BBA 46140

# THE RESPIRATORY SYSTEM OF THE AEROBIC, NITROGEN-FIXING, GRAM-POSITIVE BACTERIUM, *MYCOBACTERIUM FLAVUM* 301*

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(Received February 23rd, 1971)

SUMMARY

1. The membrane bound respiratory system of the Gram-positive, obligate aerobic, $N_2$-fixing bacterium, *Mycobacterium flavum* 301 was investigated. It contains cytochromes *a*, $a_3$, *b*, *c*, and *o*; ubiquinone (Q-8), carotenoids and NADH and succinate dehydrogenases. No membrane bound malate or lactate oxidase activity was detected.

2. Sensitivities to inhibitors emphasized the similarities between this system and mitochondrial ones. The *Mycobacterium flavum* system is sensitive to antimycin A, rotenone, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, cyanide, azide, in concentrations similar to those required in mitochondrial systems.

3. These properties put *M. flavum* into a unique position among the bacteria in regards to respiratory system.

## INTRODUCTION

*Mycobacterium flavum* 301, a Gram-positive, obligate aerobic bacterium capable of fixing $N_2$ was first isolated from Russian turf podzol soils in 1960, by FEDOROV AND KALININSKAYA[1]. Since these soils are acidic, Mycobacteria are one of the few nitrogen fixers capable of growth in such an environment.

BIGGINS AND POSTGATE[2] studied the nitrogenase system of *M. flavum* and found it to be similar to that of Azotobacter[3]. Both systems are particulate and are unable to utilize pyruvate as a source of electrons and ATP. However, the nitrogenase from *M. flavum* appears more sensitive to $O_2$ (ref. 2).

Since nothing was known about the respiratory system of *M. flavum*, it was thought a study of it would prove useful.

## MATERIALS AND METHODS

### *Chemicals*

NADH, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), antimycin A, were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; cytochrome *c*, from

Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; TMPD, tetramethylphenylenediamine.

* Parts of this work were presented at the Colloquium on Bioenergetics: Energy Transduction in Respiration and Photosynthesis, Pugnochioso, Italy, September, 1970.

Boehringer Corp., Gmbh, Mannheim, Germany; yeast extract, from Oxoid, Ltd., London, Great Britain; silicic acid from Mallinckrodt, New York, N.Y., U.S.A.; Silica gel G, from E. Merck, AG, Darmstadt, Germany; polyamide for thin layer chromatography from Macherey, Nagel and Co., 516 Duren, Germany. We are indebted to Hoffmann-LaRoche, Basle, Switzerland, for the ubiquinone homologues.

All other chemicals were obtained from British Drug Houses Ltd., Poole, Great Britain, and were the finest grade obtainable. Glass double distilled water was used throughout.

*Culture and maintenance*

*M. flavum* 301 was maintained on agar slopes containing the nitrogen-free medium with succinate as a carbon source described by BIGGINS AND POSTGATE[2]. A slope was used to inoculate 150 ml of either $N_2$-free or ammonium-containing media in a 500-ml flask. The bacteria were grown with shaking at 150 rev./min at 30° on a gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) to late log phase. 10 ml of this was used to inoculate 600 ml of media in 2-l flasks which were grown to late log phase as above.

*Preparation of electron transport particles*

The cells were harvested by centrifuging for 5 min at 27500 × *g* in an MSE-18 refrigerated centrifuge. The cells were suspended in 0.05 M sodium–potassium phosphate buffer, pH 6.8 (10 ml buffer for each 10 g wet weight of cells). They were sonicated with cooling in ice at 15-sec intervals for 3 min on an MSE sonicator at full output. The broken cell suspension was centrifuged 15 min at 19000 × *g* at 5° in an MSE-19 centrifuge to remove whole cells and debris. The supernatant was carefully removed and centrifuged 1 h at 150000 × *g* at 5° in an MSE-50 refrigerated centrifuge. The pellet was suspended in the same buffer as above. Electron transport particles were prepared fresh for each experiment. Protein was assayed by the modified biuret method of GORNALL *et al.*[4].

