Expression of Cytochrome Oxidases in *Bacillus cereus*: Effects of Oxygen Tension and Carbon Source

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The expression of three putative terminal oxidases (cytochromes aa_3 , o and d) of *Bacillus cereus* was studied in response to oxygen tension and the nature of the carbon source used for growth. High levels of cytochromes b and o were expressed irrespective of culture conditions. Oxygen deprivation caused a large drop in the levels of cytochromes c and aa_3 . Cytochrome d appeared at low oxygen tensions, in anaerobic cultures and during slow aerobic growth on poorly utilized carbon sources (casein hydrolysate). No cytochrome d was detected during fast aerobic growth in fermentable (sucrose-containing) media or when NaNO_3 was added to anaerobic cultures. Cytochromes aa_3 , o and d were reduced by physiological substrates (NADH and succinate) and by ascorbate plus tetramethyl- p -phenylenediamine (TMPD). Oxidase activity using ascorbate-TMPD as electron donor was significantly more resistant to cyanide inhibition in cells containing none or trace amounts of cytochrome aa_3 . Thus, the involvement of cytochromes o and d in the cyanide-resistant respiration of *Bacillus cereus* is suggested.

INTRODUCTION

The complexity and diversity of bacterial respiratory systems has attracted the attention of many research workers in recent years (Poole, 1983). Significant variations in the composition and organization of the electron transport chains are observed in different species, and also within the same strain in response to environmental conditions. The respiratory systems of *Bacillus* spp. have not received much attention, even though *B. subtilis* was the first prokaryote that was examined for cytochromes (Poole, 1983). Early studies on *Bacillus megaterium* (Broberg & Smith, 1967), *B. subtilis* (Tochikubo, 1971) and *B. cereus* (Felix & Lundgren, 1973) revealed the presence of cytochromes a , b and aa_3 . The presence of cytochrome o in *B. megaterium* (Broberg & Smith, 1967), *B. subtilis* (Tochikubo, 1971) and *B. cereus* has also been suggested (Escamilla & Benito, 1984). Although cytochrome d (formerly called cytochrome a_2) has rarely been found in combination with cytochrome aa_3 (Poole, 1983), its presence in *B. megaterium* and *B. cereus* has been suggested by Hoggarth *et al.* (1977) and by ourselves (Escamilla & Benito, 1984). Therefore, the regulation of the expression of the *B. cereus* respiratory system was further studied, in particular the expression of the three putative terminal oxidases (cytochromes aa_3 , o and d) in response to prevailing culture conditions.

METHODS

Organism and growth. A *Bacillus cereus* strain originally isolated by Andreoli *et al.* (1973) was grown in either modified G medium (Hanson *et al.*, 1963) containing 0.2% yeast extract and 0.4% sucrose as fermentable carbon source, or a medium (Sterlini & Mandelstam, 1969) free of fermentable sugars and containing 0.8% casein

Abbreviation: TMPD, tetramethyl- p -phenylenediamine.

hydrolysate, 0.32% L-glutamic acid, 0.21% DL-alanine, 0.12% L-asparagine and the metal salts mixture of G medium (Hanson *et al.*, 1963) (final pH 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. For oxygen-limited cultures, the medium was not sparged, but air (14 l min⁻¹) was passed through the gas phase of the vessel and agitation was adjusted to 500 r.p.m. For anaerobic growth, the medium was bubbled with oxygen-free nitrogen. Where indicated, anaerobic cultures (sucrose/yeast extract medium) were supplemented with 0.15% NaNO₃. Aerobic cultures were inoculated with 1 litre of an actively growing culture from an orbital incubator (300 r.p.m.) while oxygen-limited and anaerobic cultures were inoculated with 500 ml of a culture grown at 100 r.p.m. Cultures were harvested at the end of the exponential growth phase, and washed twice with cold 50 mM-Tris/HCl (pH 7.4) containing 5 mM-CaCl₂ and 5 mM-MgCl₂ (TCM buffer). Cells were disrupted immediately or stored under liquid nitrogen.

