An unusual cytochrome *o'*-type cytochrome *c* oxidase in a *Bacillus cereus* cytochrome *aa₃* mutant has a very high affinity for oxygen

Martha L. Contreras,¹ J. Edgardo Escamilla,¹ I. Patricia Del Arenal,¹ J. R. Dávila,² Rita D'mello³ and Robert K. Poole⁴

Author for correspondence: Robert K. Poole. Tel: +44 114 222 4447. Fax: +44 114 272 8697.
e-mail: r.poole@sheffield.ac.uk

¹ Instituto de Fisiología Celular y Depto de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, Apartado postal 70-242, México 04510, D.F., México

² Escuela de Biología, Universidad Autónoma de Puebla, Puebla, México

³ Department of Paediatrics, Imperial College School of Medicine at St Mary's, London W2 1PG, UK

⁴ Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, UK

***Bacillus cereus* strain PYM1 is a mutant unable to synthesize haem A or spectrally detectable cytochromes *aa₃* or *caa₃*. The nature of the remaining oxidase(s) catalysing oxygen uptake has been studied. Respiratory oxidase activities and the levels of cytochromes *b* and *c* increased 2.6- to 4.2-fold on transition from exponential growth, in either of two media, to sporulation stage III, as previously observed for the parent wild-type strain. NADH oxidase activity at both stages of culture was several-fold higher than ascorbate plus tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity, consistent with the TMPD⁻ phenotype of strain PYM1. Oxidase activity with ascorbate as substrate was significant even in the absence of TMPD as electron mediator, suggesting that the terminal oxidase receives electrons from a cytochrome *c*. Carbon monoxide (CO) difference spectra of membranes were obtained using various reductants (ascorbate±TMPD, NADH, dithionite) and revealed a haemoprotein resembling cytochrome *o'*. The CO complex of this cytochrome was photodissociable: the photodissociation spectrum (photolysed minus CO-ligated) exhibited a trough at 416 nm and a peak at 436 nm, together with minor features in the α/β region of the spectrum, consistent with the presence of a cytochrome *o'*-like pigment. CO recombination occurred at -85 to -95 °C. No other haemoproteins showing photoreversible CO binding under these conditions were detected. Evidence that this pigment was the oxidase responsible for substrate oxidation was obtained by photodissociating the CO complex at subzero temperatures in the presence of oxygen; this resulted in faster ligand recombination, attributed to oxygen binding, and extensive oxidation of cytochromes *c* and *b*. The oxygen affinity of the oxidase was determined by using the deoxygenation of oxyleghaemoglobin as a sensitive reporter of dissociated oxygen concentration. A single oxidase was revealed with a *K_m* for oxygen of about 8 nM; this is one of the highest affinities yet reported for a terminal oxidase.**

Keywords: *Bacillus cereus*, cytochrome *o'*-like oxidase, cytochrome *aa₃*, oxygen affinity, sporulation

INTRODUCTION

Mitochondrial cytochrome *c* oxidase (cytochrome *aa₃*) and the numerous bacterial terminal oxidases containing haem A appear to be variants on a common structural theme and members of a superfamily of oxidases

containing haem and copper at the oxygen-reactive centre (Calhoun *et al.*, 1994; Mogi *et al.*, 1994). Sequence analyses of subunits of several oxidases containing haem A reveal phylogenetic relationships between the bacterial and mitochondrial enzymes (Garcia-Horsman *et al.*, 1991; Mogi *et al.*, 1994). Moreover, DNA sequence analysis of the *Escherichia coli* *cyo* operon, encoding cytochrome *bo*, has indicated that this oxidase is a full member of the haem-copper oxidase superfamily

Abbreviations: DCPIP, dichlorophenolindophenol; LHb, leghaemoglobin; TMPD, tetramethyl-*p*-phenylenediamine.

(Calhoun *et al.*, 1994; Mogi *et al.*, 1994), albeit with different redox centres and oxidizing ubiquinol not reduced cytochrome *c*.

There have been several reports of cytochrome *o*-like cytochromes in the *Bacillaceae* (Poole, 1983). Baines *et al.* (1984) and Sone *et al.* (1990) reported the purification of two different cytochrome *o*-like pigments from the thermophilic bacterium PS3. In both cases, the purified cytochromes reacted with carbon monoxide (CO) to give spectral features resembling those of a true cytochrome *o* (see below) and tetramethyl-*p*-phenylenediamine (TMPD) or exogenous cytochrome *c* were oxidized by the purified oxidases at appreciable rates. The cytochrome purified by Baines *et al.* (1984) was obtained from cells grown under forced aeration and comprised a single subunit of molecular mass 47–48 kDa. Sone *et al.* (1990), however, purified the cytochrome from cells grown with limited aeration and found it was composed of three subunits of molecular masses 60, 30 and 16 kDa.

