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Influence of Oxygen Tension on the Respiratory Activity of *Mycobacterium phlei*

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Growth of *Mycobacterium phlei* under low oxygen tension resulted in specific activities two to twenty times lower for formate dehydrogenase, malate dehydrogenase, β-hydroxybutyrate dehydrogenase, lactate oxidase and NADH dehydrogenase than when cultures were grown under high aeration. An increase in fumarate reductase and succinate dehydrogenase occurred with *M. phlei* grown under low oxygen tension. Malate : vitamin K dehydrogenase and glucose-6-phosphate dehydrogenase activity were not significantly affected by the oxygen tension used to grow the bacteria, and neither culture contained a lactate dehydrogenase. With growth of *M. phlei* in conditions of low oxygen tension, cytochrome *a* was not detected, but cytochrome *b* was prominent in membranes and cytochrome *c* was present in the soluble fraction.

INTRODUCTION

The influence of oxygen tension on the growth of mycobacteria has been the subject of numerous reports. In submerged-stationary cultures, the rate of growth of *Mycobacterium tuberculosis* is less than in shaken cultures (Kull & Grimm, 1952; Volk & Myrvik, 1953; Guy *et al.*, 1954). The surface : volume ratio of the cultures is critical in stationary flasks because the oxygen tension is dependent on the depth of the medium, and rate of growth is dependent on oxygen tension (Moore & James, 1982). While mycobacteria grow in oxygen tensions which range from atmospheric to microaerophilic (Jenkins *et al.*, 1982; Guy *et al.*, 1954), strong evidence has been presented by Moore & James (1982) that exponential growth of *M. tuberculosis* can be achieved under conditions of extremely low oxygen tension.

Physiological characteristics of mycobacteria are also influenced by oxygen tension. In a study involving nine different cultures of mycobacteria (Gillespie *et al.*, 1986), colony characteristics, catalase production, nitrate reduction, urease production, Tween hydrolysis and acid production from carbohydrates were influenced by growth at low oxygen tensions. Virulent strains of mycobacteria in general have a greater tolerance to low oxygen tension than avirulent strains (Heplar *et al.*, 1954; Guy *et al.*, 1954), and *M. tuberculosis* can adapt to survive anaerobic exposure (Wayne & Lin, 1982). Growth of *M. tuberculosis* H37R$_v$ under low oxygen tension appears to account for the production of aldolase type I, while in oxygen-rich environments aldolase type II is produced (Jayanthi Bai *et al.*, 1975).

This is a report on the effect of low oxygen tension on the level of cytochromes, dehydrogenases and oxidases produced by *Mycobacterium phlei* with glucose as the energy source. This organism was selected because its respiratory system has been extensively characterized (Asano & Brodie, 1964), and oxygen tension is reported to affect production of NADH dehydrogenase (Herman & Weber, 1978).

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

METHODS

Cultures. Stock cultures of *M. phlei* ATCC 354 were maintained by aerobic growth on Lowenstein–Jensen slopes containing malachite green (Difco). Cultivation of aerobic cultures for enzyme studies was achieved by inoculation of 250 ml Erlenmeyer flasks containing 100 ml 0·8% nutrient broth (BBL), 0·4% yeast extract (BBL), 0·2% Tween 80 (Sigma) and 0·2% glucose (NYTG medium) with incubation at 37 °C on a gyratory shaker at 100 r.p.m. for 2 d.