*Measurement of oxidase activities*

$O_2$ uptake was measured at 30° using a Clark electrode. The reaction mixture contained 500 μmoles $KH_2PO_4$–$Na_2HPO_4$ buffer, pH 6.8, 0.6–1.2 mg particle protein, 15 μmoles substrate and glass distilled water to a final volume of 2.0 ml.

*Determination of cytochrome levels*

The concentrations of cytochromes in the electron transport particles were calculated routinely from the room temperature dithionite reduced *minus* oxidised difference spectra. Cytochromes $a_3$ and *o* were demonstrated by the CO–dithionite reduced *minus* reduced difference spectra. Concentrations of these two cytochromes were not estimated because the spectral overlap makes it difficult to obtain quantitative results. Substrate reduced *minus* oxidised spectra were also done. Extinction coefficients used were those for mammalian cytochromes: cytochrome *a*, $A_{598\,nm} - A_{623\,nm}$, $\varepsilon = 24000$ (ref. 5); cytochrome *b*, $A_{560\,nm} - A_{570\,nm}$, $\varepsilon = 20000$ (ref. 6); cytochrome *c*, $A_{550\,nm} - A_{540\,nm}$, $\varepsilon = 21000$ (ref. 7). Low temperature difference spectra (77°K) were done through the kind courtesy of Dr. Derek Bendall, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain.

*Determination of pyridine hemochromogens*

The particles were extracted with acetone–HCl to separate the proto-heme from the covalently linked meso-heme using the procedure of LANYI[8]. Difference spectra were run in NaOH–pyridine reducing with dithionite. Extinction coefficients were those used by APPLEBY[9].

*Determination of the quinone*

50 g of wet cell paste was extracted exhaustively with acetone, the lipid extracted into ethyl ether, the ether removed and the lipid purified by chromatography on a silicic acid–celite column. A $\beta$-carotene and a ubiquinone containing fraction were found. The ubiquinone containing fractions were then further purified on Silica gel G thin layer plates. Spectra of the various fractions were run in cyclohexane and the ubiquinone containing fraction rechromatographed on Silica gel G. Oxidised and reduced spectra of the quinone were done in ethanol, reducing with $KBH_4$. The homologue was determined by chromatography on polyamide thin layer plates[10] with known ubiquinone homologues as references.

*Dual wavelength spectrophotometry*

The reduction states of the individual cytochromes were determined using an Aminco-Chance dual wavelength spectrophotometer (Aminco Instrument Co., Silver Spring, Md., U.S.A.). All measurement were carried out at 25°. The system contained 500 $\mu$moles sodium–potassium phosphate buffer, pH 6.8, 2.0–4.0 mg particle protein and glass distilled water to a final volume of 2.5 ml. The reaction was initiated by the addition of substrate. The following wavelength pairs were used: cytochrome *b*, 560/570 nm; cytochrome *c*, 550/540 nm.

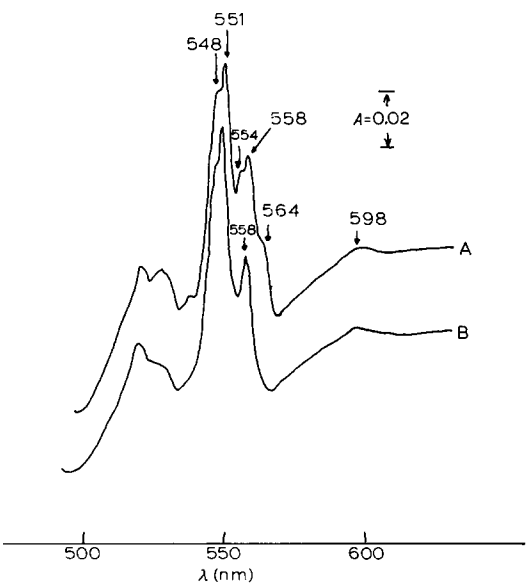


Fig. 1. Difference spectra of respiratory particles from *M. flavum* 301 at 77°K. A. NADH or succinate reduced *minus* oxidised. B. Dithionite reduced *minus* oxidised.