In this older literature, the term cytochrome 'o' was used to describe a CO- and/or oxygen-reactive cytochrome with spectral features similar to the cytochrome *o* first described by Castor & Chance (1959) and thought to have protohaem as the oxygen-reactive haem. The characteristic features of the CO difference spectrum (reduced plus CO minus reduced) are a peak at about 415 nm (due to the CO-ligated form) and a trough near 430 nm (due to the loss of absorbance at this wavelength on binding CO by the reduced form). The features are inverted in the photodissociation spectrum (photolysed, i.e. unligated, reduced minus CO-ligated); such spectra also reveal the much weaker α/β bands (Poole *et al.*, 1979, 1994). The term cytochrome *o* implies that the cytochrome is an oxidase ('o' for oxidase; Castor & Chance, 1959), although rarely since these pioneering and elegant studies has this been demonstrated in any bacterium by photochemical action spectroscopy or the binding of oxygen. Since the spectrally distinctive haem of cytochromes of the *o*-type is the ligand-binding haem, it is appropriate to indicate this in the name by a primed letter, as *o'*, in accord with IUBMB nomenclature (Poole & Chance, 1995). Puustinen & Wikström (1991) showed that this ligand-reactive haem (which they called *o*₃) in the *E. coli* oxidase is of a distinct type, related to haem A, and named it haem O.

Haem O synthesis in the *Bacillaceae* seems to occur under special conditions only, such as slight oxygen-limitation of growth as in PS3 (Sone *et al.*, 1990) or in *Bacillus subtilis* RB-829, a strain deleted for the *ctaA* gene (Svensson *et al.*, 1993). In the former case, it was demonstrated that a cytochrome *o*-type pigment replaced cytochrome *a*₃ in the ligand-reactive centre of cytochrome *caa*₃, to produce a cytochrome *cao* with higher affinity towards oxygen than that shown by cytochrome *caa*₃ (Sone & Fujiwara, 1991). We recently described (Del Arenal *et al.*, 1997) a spontaneous mutant of *Bacillus cereus* (strain PYM1) that lacks haem A and spectroscopically detectable cytochromes *aa*₃ and *caa*₃, but instead produces haem O, high quantities of a

cytochrome *o'*-like pigment and cytochrome *c* at normal or higher levels than in the parent strain. The cytochrome *o*-like pigment showed reactivity with CO and the spectral features of a typical cytochrome *o*–CO complex, but reactivity with oxygen was not demonstrated. Nevertheless, in view of the presence of haem O in cells and the spectral similarities with *E. coli* cytochrome *bo'*, we refer to it here as a cytochrome *o'*-like protein.

The present paper reports the application of low-temperature ligand exchange techniques (Poole *et al.*, 1979, 1994) to elucidate the reaction with CO and oxygen of this putative oxidase in membranes of the *B. cereus* PYM1 mutant. In addition, the apparent affinity (*K*_m) for oxygen uptake by membranes of strain PYM1 has been determined by using the deoxygenation of soybean oxyleghaemoglobin as a suitably sensitive reporter of the dissolved oxygen concentration (D'mello *et al.*, 1994, 1995, 1996). The remarkably high oxygen affinity thus obtained, together with the evidence at low temperatures that the cytochrome does react with oxygen, lead us to propose that the cytochrome *o'*-like pigment is the oxidase responsible for respiratory activity in strain PYM1. Moreover, we show that this oxidase appears to use endogenous cytochrome *c* as physiological electron donor as proposed earlier (Del Arenal *et al.*, 1997).

METHODS

Bacteria and culture techniques. *B. cereus* strain PYM1 is a spontaneous mutant, devoid of the spectrally detectable terminal oxidases, cytochromes *aa*₃ and *caa*₃. This mutant was isolated as described elsewhere (Del Arenal *et al.*, 1997) from a *B. cereus* wild-type strain originally isolated by Andreoli *et al.* (1973). Growth and sporulation were carried out as previously described (Escamilla *et al.*, 1986) by using the exhaustion technique in culture media that contained the salt mixture described for the G-medium of Hanson *et al.* (1963), plus one of the following carbon sources (all quantities expressed as w/v): (i) 0.1% sucrose plus 0.1% yeast extract (sucrose/yeast extract medium, SYM2); or (ii) 0.8% casein hydrolysate, 0.32% glutamic acid, 0.21% DL-alanine and 0.12% L-asparagine (casein hydrolysate medium, CHM; Taber, 1974; Del Arenal *et al.*, 1997). The buffering capacity of medium SYM2 was increased by doubling the amount of K₂HPO₄ recommended by Hanson *et al.* (1963) (i.e. from 0.05% to 0.1%; Del Arenal *et al.*, 1997). The final pH of CHM medium was adjusted to 7.1 with NaOH. Cultures were grown aerobically at 30 °C in a 25 l fermenter stirred at 250 r.p.m. and bubbled with 14 l air min⁻¹. The inoculum (0.5 l) for large-scale growth (20 l) was prepared by the active culture technique of Collier (1957) utilizing a minimum of five serial transfers, in the same medium, at 3 h intervals. Bacterial biomass densities in cultures and cell suspensions were estimated using OD₅₄₀ values after correction for appropriate dilutions.

Preparation of cell membrane fractions. Harvested cells were washed in 50 mM Tris/HCl, 5 mM CaCl₂ and 5 mM MgSO₄ (pH 7.4) (TCM buffer) and resuspended in the same to a concentration of 0.5 g wet wt cells ml⁻¹. Cells were disrupted in a Dyno-mill (WAB Maschinen Fabrik) with 0.1 mm diameter glass beads at 4500 r.p.m. and 4 °C. Unbroken cells

and debris were removed by centrifugation at 8000 *g* for 10 min. Membranes were recovered and washed three times with TCM buffer by centrifugation at 144000 *g* for 45 min.