M. phlei was adapted to grow under low oxygen tension by the modification of a method reported by Gillespie *et al.* (1986). *M. phlei* was inoculated into a 13 × 125 mm tube of Fluid Thioglycollate Medium (BBL) and incubated at 37 °C for 4 d. An inoculum (0·1 ml) was taken from the bottom of the microaerophilic zone and transferred to a second tube of thioglycollate medium. By the second transfer, cells of *M. phlei* grew throughout the anaerobic region in the tube of the thioglycollate medium; 1 ml of such a culture was used to inoculate 500 ml Erlenmeyer flasks filled with freshly prepared NYTG medium to which was added 0·05% sodium thioglycollate. The inoculated flasks were placed in GasPak System jars, 12 × 20 cm (BBL), along with a kit for the generation of H_2 plus CO_2 (Oxoid); the catalyst for the removal of oxygen was contained in the non-vented lid. As reflected by a methylene blue indicator system (BBL), oxygen in the jar was depleted within 2 h of the gas generating kits being activated and the jars sealed. Incubation was for 4 d at 37 °C in the dark. We are not prepared to claim anaerobic growth of a Mycobacterium at this stage, since we have not measured oxygen tension directly in the growth medium. However, differences in physiology are evident between *M. phlei* grown as described above and grown aerobically.

Inocula were from single colony isolates on NYTG media solidifed with 1·5% (w/v) agar.

Cell-free extracts. Cells of *M. phlei* were collected by centrifugation at 5000 ***g*** for 10 min at 4 °C and were washed twice in 0·05 M-Tris/HCl buffer, pH 7·5. The packed cells were suspended in 0·05 M-Tris/HCl, pH 7·5 [1 g (wet wt) cells : 2 ml buffer] and disrupted at $6·9 \times 10^4$ kPa in a French pressure cell. The viscosity of the extract was reduced by addition of 0·01 to 0·05 mg DNAase III ml^{-1} (Sigma) and the cell extract was centrifuged at 20000 ***g*** for 30 min at 4 °C.

The clarified cell-free extract was layered over a discontinuous sucrose gradient of 20% (w/v) and 60% (w/v) sucrose (Barton *et al.*, 1983) containing 0·01 M-Tris/HCl, pH 7·8. After centrifugation at 100000 ***g*** for 2 h at 5 °C, the soluble protein fraction above the 20% sucrose layer was collected; the plasma membrane fraction was then collected from the interface of the 20 and 60% sucrose layers. The soluble fraction was dialysed against 0·01 M-Tris/HCl, pH 7·6, at 4 °C to remove endogenous material which might interfere with enzyme assays. Protein was measured by the Lowry method.

Enzyme assays. Spectrophotometric assays for carbon metabolizing enzymes were done in 2 ml anaerobic cuvettes with a 1 cm lightpath. The final volume of the reaction mixtures was 1·5 ml and the methods of Gorrell & Uffen (1978) were used in the preparation of anaerobic buffers, electron acceptors, substrates and reaction mixtures. The cell fraction, electron acceptor solution and buffer were placed in the spectrophotometer cuvette under a steady flow of purified N_2. Before addition of the electron donor, the cuvette was fitted with a serum stopper containing a needle for gas to exit and the cuvette was flushed for an additional 10 min with N_2. The reaction was initiated by the addition of the electron donor from an anaerobic solution through the use of a gas-tight syringe.

Incubation was at 25 °C and changes in absorbance at specified wavelengths were followed for 15 min. Enzyme activities were calculated from initial velocities where the changes in absorbance were linear and at the greatest rate. Corrections for endogenous activity in cell-free fractions were made by subtracting the values for controls, which contained no added electron donors, from the experimental values.

Reduction of 2,6-dichlorophenolindophenol (DCIP) was followed at 610 nm and used to measure malate : vitamin K dehydrogenase (EC 1.1.99.16) as described by Asano & Brodie (1964), formate dehydrogenase (EC 1.2.1.2) as described by Dehyle & Barton (1977), succinate dehydrogenase (EC 1.3.99.1) as described by Reddy & Weber (1986) and lactate oxidase (EC 1.13.12.4) as described by Lockridge *et al.* (1972). All the published procedures were modified in that 0·06 M-MOPS/NaOH, pH 7·5, was used as the buffer in the reaction mixtures.

