for comparison, horse-heart cyt c is shown in lane A). Fig. 7 lane C shows the cyt c-type profile of membranes from KF707 cells grown in the presence of tellurite. The profile of cyt c-type species is clearly different from that seen in control membranes (lane B) since the amount of stained haems is considerably decreased; in particular, subunits of M_r 33000, 17000 and 10000 are barely detectable, in line with our observation that the ascorbate/cyt c oxidase activity and the cyt c-type haem complement of membranes from tellurite-grown cells are decreased by 50% and 75%, respectively.

**Redox chain of membranes from cultures harvested in the early exponential growth phase in LB medium plus tellurite**

The functional, spectroscopic and thermodynamic data presented in the preceding paragraphs were obtained in membranes from KF707 cells harvested in the late exponential or stationary growth phase (OD_{660} 2). To test whether the changes of the respiratory chain were due to tellurite reduction activity occurring from mid-exponential phase onwards or were the result of a metabolic cell adaptation from the beginning of growth, membranes from cells grown in the presence or absence of tellurite were harvested at the beginning of the exponential growth phase (OD_{660} 0.5) and analysed. Interestingly, most of the features seen in membranes from cells harvested at the stationary growth phase (OD_{660} 2) were already apparent at the beginning of the exponential phase. The data in Table 2 summarize some of our results. In particular, tellurite-grown cells have a strong cyanide-resistant oxidative activity (59% and 64% of activity with 10 µM KCN and/or 5 µM antimycin A, respectively) while most of the NADH-dependent respiration in control cells is catalysed by the cyt c oxidase (only 22% of activity with 10 µM KCN); in line with this, the cyt c oxidase activity is almost three times lower in membranes from tellurite-grown cells than in the control, in parallel with a consistent decrease (70–75%) of both soluble and membrane-bound cytochromes c.

Further studies indicated that changes of the redox-chain components in membranes from cells grown in LB medium supplemented with tellurite can be reversed by transfer of cells into LB medium without tellurite. This conclusion was obtained by re-inoculating cells grown in LB+tellurite (35 µg ml^{-1}), and harvested at the stationary growth phase, into LB liquid medium without tellurite. Membranes isolated from the latter type of cultures during either the early- or late-exponential growth phase showed respiratory activities, inhibitor sensitivities, difference redox spectra, and redox titrations identical to those reported for those from cultures re-inoculated several times into LB media without tellurite (data not shown). These results were interpreted as showing that the effects induced by tellurite on the functional and structural organization of the KF707 redox chain are likely to be the result of a metabolic pressure and not the result of mutations generated by tellurite, although this possibility, at present, cannot be excluded *a priori*.

**DISCUSSION**

In this study we have investigated the structural, thermodynamic and functional aspects of the respiratory chain of *P. pseudoalcaligenes* KF707 cells grown in the presence or absence of the toxic oxyanion tellurite.

The main conclusions concerning the functional aspects of the respiratory chain of cells grown in the absence of tellurite can be summarized as follows: (a) the respiratory chain of KF707 is branched because three oxidative pathways can be distinguished by their cyanide sensitivities; (b) a first branch occurs before the antimycin-A-sensitive site (cyt bc_1 complex) because the NADH-dependent respiration is not completely blocked (75% inhibition) by this inhibitor; (c) the second branch is at the level of cytochromes c (most likely soluble cyt c + membrane-bound cyt c_1; see below) because two cyt c oxidases can be distinguished by their cyanide sensitivities; (d) the most cyanide-sensitive cyt c oxidase contributes only 20% of the total respiratory activity of cells harvested at the stationary growth phase.