Assay of respiratory activities. NADH oxidoreductase activity was measured as described by Escamilla *et al.* (1986) at 30 °C in 3 ml of a buffer containing 50 mM potassium phosphate (pH 7.4), 0.08 mM 2,6-dichlorophenolindophenol (DCPIP), 0.1 mM KCN (to inhibit electron transfer to oxygen) and about 0.5 mg membrane protein. The reaction was started with 0.5 mM NADH (final concentration). An absorbance coefficient of 21 mM⁻¹ cm⁻¹ at 600 nm was used for DCPIP.

Oxidase activities were measured as described by Escamilla *et al.* (1986) at 30 °C in a model 53 Oxygen Meter (Yellow Spring Instruments) in 3 ml of a buffer containing 50 mM potassium phosphate, 3.0 mg of membrane protein and one of the following electron donors: 0.5 mM NADH, 3.0 mM sodium ascorbate, or 3.0 mM sodium ascorbate plus 0.1 mM TMPD. The reaction was started by addition of substrate. The pH of the reaction mix was 6.8 for assays with ascorbate, and ascorbate plus TMPD, or 7.4 for NADH.

Spectral analysis of cytochromes at room temperature and 77 K. Electronic absorption difference spectra of membranes suspended in TCM buffer supplemented with 50% glycerol were determined as described earlier (Escamilla *et al.*, 1987) in an SLM DW2000 spectrophotometer at room temperature (10 mm pathlength cuvettes) or at 77 K (2 mm pathlength). CO difference spectra at room temperature were recorded in a Johnson Foundation SDB3 dual wavelength spectrophotometer in cuvettes of 10 or 2 mm pathlength. The concentrations of cytochromes were calculated from difference spectra (dithionite-reduced *minus* oxidized) of membranes at room temperature (not shown), using the wavelength pairs and absorption coefficients given by Escamilla *et al.* (1987).

Photodissociation and ligand-exchange spectroscopy. Membrane particles (8 mg protein ml⁻¹) were prepared in potassium phosphate buffer (0.1 M, pH 7.0) containing 30% (v/v) ethylene glycol. Samples were reduced with a few grains of sodium dithionite, 5 mM NADH, 10 mM ascorbate, or 10 mM ascorbate plus 1.0 mM TMPD in cuvettes of 2 mm pathlength as described by Chance *et al.* (1975a) and illustrated in the paper by Jones & Poole (1985). After 30 min incubation, preparations were bubbled with CO (2 min) at room temperature. If oxygen was to be present, the sample was then cooled in an ethanol/dry ice bath to -23 °C and allowed to equilibrate for 5–10 min in the dark. Oxygen was introduced by stirring the sample in the cuvette with vertical strokes of a closely fitting coiled wire for 30 s. Samples were then quickly frozen in an ethanol/dry ice bath at -78 °C and kept for at least 5 min in the dark before further equilibration (10–20 min) at the desired temperature in the sample compartment of a Johnson Foundation SDB3 dual-wavelength spectrophotometer, using the liquid nitrogen cryostat described by Jones & Poole (1985). The sample was scanned twice between 400 and 800 nm and the difference plotted to generate a baseline. The preparation was then exposed to actinic light (the focused beam of a 150 W projector) for 2 min, scanned repetitively at the noted times and, at every scan, the pre-photolysis spectrum stored in the digital memory was subtracted from the incoming spectrum.

Determination of oxygen affinity. This was performed using the sensitive leghaemoglobin (LHb) method as adapted and described in detail by D'mello *et al.* (1994). Oxygenated soybean LHb (a kind gift of Dr F. Bergersen, CSIRO, Canberra, Australia) was prepared using published methods

(Appleby & Bergersen, 1980). The concentration of LHb was determined from the absolute spectrum of the oxygenated form. Samples of LHb were diluted to 10–20 µM in 25 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA, which had been previously sparged with a gas mixture of 1% oxygen in argon. Other methods and the calculations described by Appleby & Bergersen (1980) were used, except that deoxygenation was monitored by a time-shared multi-wavelength spectrophotometer (Chance *et al.*, 1975b). The instrument was used in the dual-wavelength mode with interference filters (Omega Optical) transmitting maximally at 560 nm (bandpass 2.5 nm) and 575 nm (bandpass 2.0 nm) at a chopping frequency of 100 Hz. A glass optical cuvette (1.3 ml, 10 mm pathlength; Radley's) was completely filled with the oxyleghaemoglobin solution (15 µM). The cuvette was sealed with a stopper drilled with a fine hole, through which a Hamilton syringe could be inserted. Substrate (0.5 M NADH, 10 µl) was injected into the cuvette and the stability of the oxygenated form of LHb checked by monitoring ΔA for 5–10 min. After addition of membranes (5–50 µl), deoxygenation of the oxygenated LHb was continuously monitored by plotting the ΔA (575 *minus* 560 nm). Recorder traces were scanned and analysed with Flexitrace software. The rates of oxygen consumption (*V*) and the mean concentrations of free dissolved oxygen (*S*) were calculated using the method of Bergersen & Turner (1979). Graphs were drawn using Cricket Graph and lines fitted using a linear regression feature of that software. The value of *K* (= *k*, oxygen dissociation rate/*k'*, association rate) used in these calculations was 50×10^{-9} M for oxygenated LHb (Appleby & Bergersen, 1980). Plots of *V* versus *V/S* (Eadie-Hofstee) were used to calculate *K_m* values.