Measurements at 340 nm were used to follow pyridine nucleotide coupled reactions of fumaric reductase (EC 1.3.1.6) as described by Harvey & Lascelles (1980), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) as described by Hohorst (1965*a*), malate dehydrogenase (EC 1.1.1.49) as described by Asano & Brodie (1964), β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) as described by Bock & Fleisher (1974), malic enzyme (EC 1.1.1.40) as described by Singer & Lusty (1965) and lactate dehydrogenase (EC 1.1.1.27) in the forward and reverse reactions using the procedures of Hohorst (1965*b*). NADH dehydrogenase (EC 1.6.99.3) was assayed in aerobic cuvettes according to the method of Murthy & Brodie (1964). The high level of NADH dehydrogenase in aerobic reactions meant that all other pyridine nucleotide coupled reactions had to be done under anaerobic conditions. The only modification to the published procedures was the use of 0·06 M-MOPS/NaOH, pH 7·5, as the buffer.

Cytochrome assays. Reduced *minus* oxidized difference spectra on cell fractions were done according to the procedures of Falk (1964). Potassium ferricyanide (15 μmol) was added to the reference cuvette to fully oxidize cytochromes and 2 mg of sodium dithionite ml^{-1} was added to the experimental cuvette to reduce the cytochromes. Absorbance was recorded from 630 to 400 nm with a Varian-Cary 219 recording spectrophotometer. These experiments for cytochrome analysis used 25 mg protein ml^{-1} of clarified cell extract, 16 mg protein ml^{-1} of membranes and 5 mg protein ml^{-1} of soluble fraction.

RESULTS

The oxygen tension under which *M. phlei* was grown influenced the activities of some dehydrogenases and affected electron transport systems. Formate dehydrogenase, malate dehydrogenase and lactate oxidase activities were higher in cells grown aerobically than in cells grown under low oxygen tension (Table 1). Succinate dependent reduction of DCIP was fourteen times greater in cells grown under low oxygen tension than in cells grown in aerobic culture. Malate : vitamin K dehydrogenase activities were similar in cells grown under both growth conditions. The oxygen tension during growth did not affect the distribution of these enzymes in the soluble or membrane fraction.

The effect of oxygen on *M. phlei* was also evident from the activities of NAD coupled dehydrogenases (Table 2). NADH dehydrogenase, malate dehydrogenase and β-hydroxybutyrate dehydrogenase activities were 6 to 25 times greater in cells grown in aerobic culture than in cells from low oxygen tension. Glucose-6-phosphate dehydrogenase activity was essentially unaffected by the oxygen tension under which cells were grown. Approximately four times greater activity of fumarate reductase was observed in cells under low oxygen tension than in cells grown aerobically. While the greatest activity of fumarate reductase was seen in the membrane fraction, some activity was observed also in the soluble fraction (Table 2). Lactate dehydrogenase and malic enzyme were not detected in either culture of *M. phlei.*

Growth of *M. phlei* under aerobic conditions resulted in the production of *a*, *b* and *c*-type cytochromes (Fig. 1). Cytochromes of the *a*-type, which had an α-peak at 598 nm in the membranes of aerobically grown cells, were absent from cells grown under conditions of limited oxygen tension. In cells grown under low oxygen tension, peaks at 562, 530 and 430 nm indicated

Table 1. *Comparison of the activities of enzymes which reduce DCIP in M. phlei grown aerobically or under low oxygen tension*

The reaction mixture (1·5 ml) contained 0·06 M-MOPS/NaOH, pH 7·5, 0·03 M-substrate (sodium formate, sodium malate, sodium lactate or sodium succinate), 0·074 mM-DCIP, and clarified cell extract (5 mg protein), membrane fraction (3 mg protein) or soluble fraction (2 mg protein). Malate : vitamin K reductase was differentiated from malate dehydrogenase by measuring the increase in DCIP reduction in the presence of 0·04 mM-FAD. Incubation was at 25 °C for 10 min and specific activity is expressed as nmol DCIP reduced min^{-1} (mg protein)$^{-1}$. Each assay was done at least three times with results expressed as mean values. Replicate values did not vary more than 5% from the mean.