The thermodynamic and SDS-PAGE analyses of membrane fragments from KF707 revealed a cyt c composition similar to that observed in other species of *Pseudomonas* and *Rhodobacter* (Zannoni, 1989, 1995; Hochkoeppler et al., 1995). Indeed, at least four membrane-bound c-type cytochromes (operationally defined as cyt c_1, c_2, c_3, and c_4) of M_r 33000, 32000, 27500 and 24000 can be distinguished by TMBZ-SDS-PAGE analysis along with a soluble c-type species with its z band in the reduced form at 551± nm isolated from the soluble fraction (E_{m,7} +291 mV, M_r 10000) and a membrane-bound c-type haem of unknown function and M_r 17000. Unfortunately, only one membrane-bound c-type haem is thermodynamically resolvable [E_{m,7} +379±6 mV, 0.3 nmol (mg protein)^{-1}] whereas all the rest of the cyt c type complement titrates as a single unresolved component at pH 7±0 with E_{m} of +300±1 mV (n=2) contributing about 80% of the total signal at 552–540 nm. The b-type complement of strain KF707 contains at least five haems with E_{m,7} of +395±4, +318±3, +203±6, +124±8 and +64±2 mV. These latter two E_{m,7} values are extrapolated from data obtained at pH 9±0 (30 mV slope per pH unit from 7±0 to 9±0) while the b-type haem at +203 mV can only be seen at pH 9±0 in cells grown in the presence of tellurite (see below) thanks to a 60 mV shift to lower values of haems at +124 and +64 mV (pH 7±0). By analogy with previously published data on b-type species present in the respiratory chain of *R. capsulatus* and *R. sphaeroides* (Zannoni, 1995) the two high-potential haems at +395 and +318 mV are likely to be involved in oxygen dioxide reducing activities,
although it is difficult to state whether they belong to the same redox complex, i.e. the \textit{cbb}_{\text{D}}\text{-}type cyt oxidase, or they are part of two different oxidases. Indeed, an interesting but still unexplained result of this study concerns the presence of two cyt \textit{c} oxidase activities, this conclusion being based on the cyanide titration patterns of NADH- and reduced cyt \textit{c}-dependent oxidation activities. Thus, the actual features of the \textit{b}\text{-}type haems catalysing these two oxidases remain at present elusive.