RESULTS

Oxidase activities and cytochrome complement of strain PYM1

Previous studies (Escamilla *et al.*, 1986, 1987) have shown that a *B. cereus* wild-type strain expresses the highest respiratory activities and cytochrome levels during the early stages of sporulation. We therefore compared membrane particles prepared from mutant PYM1 cells cultured on SYM2 and harvested either during exponential growth (OD₅₄₀ 2.5, after correction for appropriate dilution) or at stage III of sporulation.

Table 1 shows that respiratory oxidase activities and cytochrome levels in strain PYM1 increased significantly (2.6- to 4.2-fold) during the transition from vegetative growth to sporulation stage III, in agreement with the increment demonstrated in the wild-type strain during the same period of development (Escamilla *et al.*, 1986, 1987). Similar behaviour was shown for strain PYM1 cultured in medium supplemented with casein hydrolysate (CHM; not shown). The activity of NADH oxidase, at both stages of culture, was several-fold higher than the activity of ascorbate/TMPD oxidase, in agreement with the TMPD⁻ phenotype sought in isolating mutant PYM1 (Del Arenal *et al.*, 1997).

It has been reported that TMPD oxidase activity in membranes of *B. subtilis* (Van der Oost *et al.*, 1991) and in *Bacillus firmus* OF4 (Gilmour and Krulwich, 1997) is mostly attributable to cytochrome *caa*₃. However, cytochromes *aa*₃ and *caa*₃ were not detected in mem-

Table 1. Respiratory activities and cytochrome content of membrane particles prepared from vegetative and sporulating cells of *B. cereus* PYM1Vegetative cells (OD_{540} 2.5) and cells at stage III of sporulation (SPOIII) were cultured on SYM2.

	Vegetative, exponential-phase	SPOIII phase	Ratio: SPOIII phase/exponential phase
NADH-DCPIP oxidoreductase activity [nmol NADH min ⁻¹ (mg protein) ⁻¹]	67	200	3.0
Oxidase activity [ng atom O min ⁻¹ (mg protein) ⁻¹] with:			
NADH	31	104	3.4
Ascorbate	8.0	21	2.6
Ascorbate/TMPD	10.7	30	2.8
Cytochrome content [nmol (mg protein) ⁻¹]*			
<i>c</i>	0.07	0.3	4.2
<i>b</i>	0.21	0.45	2.1
<i>o'</i> -like	0.10	0.32	3.2
<i>aa</i> ₃	ND	ND	—

ND, Not detected.

*Cytochrome concentrations were calculated from dithionite-reduced *minus* persulphate-oxidized spectra or CO difference spectra at room temperature, using wavelength pairs and absorption coefficients reported elsewhere (Del Arenal *et al.*, 1997). **Fig. 1.** Cytochrome reduction by various substrates in membranes from *B. cereus* strain PYM1 cultured on SYM2 and harvested at: mid-exponential phase (OD_{540} 2.5, traces C); early stationary phase (OD_{540} 5.0, traces B); or sporulation stage III (traces A). Membranes (6.0 mg protein ml⁻¹) were incubated for 30 min at room temperature with the reductants sodium dithionite (a), 5.0 mM NADH (b), 10 mM ascorbate (c), or 10 mM ascorbate plus 1.0 mM TMPD (d). In all cases, reference spectra were obtained by oxidation of the samples with a few grains of ammonium persulphate. Spectra were recorded at 77 K using cuvettes of 2 mm pathlength with a spectral bandwidth of 3 nm. Bars represent ΔA of 0.1.

branes of strain PYM1, as judged by the absence of absorbance peaks near 600 nm in reduced *minus* oxidized spectra (Fig. 1; see later). Therefore, the TMPD

oxidase activity observed in these membranes presumably reflects the expression of an alternative oxidase such as the cytochrome *o'*-like pigment (determined as

Fig. 2. CO difference spectra at room temperature (a) and photodissociation spectra of the CO compound (b) of the cytochrome *o'*-like pigment in membranes obtained from exponential-phase cells of strain PYM1 grown on CHM. Membranes (8.0 mg protein ml⁻¹) were reduced by dithionite (traces A), NADH (traces B), ascorbate plus TMPD (traces C), or ascorbate alone (traces D). After 30 min reduction, CO was bubbled into the samples for 3 min. Reduced-CO *minus* reduced spectra were recorded at room temperature. For photodissociation spectra (b), the reduced, CO-ligated preparations were frozen and scanned at -105 °C. Flat baselines (not shown) were obtained by subtracting the reduced-CO spectrum from a second scan of the same preparation. Each sample was then photolysed at -105 °C and scanned against the reduced-CO spectrum stored in the digital memory of the spectrophotometer. Bars represent ΔA of 0.016 in (a) and 0.04 in (b).

the CO compound, see Table 1), which is absent in the wild-type strain as judged by haem O assays (Del Arenal *et al.*, 1977).