Cell disruption and preparation of membrane fractions. Protease activity was prevented by adding phenylmethylsulphonyl fluoride (15 µg ml⁻¹) to the cell suspension (80 g wet wt in 200 ml of TCM buffer) before disruption. This was done in a Ribi Cell-Fractionator at 207 MPa as described by Escamilla & Benito (1984). Non-disrupted cells were removed by centrifugation at 1500 g for 5 min and membranes were pelleted from the supernatant by centrifugation at 144000 g for 45 min; they were washed twice with TCM buffer under the same conditions. The membranes were used immediately for assay of enzymic activities.

Respiratory activities. Oxidoreductase and oxidase activities were determined as described by Escamilla & Benito (1984). Succinate oxidoreductase was measured at 30 °C in 3 ml of a mixture containing 20 mM-disodium succinate (pH 7.5), 50 mM-potassium phosphate (pH 7.5), 1.1 mM-phenazine methosulphate (PMS), 0.08 mM-sodium 2,6-dichlorophenolindophenol (DCPIP), 0.1 mM-KCN, and membranes (0.25 mg protein). NADH oxidoreductase was measured under the same conditions except that succinate and PMS were replaced by 0.5 mM-NADH. An extinction coefficient of 21 mm⁻¹ cm⁻¹ at 600 nm was used for DCPIP.

Oxidase activities with NADH or succinate as substrates were estimated at 30 °C in a model 52 Oxygen Meter (Yellow Spring Instruments). The reaction vessel contained 3 ml 50 mM-potassium phosphate (pH 7.4) and membranes (1.5 mg protein); the reaction was started by the addition of 40 mM-succinate or 0.5 mM-NADH (final concentrations). Cytochrome *c* oxidase was determined under the same conditions except that the pH was 6.8 and 10 mM-sodium ascorbate plus 0.1 mM-TMPD were used as electron donors.

Spectral analysis of cytochromes. Membranes were suspended in 50 mM-Tris/HCl buffer (pH 7.4) containing 5 mM-CaCl₂, 5 mM-MgCl₂ and 50% (v/v) glycerol, and analysed in an SLM-Aminco DW-2c II spectrophotometer at room temperature (1.0 cm light-path cuvettes) or under liquid nitrogen (2.0 mm light path cuvettes). The concentrations of cytochromes were estimated from difference spectra (dithionite-reduced *minus* persulphate-oxidized or dithionite + CO *minus* dithionite reduced) of membranes at room temperature. A spectral band width of 2.0 nm was used for spectra recorded at room temperature and 77 K. The following wavelength pairs and absorption coefficients were used: cytochrome *aa*₃, 603–630, 16.6 mm⁻¹ cm⁻¹ (Sone, 1986); cytochrome *b*, 563–575, 20 mm⁻¹ cm⁻¹ (De Vrij *et al.*, 1983); cytochrome *c*, 550–540, 19 mm⁻¹ cm⁻¹ (De Vrij *et al.*, 1983); cytochrome *aa*₃-CO, 590–605, 8.1 mm⁻¹ cm⁻¹ (Yoshida & Fee, 1984); cytochrome *o*-CO, 415–430, 145 mm⁻¹ cm⁻¹ (Kita *et al.*, 1984); cytochrome *d*, 630–650, 18.8 mm⁻¹ cm⁻¹ (Kita *et al.*, 1984).

RESULTS AND DISCUSSION

Effect of carbon source and aeration on growth

The growth properties of *B. cereus* in a medium containing sucrose/yeast extract or casein hydrolysate under different aeration conditions were examined. Under forced aeration, the fastest growth rate (generation time 28 min) was obtained in the sucrose/yeast extract medium. When casein hydrolysate was the only carbon source, growth rate decreased significantly (generation time 60 min). Limited aeration and anaerobiosis caused a dramatic drop of growth rate, generation times being 182 min and 290 min respectively, in sucrose/yeast extract medium. Supplementation of the medium with 0.15% NaNO₃ improved growth under anaerobic conditions (generation time 170 min). Although a minor role in the generation of ATP has been assigned to the respiratory system of *B. cereus* during vegetative growth on fermentable carbon sources (Goldman & Blumenthal, 1964), it seems that an adequate supply of oxygen is needed for fast growth. On the other hand, it has been postulated that anaerobic growth of other bacteria in the presence of NO₃⁻ is limited by the accumulation of NO₂⁻ (Meijer *et al.*, 1979).