Enzyme	Cell preparation	Specific activity: Aerobic	Specific activity: Low oxygen tension
Formate dehydrogenase	Clarified extract	0·68	0·34
	Membrane	0·99	0·48
	Soluble	<0·01	<0·01
Malate dehydrogenase	Membrane	2·92	0·06
	Soluble	<0·01	<0·01
Malate : vitamin K dehydrogenase	Membrane	0·92	0·47
	Soluble	3·80	3·47
Lactate oxidase	Clarified extract	10·98	0·50
Succinate dehydrogenase	Clarified extract	1·23	17·12
	Membrane	1·42	19·99
	Soluble	0·11	1·62

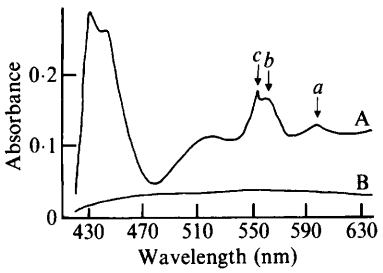

Fig. 1

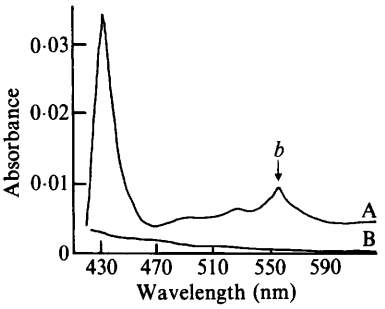

Fig. 2

Fig. 1. Difference spectra of cell-free extracts from *M. phlei* grown in aerobic culture. Cuvettes contained 25 mg protein ml^{-1} of clarified extract from aerobic culture. A, Reduced *minus* oxidized spectrum (the α-peaks of cytochrome *a* (597 nm), *b* (562 nm) and *c* (553 nm) are indicated). B, Oxidized *minus* oxidized control.

Fig. 2 Difference spectra of membrane fraction from *M. phlei* grown at low oxygen tension. Cuvettes contained 16 mg membrane protein ml^{-1}. A, Reduced *minus* oxidized spectrum showing cytochrome *b* with absorbance peaks at 562, 530 and 430 nm. B, Oxidized *minus* oxidized control.

Table 2. *Comparison of the activities of pyridine nucleotide coupled dehydrogenases and reductase in M. phlei grown aerobically or under low oxygen tension*

The reaction mixture (1·5 ml) contained 0·06 M-MOPS/NaOH, pH 7·5, 0·03 M-substrate (sodium malate, sodium fumarate, or sodium salts of β-hydroxybutyrate or glucose 6-phosphate), 1·4 mM-NAD(P) or NAD + H^+, 0·25 M-β-mercaptoethanol, and clarified cell extract (6 mg protein), membrane fraction (2 mg protein) or soluble fraction (3 mg protein). NADH dehydrogenase was measured by the addition of 1·0 mM-NADH, the elimination of β-mercaptoethanol from the above assay mixture and incubation of the reaction under aerobic conditions. Reaction mixtures were incubated at 25 °C for 10 min and specific activities are expressed as nmol NAD(P) reduced or NADH + H^+ oxidized min^{-1} (mg protein)$^{-1}$. Each assay was done at least three times with results expressed as mean values. Replicate values did not vary more than 5% from the mean.

Enzyme	Cell preparation	Specific activity: Aerobic	Specific activity: Low oxygen tension
NADH dehydrogenase	Clarified extract	12·18	1·21
Malate dehydrogenase	Membrane	9·49	0·39
	Soluble	<0·01	<0·01
β-Hydroxybutyrate dehydrogenase	Membrane	<0·02	<0·01
	Soluble	60·69	11·83
Glucose-6-phosphate dehydrogenase	Clarified extract	10·30	10·85
Fumarate reductase	Membrane	1·93	7·19
	Soluble	0·63	4·56

that *b*-type cytochromes were present in the membrane fraction (Fig. 2) while peaks at 553, 520 and 408 nm indicated that *c*-type cytochromes were present in the soluble fraction (Fig. 3).