Even though cytochromes *aa*₃ and *caa*₃ were absent in strain PYM1, the expression levels and pattern of regulation of cytochrome *c* were normal (Table 1), and similar to the wild-type strain (Del Arenal *et al.*, 1997), thus suggesting a functional role for cytochrome *c* in strain PYM1. This view is supported by the fact that ascorbate alone (i.e. without the electron mediator TMPD) supported significant and similar respiratory activities in both vegetative and sporulating cells (Table 1). These data are consistent with parallel decreases in both respiration rates and cytochrome oxidation states during titration with cyanide (Del Arenal *et al.*, 1997). Furthermore, NADH oxidation inhibited by 2-n-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) was restored by adding TMPD, suggesting donation of electrons to a *c*-type cytochrome downstream of (mena)quinol (not shown).

Substrate reduction patterns

The cytochromes reduced by various substrates are shown in Fig. 1 in spectra recorded at 77 K. Dithionite (Fig. 1a), NADH (b), ascorbate (c) and ascorbate plus TMPD (d) were each used to reduce membranes isolated from strain PYM1 cultured on SYM2 and harvested during exponential growth (OD_{540} 2.5, traces C), early stationary phase (OD_{540} 5.0, traces B) and sporulation stage III (traces A). As reported earlier for dithionite reduction of PYM1 whole cells (Del Arenal *et al.*, 1997), membranes showed qualitatively similar reduction patterns with all electron donors tested and at all stages of culture tested. The total concentration of cytochrome(s) *c* (bands at 416, 520 and 548 nm), as measured by dithionite reduction (Fig. 1a) and the level of

reduction of cytochrome *c* (relative to that of other cytochromes) obtained with the other substrates (Fig. 1b–d) were significantly higher for sporulating cells (traces A in each of Fig. 1a–d) than for other cell types. Van der Oost *et al.* (1991) have reported that ascorbate alone may be used to identify those terminal oxidases that use endogenous cytochrome *c* as electron donor (e.g. cytochrome *caa*₃ in *B. subtilis*). Here, bands at 553 and 560 nm were seen in the presence of ascorbate alone (Fig. 1c), thus suggesting the reduction of a cytochrome *b*- or *o*-like pigment by this substrate without the requirement for TMPD as mediator.

CO difference and photodissociation spectra

Membranes obtained from vegetative PYM1 cells, reduced by the same set of electron donors tested in Fig. 1, were reacted with CO and the reduced plus CO *minus* reduced spectra were recorded at room temperature. The CO difference spectra thus obtained (Fig. 2a) had the same spectral features, again irrespective of the electron donor used (traces A–D). The major spectral features were a peak at 417 nm and a trough at 433 nm. The α/β regions of the spectra exhibited peaks at about 540 nm and 568 nm, and a trough at 558 nm. These features are consistent with the presence of a cytochrome *o'*-CO complex, similar to that shown in *E. coli* (Poole *et al.*, 1979, 1994). No clear indication of the presence of other potential terminal oxidase–CO adducts was seen.

CO difference spectra (as in Fig. 2a) reveal all CO-reactive haemoproteins, including hydroperoxidases and globins as well as putative oxidases. To identify oxidases, which, in general, exhibit relatively slow CO recombination kinetics at subzero temperatures, low-temperature photodissociation spectra were recorded. When the above CO-ligated and anoxic preparations, frozen at -105 °C, were exposed to actinic light, the

bound CO was photodissociated allowing recording of the photodissociation spectra (photolysed *minus* pre-photolysis, Fig. 2b). The spectra were similar for all the reductants tested (i.e. dithionite, NADH, ascorbate/TMPD or ascorbate alone; traces A–D, respectively, in Fig. 2b). Troughs were seen at 418, 535 and 568 nm. A prominent peak near 436 nm, a peak at 550 nm with a shoulder near 557 nm and a discrete peak at 577–580 nm (not labelled) were also seen. These absorbance maxima arise from generation of the unligated, reduced cytochrome following dissociation of CO. These spectral features resemble the photodissociation difference spectrum of cytochrome *o'* of *E. coli* (Poole *et al.*, 1994), except for the red-shifted absorbance maximum in the Soret region in some spectra (432–436 nm) relative to that for *E. coli* cytochrome *o'* (432 nm).

To assign tentatively a spin type to the *o'*-type oxidase, we adopted the proposal of Wood (1984), who pointed to the gross differences in CO difference spectra for high-spin CO-binding haemoproteins (e.g. reduced myoglobin) and low-spin CO-binding haemoproteins (e.g. the reduced pyridine haemochrome of myoglobin). The former have a high γ/α ratio, i.e. a high ratio of absorbance in the Soret region to that in the α regions. To determine this ratio, we used low concentrations of membranes (i.e. 2.0 mg protein ml⁻¹, data not shown) to obviate artefacts due to excessive light scattering at the lowest (Soret) wavelengths. The ratio of absorbance change in the Soret region (434 nm peak *minus* 418 nm trough) to that in the α region (567 nm trough *minus* 580 nm, i.e. the 'peak-trough' (as defined by Wood, 1984) was 26–28. This high value (close to the value of 30 as proposed by Wood, 1984) suggests the presence of a high-spin *o'*- or *b*-type cytochrome in membranes of *B. cereus* mutant strain PYM1.

Membrane preparations obtained from stationary and sporulating cells gave results similar (not shown) to those described for vegetative cells in Fig. 2(a–b); therefore subsequent experiments were performed on preparations from vegetative cells only.