RESULTS

Room temperature difference spectra of whole cells and of respiratory particles from *M. flavum* grown on either ammonia or $N_2$ indicated the presence of mammalian type cytochromes *a*, *b*, *c*, and the presence of two CO-binding pigments with a trough at 443 nm, plateau at 430 nm and a peak at 415 nm, indicative of cytochromes $a_3$ and *o*. Low temperature difference spectra (77°K) of particles reduced with succinate or with NADH (see Fig. 1) showed maxima at 598 nm (assigned to cytochrome *a*), 558 nm (assigned to cytochrome *b*), and a split band at 551 and 548 nm (assigned to cytochrome *c*). The difference spectra obtained with dithionite as reducing agent gave two additional maxima at 564 and 554 nm (see Fig. 1). Cytochrome contents in the electron transport particles were calculated from room temperature difference spectra and are given in Table I for both ammonia and $N_2$-grown cells.

The respiratory particles oxidise NADH, succinate, mammalian cytochrome *c* + ascorbate and tetramethylphenylenediamine (TMPD) + ascorbate. There is no membrane bound malate or lactate oxidase activity. Typical oxidase activities for $N_2$-free and ammonia-grown cells are given in Table I.

TABLE I

CYTOCHROME CONTENT AND OXIDASE ACTIVITIES OF RESPIRATORY PARTICLES FROM *M. flavum* 301 GROWN ON $(NH_4)_2SO_4$ OR ATMOSPHERIC $N_2$

Cytochrome contents are expressed in μmoles/g protein. Oxidase activities are expressed as μatoms $O_2$/min per mg protein.

| *Cytochrome* | | | *Nitrogen source* | *Oxidase activity* | | | |
|---|---|---|---|---|---|---|---|
| *a* | *b* | *c* | | *Succinate* | *NADH* | *Ascorbate +* | |
| | | | | | | *Cytochrome c* | *TMPD* |
| 0.03 | 0.30 | 0.38 | $N_2$ | 0.059 | 0.170 | 0.151 | 0.468 |
| 0.04 | 0.38 | 0.57 | Ammonia | 0.132 | 0.176 | 0.108 | 0.478 |

TABLE II

AEROBIC STEADY STATES AND REDUCED STATES OF CYTOCHROMES *b* AND *c* IN RESPIRATORY PARTICLES OF *M. flavum* 301

Steady states were measured in an Aminco-Chance dual wavelength spectrophotometer at the following wavelength pairs: *b*, 560/570 nm; *c*, 550/540 nm. Experimental conditions were as described in the text. The values are the averages for ten experiments. Similar results were obtained with cells grown on either $N_2$ or $(NH_4)_2SO_4$.

| *Substrate* | *Cytochrome* | *Aerobic steady state* (%) | *Reduced state* (%) |
|---|---|---|---|
| NADH | *b* | 44 | 80 |
| | *c* | 32 | 90 |
| Succinate | *b* | 42 | 80 |
| | *c* | 41 | 90 |

Determination of the pyridine hemochromogens in the respiratory particles from ammonia-grown cells confirmed the presence of proto- and meso-heme groups in the ratio of about 1:2.

Table II shows the aerobic steady state and reduced state levels of cytochromes *b* and *c* in the particles.

TABLE III

EFFECTS OF INHIBITORS ON THE OXIDASE ACTIVITY OF RESPIRATORY PARTICLES FROM *M. flavum* 301

Oxidase activities were measured on a Clark oxygen electrode using NADH or succinate as substrate. Experimental conditions were as described in the text. The results are representative of eight experiments.

| *Inhibitor* | *Concentration necessary for 50% inhibition* |
|---|---|
| Antimycin A | 10 nmoles/mg protein |
| HQNO | 8 nmoles/mg protein |
| Rotenone | 6 nmoles/mg protein |
| KCN | 30 $\mu$M |
| $NaN_3$ | 1 mM |

Table III lists the results of studies with inhibitors. The NADH and succinate oxidase systems are both sensitive to cyanide and azide at concentrations compatible with a cytochrome oxidase as terminal electron acceptor. Both are also sensitive to HQNO and antimycin A, 50% inhibition being achieved at concentrations similar to those required in mitochondrial systems. The NADH oxidase system is sensitive to rotenone.