DISCUSSION

Some marked differences between *M. phlei* grown under low oxygen tension and *M. phlei* grown aerobically were evident in this study. When oxygen was the final electron acceptor a respiratory chain containing many of cytochromes was present, and multiple dehydrogenases generated electrons for the electron transport system (Asano & Brodie, 1964). Under oxygen-limited conditions, electron acceptors other than oxygen must function. In this study,

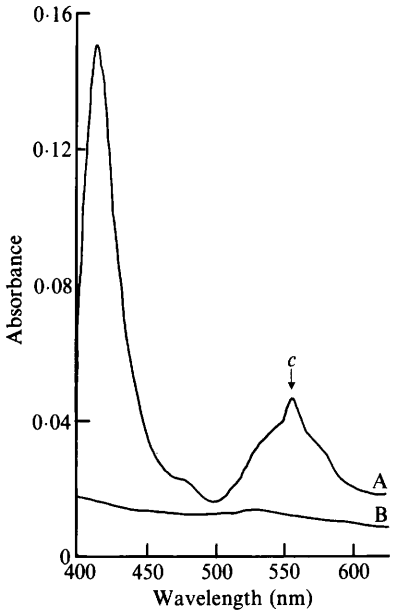

Fig. 3. Difference spectra of soluble fraction from *M. phlei* grown at low oxygen tension. Cuvettes contained 5 mg protein ml^{-1}. A, Reduced *minus* oxidized spectrum showing cytochrome *c* with absorbance peaks at 553, 520 and 408 nm. B, Oxidized *minus* oxidized control.

diminished activities of lactate oxidase, NADH dehydrogenase, formate dehydrogenase, malate dehydrogenase and β-hydroxybutyrate dehydrogenase were observed in *M. phlei* grown under low oxygen tension. Conversely, fumarate reductase activity was elevated. A high level of DCIP reduction with succinate in cell-free fractions could reflect, in part, contribution of fumarate reductase measured in the reverse direction. Perhaps fumarate functions as a final electron acceptor in *M. phlei* when it is growing under low oxygen tension, in a manner similar to *Escherichia coli* and *Vibrio succinogenes* (Jones, 1982). Since fumarate reductase systems in bacteria are cytochrome *b* linked (Jones, 1982), cytochrome *b* detected in membranes of *M. phlei* grown under low oxygen tension may be important for fumarate reduction. If succinate is an end-product of respiration, this could explain acid production from carbohydrates with mycobacteria grown under low oxygen tension (Gillespie *et al.*, 1986).

Lactate oxidase activity was twenty times greater in aerobic cultures of *M. phlei* than in *M. phlei* grown under low oxygen tension. This dependence of lactate oxidase synthesis on oxygen is consistent with the inducible character of this enzyme in mycobacteria (see Ratledge, 1976, for a review). Since lactate dehydrogenase was absent in the cultures of *M. phlei*, it is conceivable that appropriate conditions may be present in the cells to allow conversion of lactate to pyruvate as has been reported in controlled anaerobic experiments (Lockridge *et al.*, 1972). The low levels of lactate oxidase in cells grown under low oxygen tension make it unlikely that pyruvate would be produced by this enzyme under such conditions.

From this study it is evident that the oxygen tension under which *M. phlei* grows has an influence on the type of cytochromes and respiratory coupled dehydrogenases produced. It will be important in future studies to determine the extent to which fermentation and fumarate respiration function in mycobacteria grown under low oxygen tension. Such studies will provide valuable information on the energetic capabilities of mycobacteria, help in the understanding of what are increasingly important microbes that can adapt to changes in oxygen tension and maybe elucidate the range of environments where mycobacteria may be found.

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