Kinetics of CO rebinding

The progress of CO rebinding to the cytochrome *o'*-like pigment of *B. cereus* PYM1 was studied by photolysis of the CO compound at subzero temperatures, using the technique described by Poole *et al.* (1979). Membrane particles from vegetative mutant PYM1 cells cultured on CHM were reduced with NADH and bubbled with CO. Anoxic samples were frozen at the desired temperatures (e.g. –95 °C; Fig. 3) and photolysed as described in Methods. Such spectra showed a peak at 432 nm and a trough at 420 nm, consistent with the presence of cytochrome *o'* (Poole *et al.*, 1979, 1994), and the approach of successive scans to the baseline (not shown), i.e. the spectrum of the ligated enzyme. Furthermore, the absorbance changes in the γ region at –75 °C and –85 °C (not shown), and –95 °C (Fig. 3) were symmetrical with respect to the baseline, i.e. changes at 420–444 nm and 432–444 nm were equal in magnitude

Fig. 3. Kinetics of the binding of CO following photolysis at –95 °C in the absence of oxygen. NADH-reduced, CO-ligated membranes (8.0 mg protein ml⁻¹) from exponentially growing cells of strain PYM1, grown on CHM, were photolysed and repetitively scanned at the times indicated (min) alongside the spectra. The reference spectrum of the NADH-reduced, CO-ligated sample was subtracted to obtain the difference spectra shown. The bar represents ΔA of 0.01.

Table 2. Velocity of the rates of oxygen and CO binding to the cytochrome *o'*-like oxidase at subzero temperatures

Spectra were recorded repetitively at timed intervals after photolysis and at the temperatures shown. The progress of the reaction was determined from measurements at 432 (peak) and 420 (trough) nm, and half-times of recombination ($t_{1/2}$) assessed from semi-logarithmic plots of ΔA in the initial phase of the reaction.

Reaction with O ₂		Reaction with CO	
Temperature (°C)	$t_{1/2}$ (min)	Temperature (°C)	$t_{1/2}$ (min)
–90	5.0	–65	5.2
–100	6.7	–75	9.0
–105	7.5	–85	13.0
–114	8.1	–95	22.00

but in opposite directions. This is also in agreement with the behaviour described for *E. coli* cytochrome *o'* (Poole *et al.*, 1979, 1994). Recombination rates of CO with the cytochrome *o'*-like pigment are given in Table 2. Below

Fig. 4. Kinetics of the reaction of oxygen with the cytochrome *o'*-like pigment at -114°C (a) and oxidation of cytochrome *c* at -75°C following photolysis of the CO adduct in the presence of oxygen (b). Membrane preparations reduced by 5 mM NADH and treated with CO were supplemented with oxygen by vigorous stirring at -23°C . Spectra were scanned at the temperatures noted, stored in the digital memory of the spectrophotometer and subtracted from a subsequent scan of the unphotolysed sample to give the reduced plus CO minus reduced plus CO baselines (not shown). The samples were then photolysed and repetitively scanned at the times indicated (min) alongside the traces against the stored pre-photolysis spectrum. The lowest traces in both panels represent the last scan minus the first scan taken after photolysis; these spectra are offset downwards for clarity. Samples contained 8.0 mg of membrane protein ml^{-1} . Bars represent ΔA of 0.025 (a) and 0.01 (b) except for the lowest trace, where it represents 0.005 in both (a) and (b).

-95°C , the reaction with CO was so slow as to make a detailed analysis of the recombination problematic.

The reaction with oxygen at subzero temperatures

When oxygen was introduced to the CO-ligated sample (reduced by NADH) at about -23°C and the sample was photolysed by white light at -114°C , the photodissociation spectrum recorded within the first minute after photolysis (Fig. 4a) showed the same features as the post-photolysis spectrum taken at -105°C under anoxic conditions (Fig. 2b trace B), except for variation by 2–4 nm in the Soret signals. This result is interpreted as photolysis of the CO adduct, irrespective of the absence or presence of oxygen, to give the unligated oxidase haem, which then reacts with CO or oxygen. The progress of the reaction with oxygen was followed by repetitive scanning at the times (given in min) shown in Fig. 4(a). The absorption changes towards the baseline (not shown) were indicative of ligand binding. That this ligand was oxygen and not CO was demonstrated by comparison with the much slower reaction time course following photolysis under anoxic conditions (Table 2). The decrease in absorbance at 432 nm is attributable to

the reaction of the cytochrome *o'*-like pigment with oxygen to give a light-insensitive oxy-complex similar to that observed in *E. coli* (Poole *et al.*, 1979). The asymmetry in the approach of the 419 nm trough and the 432 nm peak to the baseline is marked (cf. Fig. 3) and reflects the greater similarity of the oxygenated form to the CO adduct at 432 nm than at 419 nm, where the maximal absorption for the CO adduct is found. The α/β regions of the first spectrum after photolysis showed troughs at 530 and 567 nm and a broad peak extending from 549 to about 556 nm, thus approximating to the reduced minus CO-reduced difference spectrum seen after photolysis of the CO adduct under anaerobic conditions (see Fig. 2b). Successive scans revealed a deepening of the signal at 567 nm and development of a second trough at 556 nm, whereas little change occurred at about 530 nm. This behaviour is also in agreement with the oxygen reaction of the cytochrome *o'* in *E. coli* (Poole *et al.*, 1979, 1994) and of the cytochrome *o'*-like pigment of *B. subtilis* FG83 (James *et al.*, 1989). When the first post-photolysis spectrum (0 min) was subtracted from the last spectrum, recorded 130 min later, the resulting difference spectrum (offset in Fig. 4a) revealed troughs at 549 and 556 nm, suggesting that significant

oxidation of *c*- and *b*-type cytochromes had occurred during the experiment. The 432 nm trough indicates substantial oxidation of cytochrome *b* relative to the CO-ligated state.