Respiratory activities

Dehydrogenase and oxidase activities of membranes from cells grown under the conditions described above were compared (Table 1). Cells grown with vigorous aeration in medium

Table 1. Respiratory activities and cytochromes in membranes of *B. cereus* grown under different culture conditions

SYE, sucrose/yeast extract medium; CH, casein hydrolysate medium.

Activity	Medium ...	Aerobic		Oxygen-limited	Anaerobic	
		SYE	CH		SYE - NO ₃ ⁻	SYE + NO ₃ ⁻
Dehydrogenase*						
NADH		55	150	45	32	40
Succinate		86	360	80	71	94
Oxidase						
NADH		20	45	15	5	7.5
Succinate		7.5	58	6	4	4.5
Ascorbate-TMPD		17	38	10	7	5
Cytochrome†						
<i>b</i>		0.12	0.29	0.18	0.14	0.13
<i>c</i>		0.05	0.15	0.02	0.02	0.02
<i>aa</i> ₃		0.072	0.21	0.03	0.01	0.02
<i>d</i>		ND	0.01	0.013	0.013	ND
<i>o</i> ‡		0.044	0.154	0.066	0.08	0.08
<i>a</i> ₃ ‡		0.05	0.16	ND	ND	ND

ND, Not detected or too low to be calculated (see Figs 2 and 3).

* Dehydrogenase activity is expressed as nmol DCPIP reduced min⁻¹ (mg membrane protein)⁻¹. Values are means of three different cultures. The SD values were less than 15% of the figures given.

† Cytochrome concentration are given as nmol (mg membrane protein)⁻¹ and were calculated from (dithionite-reduced *minus* persulphate-oxidized) or (dithionite-reduced + CO *minus* dithionite-reduced) difference spectra at room temperature. The wavelength pairs and millimolar absorption coefficients given in Methods were used. Data are means of at least four different cultures. The SD values were less than 20% of the figures given.

‡ Measured in CO difference spectra.

supplemented with casein hydrolysate exhibited the highest levels of NADH and succinate oxidoreductases as well as oxidase activities for NADH, succinate and ascorbate plus TMPD. In contrast, cells cultured in well-aerated sucrose/yeast extract medium exhibited significantly lower activities (Table 1); the availability of a fermentable carbon source, such as sucrose, may repress the respiratory system. Significantly lower activities were obtained when oxygen was limited or excluded (Table 1) during culture in sucrose yeast extract medium.

Cytochromes

Oxygen tension and the nature of the carbon source in culture media had marked effects on the expression of the cytochrome system of *B. cereus* (Figs 1 and 2). As reported previously (Escamilla & Benito, 1984) the dithionite-reduced *minus* persulphate-oxidized spectra at 77 K of membranes from cells grown in well-aerated sucrose/yeast extract medium (Fig. 1*a*) showed the presence of cytochrome *b* with a maximum at 559 nm and a shoulder at 562 nm tentatively identified as cytochrome *o*. A shoulder at 550 nm for cytochrome *c* was also seen, while cytochrome *aa*₃ was well-defined at 601 nm. Absorbance at 632 nm, which would have suggested the presence of cytochrome *d*, was not observed. On the other hand, a clear peak at 632 nm was present in membranes of cells grown in well-aerated casein hydrolysate medium as well as in oxygen-limited and anaerobic cultures in sucrose/yeast extract medium (Fig. 1*b-d*). The addition of 0.15% NaNO₃ to the anaerobic cultures interfered with the expression of cytochrome *d* (Fig. 1*e*), in agreement with work on *Haemophilus parainfluenzae* (Sinclair & White, 1970), in which it was shown that NO₃⁻ represses the anaerobic expression of cytochrome *d*. A lowering of the oxygen tension in the culture produced a drastic drop in the content of cytochromes *aa*₃ (Fig. 1*c*). This has been reported for other bacteria containing cytochrome *aa*₃