The detailed mechanism of tellurite reduction by bacterial cells (a four-electron reaction) is as yet unknown (Turner, 2001). Early work by Moore & Kaplan (1994) indicated that the extent of reduction in \textit{R. sphaeroides} was inversely related to the oxidation state of the carbon source and it was also dependent on FADH\textsubscript{2} activity, while recent work on several tellurite-resistant Gram-negative bacteria (\textit{genera Pseudomonas, Agrobacterium, Erwinia and Escherichia}) suggested an active role of the respiratory electron-transport chain in the accumulation of elemental tellurium and that the position of the catalytic centres of terminal membrane-bound oxidases correlates with the periplasmic or cytoplasmic location of tellurium crystallite granules (Trutko et al., 2000). On the other hand, the authors of the latter study also reported that specific activation of cyt \textit{c} activity in cells of \textit{P. aeruginosa} lowers the tellurium content of cells, which clearly indicates that reduction of tellurite and oxygen dioxide compete for the same pool of reducing equivalents before the cyt \textit{c} oxidase level. This reasoning is also consistent with the expected electrochemical properties of tellurite in aqueous solution at pH 7-0 (standard reduction potential at basic pHs of the couple Te/TeO\textsubscript{2}\textsuperscript{2+} = -0.42 V); based on the dissociation constants of tellurous acid (3 \times 10\textsuperscript{-2} and 2 \times 10\textsuperscript{-8} for \textit{k}_{1} and \textit{k}_{2}, respectively), potassium tellurite at pH 7-0 should be mainly present in the form of HTeO\textsubscript{2}\textsuperscript{2+} and TeO\textsubscript{2}\textsuperscript{2+} (10\textsuperscript{4}/1 ratio) with no Te\textsuperscript{4+} present due to its instability in water; this means that the position of the redox potentials of the redox couples free to react with the respiratory components would be too low (estimated at -0.12 V at pH 7-0) to be reduced by the catalytic centres of membrane-bound oxidases (Poole, 1988) as also previously suggested by others (Trutko et al., 2000). On the other hand, it is reasonable to presume that the periplasmic pH of growing cells would be acidic (at least two pH units lower than cytosolic pH) due to proton extrusion. This local pH might therefore affect the formation of the different forms of tellurium, shifting to more positive values the potentials of the redox couples present so as to be suitable oxidants for the redox-chain components. Keeping these considerations in mind, it is therefore particularly difficult to predict the interaction of TeO\textsubscript{2}\textsuperscript{2+} with the respiratory redox complexes. At the present experimental stage we are therefore tempted to suggest that the most likely thermodynamic interaction, if any (see also below) would be at the quinone pool level (\textit{E}_{m,7} of the redox couples Q\textsuperscript{-}/Q and Q/H\textsubscript{2} of -200 and +90 mV, respectively) in the light of the following results obtained with membranes from KF707 cells grown in the presence of tellurite, namely: (a) the cyt \textit{c} oxidase activity is drastically decreased (50\%) while the cyanide-resistant oxidase activity is enhanced, catalysing most (60–65\%) of the NADH-dependent respiration; (b) cyt \textit{c}- and \textit{b}-type components linked to the upper part of the redox chain are 75\% and 50\% decreased, respectively; SDS-PAGE analysis shows that this drastic decrease involves specifically the soluble cyt \textit{c}-type subunit (\textit{M}_{r} 10000), and the membrane-bound subunits with molecular masses of 33 and 17 kDa; (c) the amount of cyt \textit{b}_{303}, plus \textit{b}_{218} is reduced while a new cyt \textit{b} (\textit{E}_{m,9} = +203 mV) is present; this suggests that the haems with the highest potential are involved in cyt \textit{c} oxidase activity while cyt \textit{b}_{203} would play a role in the cyanide-resistant oxidase. In addition to these specific effects on the respiratory components we have also shown that: (a) tellurite is accumulated by cells of KF707 as granules of elemental tellurium, (b) tellurite reduction to tellurium and cytoplasmic accumulation of tellurium are phenomena restricted to the mid–late-exponential and stationary growth phases although functional and structural modifications of the respiratory chain are already evident in membranes from cells harvested at the beginning of the exponential growth phase (OD\textsubscript{600} 0.5), and (c) the changes observed in membranes from cells grown in the presence of tellurite can be reversed by transferring cells into LB medium without tellurite. These observations, taken together, tend to indicate that the functional and structural variations of the respiratory chain of \textit{P. pseudoalcaligenes} KF707 cells grown in the presence of tellurite and the accumulation of elemental tellurium are separate events. This raises the question whether the modifications observed are specifically required for survival in the presence of tellurite or simply reflect toxic effects of the oxyanion on metabolic pathways not directly related to the respiratory system. We have observed that KF707 cells pre-adapted to grow in the presence of tellurite start growing immediately when transferred into media containing tellurite; conversely, KF707 cells grown in media with no tellurite show a lag period of about 20 h before they start growing. On the other hand, KF707 cells pre-adapted to grow in the presence of tellurite do not show any initial lag period in LB broth and they have an orthodox redox chain after growth in LB broth for a few generations (OD\textsubscript{600} \textless 0.5). This suggests that the variations observed at the level of the respiratory chain in KF707 cells grown in the presence of tellurite are the result of its direct and/or indirect effect on respiratory genes, a phenomenon not necessarily linked with the capacity to grow in the presence of tellurite. In this respect, it is difficult to explain the general decrease of the haem complement in terms of specific metabolic requirements for survival in the presence of tellurite. Further studies are therefore necessary to establish whether modifications of the redox-chain complement are indicative of an active role of respiration in tellurite reduction, as suggested by Trutko et al. (2000), or they constitute, as the present study suggests, a secondary effect of tellurite on metabolic functions such as the thiol-redox buffering system (Turner et al., 1995).
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