The components responsible for electron transfer to the terminal oxidase were further investigated by obtaining photodissociation spectra at higher temperatures in the presence of oxygen, where the oxidation of the oxidase is achieved more rapidly and is followed by sequential oxidation of upstream components. Following photolysis at -75°C (Fig. 4b), the 432 nm peak of the reduced form of the cytochrome *o*-like pigment was seen only transiently. Successive scans taken after loss of the 432 nm peak showed no changes in the 430–440 nm region of the spectra that would indicate oxidation of an *a*-type cytochrome; instead, in the α/β region, the small peak seen at 550 nm in the first post-photodissociation spectrum, disappeared and was succeeded by the formation of a deep trough at the same wavelength. Indeed, the last (90 min) spectrum *minus* the first (0 min) post-photodissociation difference spectrum (offset in Fig. 4b) showed that the major change that occurred during this period was the formation of a deep trough at 550 nm, again suggesting extensive oxidation of a *c*-type cytochrome. Most of the deepening of the signals at 556 and 567 nm occurred before the first post-photolysis spectrum was recorded (see Fig. 4a) and are seen as small shoulders at these wavelengths on the deep 550 nm trough of cytochrome *c*. The Soret trough at 420 nm can also be assigned to cytochrome *c*. These results strongly support our previous view (Del Arenal *et al.*, 1997) that a cytochrome *c* acts as electron donor to the cytochrome *o'*-like pigment present in *B. cereus* strain PYM1.

The reaction of the cytochrome *o'*-like pigment with oxygen was measured as the diminution of the peak height at 432 nm using 444 nm as reference wavelength in successive difference spectra recorded after photolysis of the CO compound. The reaction proceeded with pseudo-first-order kinetics to give the half-times shown in Table 2, where the much faster kinetics in the presence of oxygen are evident.

Oxygen affinity of the cytochrome *o'*-like pigment in strain PYM1

Experiments involving CO/O₂ ligand exchange at sub-zero temperatures revealed a cytochrome *o'*-like pigment as sole candidate to carry out the function of terminal oxidase in *B. cereus* strain PYM1. To determine the oxygen affinity of this component and seek confirmation that further components could not be identified on the basis of their oxygen affinity, we took advantage of the fact that oxygenated LHB provides dispersed free oxygen at suitably low concentrations and its optical spectra during deoxygenation indicates the mean oxygen concentration (Appleby & Bergersen 1980; Bergersen & Turner 1979, 1980). Measuring absorbance changes at 560 *minus* 575 nm monitors the oxy-deoxy transition that accompanies respiration and oxygen removal from solution. Experiments with LHB as a source of oxygen

Fig. 5. Determination of the oxygen affinity of the cytochrome *o'*-like pigment of *B. cereus* strain PYM1 by deoxygenation of oxyleghaemoglobin. Absorbance data were used to calculate the Eadie-Hofstee plot shown. The K_m value calculated from the slope was 8.3 nM. Samples contained potassium phosphate buffer (pH 7.0), 0.1 mg membrane protein and 10–20 μM LHB in a final volume of 1.3 ml. The reaction was started by addition of 0.5 mM NADH (final concentration).

with membranes of vegetative cells of strain PYM1, respiring 5 mM NADH, revealed a single kinetic component in the Eadie-Hofstee plot (Fig. 5), with a K_m value of 8.3 nM (mean of five replicate determinations). This value indicates a remarkably high affinity for oxygen. The number of data points accessible by this method and the linearity of the plot strongly suggests the absence of any other oxidase component.

DISCUSSION

The results presented in this paper demonstrate the presence of a CO-binding cytochrome *o'*-like pigment that is expressed in the spontaneous mutant PYM1 of *B. cereus* (Del Arenal *et al.*, 1997). This strain produces haem O but lacks spectrally detectable cytochromes *aa*₃ and *caa*₃, the converse of the situation in the wild-type strain. The cytochrome *o'*-like pigment could be reduced by physiological and non-physiological electron donors (Fig. 1) and the reduced form was reactive with CO, producing a CO adduct (Fig. 2a) that fulfilled the spectral criteria of a typical cytochrome *o'*-CO complex, as described for *E. coli* (Poole *et al.*, 1979, 1994). This CO compound was photo-sensitive (Fig. 2b) and its photodissociation spectra also agreed with those described for the cytochrome *o'* of *E. coli* (Poole *et al.*, 1979, 1994). Upon photodissociation at temperatures above -95°C under anoxic conditions, CO-rebinding occurred with the expected slow first-order kinetics and with the symmetrical return of the 420 nm trough and 432 nm peak to the baseline (Fig. 3). The calculated half-time for the binding of CO was about fourfold greater than the reaction with oxygen at -90 to -100°C