Fig. 1. Difference spectra (dithionite-reduced *minus* persulphate-oxidized) of membranes from vegetative cells cultured under different conditions: (a) aerobic growth in sucrose/yeast extract medium; (b) aerobic growth in casein hydrolysate medium; (c) oxygen-limited growth in sucrose/yeast extract medium; (d) anaerobic growth in sucrose/yeast extract medium; (e) anaerobic growth in sucrose/yeast extract medium supplemented with 0.15% NaNO_3 ; (f) aerobic growth in casein hydrolysate medium supplemented with 0.1 mM-KCN. Spectra were recorded at 77 K. Membrane protein concentrations were as follows (mg ml^{-1}): (a) 13; (b) 7.5; (c-f) 12.

(Poole, 1983). Under anaerobic conditions, only trace amounts of cytochrome aa_3 (602 nm peak) were detected (Fig. 1d, e), whereas the peak height of cytochrome b (559 nm) was not significantly affected by the oxygen tension (Fig. 1c, d, e). Cells cultured in casein hydrolysate medium under forced aeration contained diverse cytochromes at high concentrations (Fig. 1b). Interestingly, levels of cytochrome d in these cells increased on addition of 0.25 mM-KCN to the culture (Fig. 1f); this is in agreement with previous reports in other bacteria containing cytochrome d (Ashcroft & Haddock, 1975).

The CO difference spectra (Fig. 2) of dithionite-reduced membranes revealed the presence of a CO-complex resembling that of cytochrome o , with a peak at 575 nm, a peak at 540 nm and a Soret peak at 419 nm. The troughs appeared at 560 nm and 430 nm. This CO-complex was present in cells grown under all the conditions studied (Fig. 2a-e). Increased concentrations of this presumptive cytochrome o were found in cells grown in well-aerated casein hydrolysate medium and oxygen-limited sucrose/yeast extract medium (Fig. 2b, c). In contrast, cytochrome aa_3 , measured as its CO-complex (445 nm trough, 592 nm peak), fell dramatically in cells cultured at low oxygen tensions or anaerobically (Fig. 2c-e).

It is known that the reduced peak of cytochrome d at 632 nm is shifted to higher wavelengths on bubbling with CO (Poole, 1983). This peak was observed at 639 nm in cells grown in aerobic casein hydrolysate medium, as well as in cells obtained from oxygen-limited and anaerobic sucrose yeast extract medium (Fig. 2b-d). The cytochrome d -CO complex was not detected in anaerobic cultures supplemented with 0.15% NaNO_3 (Fig. 2e).

Fig. 2. CO difference spectra of dithionite-reduced membranes of vegetative cells cultured as follows: (a) aerobically in sucrose/yeast extract medium; (b) aerobically in casein hydrolysate medium; (c) under oxygen-limited conditions in sucrose/yeast extract medium; (d) anaerobically in sucrose/yeast extract medium; (e) anaerobically in sucrose/yeast extract medium supplemented with 0.15% NaNO_3 . Membrane suspensions were reduced with a few grains of dithionite, mixed thoroughly and divided into two equal portions. Contents of sample cuvettes were bubbled with CO for 5 min and subsequently the spectra were recorded at room temperature. Membrane protein concentrations were as follows (mg ml^{-1}): (a, d and e) 12; (b) 7.5; (c) 8.5.

The addition of NADH, succinate or ascorbate plus TMPD to membranes from cells cultured under all the conditions described showed qualitatively the same reduction pattern (data not shown) obtained with dithionite (Fig. 1). Notably, cytochrome aa_3 (602 nm), a cytochrome b (562 nm) and cytochrome d (632 nm) were detected regardless of the substrate used for reduction.