(Table 2) and this result agrees with the kinetics observed for the cytochrome *o'* of *E. coli* (Poole *et al.*, 1979). In cytochrome *c* oxidase, for example in *Rhodobacter sphaeroides*, reduced cytochrome *a₃* is the primary site of CO binding and oxygen reduction but, on photolysis of the *a₃*-CO adduct, CO is transiently transferred to Cu_B which is located only about 0.5 nm away. At sufficiently low temperatures, the Cu_B-CO adduct is stable and can be studied by Fourier-transform infrared (FTIR) spectroscopy (Mitchell *et al.*, 1996). FTIR spectroscopy has also shown that the cytochrome *bo'*-type oxidase of *E. coli* has a haem-copper binuclear centre similar to that in cytochrome *c* oxidase (Hill *et al.*, 1992). Further evidence for the involvement of copper in CO binding kinetics comes from experiments in which the oxidase copper content is minimized by growth of *E. coli* in copper-depleted medium (Cicognani *et al.*, 1992) with consequent increases in CO-binding rates. Recently, Muntyan *et al.* (1998) have confirmed and extended these studies and shown that a variety of oxidases from several bacterial species exhibit much faster CO recombination after photolysis when copper is depleted. Thus, the low-temperature kinetic behaviour described here for *B. cereus* cytochrome *o'* suggests that it too has a copper atom sufficiently close to the ligand-reactive haem that it can transiently bind CO after low-temperature photodissociation.

Although the ability of the cytochrome *o'*-like pigment to act as terminal oxidase has not been established using the classical criterion of photochemical action spectroscopy (Castor & Chance 1959), this cytochrome otherwise exhibited appropriate criteria for it to be considered as a terminal oxidase, since (1) it forms a photodissociable CO compound, (2) it reacts with oxygen and catalyses the oxygen-dependent oxidation of cytochrome *c* and (3) it has a very low K_m for oxygen. Thus its designation as a cytochrome *c* oxidase seems appropriate. This is in agreement with previous reports for *B. subtilis* W23 (de Vrij *et al.*, 1987) and the bacterium PS3 (Baines *et al.*, 1984; Sone *et al.* 1990) claiming that *c*-type cytochromes might act as electron donors to cytochrome *o'*-like pigments in the *Bacillaceae*. Supporting this view was the observation that membranes of strain PYM1 carried out a significant respiratory activity with ascorbate alone (Table 1). This activity was 70–80% of that shown with ascorbate plus TMPD and 30% of that activity supported by NADH oxidation (Table 1). Moreover, the absence of absorption bands of other recognized terminal oxidases (i.e. cytochromes *aa₃*, *caa₃* and *d*), in all the spectral studies performed lead us to conclude that the cytochrome *o'*-like pigment is the major or sole oxidase responsible for the respiratory activity displayed by strain PYM1 of *B. cereus*. However, we cannot rule out the possibility that a minor amount of an oxidase with a very high turnover number might also contribute (see Poole & Hill, 1997 for an illustration).

Oxygen-uptake kinetics obtained by using LHb as reporter of oxygen concentrations showed the presence of a single oxidase component with a very high affinity

for oxygen (K_m 8.3 nM; see Fig. 5). Use of oxygenated globins has previously demonstrated K_m values in the submicromolar range for cytochrome *o'* of *E. coli* (D'mello *et al.*, 1995). The K_m reported here for the cytochrome *o'*-type oxidase of *B. cereus* is amongst the lowest reported; the K_m for cytochrome *bd* in *E. coli* is 3–8 nM, and that of the symbiosis-specific '*cbb₃*-type' oxidase of *Bradyrhizobium japonicum* is 7 nM (Preisig *et al.*, 1996), all measurements being made using the procedure adopted in the present work. The high affinity of the cytochrome *o'*-like oxidase in mutant strain PYM1 suggests that this oxidase should be able to support aerobic respiration during growth at very low dissolved oxygen tensions. This raises the question of the possible role for the haem-A-containing oxidases present in the wild-type strain. However, recent work on various bacteria has suggested that certain oxidases may have key physiological roles other than in respiratory electron transport. For example Cyd[−] mutants of *E. coli* lacking cytochrome *bd* have a pleiotropic phenotype that includes poor survival in stationary phase and inability to grow in the presence of siderophores or synthetic iron chelators (see Cook *et al.*, 1998 and references therein). None of these phenotypes can be attributed directly to the loss of the oxidase activity *per se*, even though cytochrome *bd* has an exceptionally high oxygen affinity, because the phenotypes are observed at oxygen concentrations orders of magnitude higher than the K_m of the remaining oxidase. Similarly, a *Staphylococcus aureus* mutant unable to survive long-term starvation has been shown (Clements *et al.*, 1999) to harbour a mutation in *ctaA* which results in inability to synthesize cytochrome *aa₃*. The basis of these growth and survival defects in certain oxidase-deficient mutants is unknown.

The oxidase described here warrants further study. The issues to be addressed include: (i) its molecular mass and subunit composition compared to the cytochromes *aa₃* and *caa₃* previously described in *B. cereus* (Del Arenal *et al.*, 1997); (ii) molecular association between the oxidase and its proposed electron donor, cytochrome *c*; and (iii) identification and molar ratio of metals and haems associated with the purified oxidase, particularly the proper identification of haem O associated with the CO-binding and oxygen-activating centre of the enzyme. All these issues are currently under study.

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