The concentrations of each of the cytochromes were calculated (Table 1). Cells grown under vigorous aeration in casein hydrolysate medium contained cytochromes at the highest concentrations. Lowering the oxygen tension of the culture decreased the concentrations of cytochromes aa_3 and c , whereas the concentrations of cytochromes b , o and d increased significantly.

The effect of cyanide on substrate-dependent respiration (Fig. 3) was determined for membranes prepared from cells cultured under the various conditions described. The oxidation of ascorbate plus TMPD by aerobic cells grown in casein hydrolysate medium was inhibited 80% by $20 \mu\text{M}$ -KCN. This concentration caused 50% inhibition of respiration in aerobic cells grown in sucrose/yeast extract medium, and about 20% inhibition in membranes of oxygen-limited and anaerobically grown cells. Thus, those cells that contained the highest respiratory activities were the most sensitive to cyanide.

Oxygen-limited and anaerobically grown cells that contained low levels of cytochromes aa_3 and c and exhibited a low capacity to oxidize ascorbate plus TMPD (Table 1) were more resistant to cyanide. Indeed, all cell types showed a certain proportion of cyanide-insensitive TMPD oxidase (Fig. 3). This cyanide-resistant respiration was most apparent when NADH was the substrate. In the case of cells grown anaerobically in the presence of NO_3^- this cyanide resistance could not be correlated with the detection of cytochrome d (Fig. 1e). However, Hoggarth *et al.* (1977) in *B. megaterium* and Escamilla & Benito (1984) in *B. cereus* demonstrated

Fig. 3. Effect of cyanide on ascorbate-TMPD oxidase activity of membranes of *B. cereus* cultured aerobically in casein hydrolysate medium (■), aerobically in sucrose/yeast extract medium (▲), under oxygen-limited conditions in sucrose/yeast extract medium (△), anaerobically in sucrose/yeast extract medium (●) and anaerobically in sucrose/yeast extract medium supplemented with 0.15% NaNO_3 (○). ---, NADH oxidase activity of cells grown aerobically in casein hydrolysate medium. Membranes (1.0 mg protein) were preincubated (3 min at 30 °C) with the indicated KCN concentrations. The reaction was started by addition of substrate.

(in cyanide difference spectra at 77 K) that the putative cytochrome *o* (562 nm peak) remains oxidized by air, while cytochromes *c* and aa_3 show almost complete reduction. Thus, it is possible that both cytochrome *o* and cytochrome *d* play a role in the cyanide-resistant respiration of *B. cereus* and *B. megaterium*. The expression of cytochrome *d* in *B. cereus* was in agreement with its pattern of regulation reported in other bacteria. Its appearance (Figs 1 and 2) is enhanced by low oxygen tensions (Scott & Poole, 1982), aerobic growth on slowly metabolizable carbon sources (Haddock *et al.*, 1976; Sweet & Peterson, 1978) and during anaerobic growth (Haddock *et al.*, 1976), and is repressed by the inclusion of NO_3^- during anaerobic growth (Sinclair & White, 1970). The presence of cytochrome *d* in *Bacillus* spp. was suggested by Taber (1974), who detected a new absorbance maximum at 627 nm (reduced minus oxidized spectra) in mutants of *B. subtilis* lacking cytochrome *a*; Hoggarth *et al.* (1977) proposed the presence of cytochrome *d* in *B. megaterium*. Studies on *B. cereus* ATCC 4342 (Felix & Lundgren, 1973) and a highly cyanide resistant strain of *B. cereus* (Mc. Feters *et al.*, 1970) showed a discrete absorbance around 630 nm.

The role of cytochromes aa_3 and *o* as terminal oxidases has been demonstrated in aerobically grown cells of *B. subtilis* (Edwards *et al.*, 1981) and in the thermophilic bacterium PS3 (Poole *et al.*, 1982). Such studies remain to be done on *B. cereus* and *B. megaterium*.

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