Studies of aerobic steady states at 77°K in the presence and absence of antimycin A showed that the two maxima at 564 nm and 554 nm, normally not reducible by substrate, have now been reduced in the presence of inhibitor (see Fig. 2).

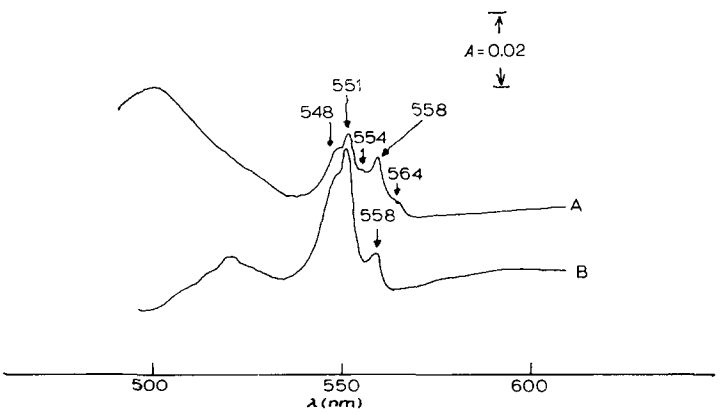


Fig. 2. Difference spectra of respiratory particles from *M. flavum* 301 at 77°K in the aerobic steady state in the presence and absence of antimycin A. The particles were reduced with NADH. A. No antimycin A. B. Antimycin A, 20 nmoles/mg protein.

Spectra of the fractions obtained from thin layer chromatography of the quinone from *M. flavum* indicated the presence of an ubiquinone and no detectable menaquinone. The oxidised and reduced spectra in ethanol are typical of an ubiquinone[12]. The maximum is at 276 nm for the oxidised form and at 290 nm for the reduced form with isosbestic points at 294 nm and 306 nm. Chromatography on polyamide thin layer plates with known ubiquinone homologues as references indicated the *M. flavum* ubiquinone is Q-8 ($R_F$ = 0.52 for *M. flavum* quinone and for an authentic sample of Q-8). In the cells grown under $N_2$-fixing conditions, there was also a small quantity of an ubiquinone like compound that ran with Q-8 on Silica gel G, but ran below it on polyamide plates with an $R_F$ = 0.37 ($R_F$ = 0.42 for Q-10 in this system).

## DISCUSSION

*M. flavum* 301 possesses a mitochondrial type respiratory system which has some unusual features. Its sensitivities to low concentrations of antimytin A and rotenone are unusual in bacteria. To our knowledge, the only other bacterial system showing such sensitivities is that of *Micrococcus denitrificans*[12], and as in this system, the system from *M. flavum* also shows more than 50–60 % inhibition with antimycin A or HQNO only at very high concentrations.

The detection of ubiquinone in this organism is surprising. As far as is known this is the only Gram-positive organism which contains ubiquinone. BISHOP *et al.*[13] studied the distribution of quinones in a large number of bacteria and found that Gram-positive organisms contain only menaquinone; Gram negatives, only ubiquinone, with the exceptions of *Escherichia coli* and *Proteus vulgaris* which contain both. Therefore, the presence of ubiquinone in *M. flavum* is interesting from a taxonomic and an evolutionary viewpoint.

Another similarity to mitochondrial systems is the lack of membrane bound malate or lactate oxidases which are often found in bacterial respiratory systems. The presence of three cytochromes *b* is reminiscent of plant mitochondria[14].

Some dissimilarities are the presence of cytochrome *o*, a terminal oxidase quite common in microorganisms. The different ratio of cytochrome *a* to cytochromes *b* or *c* in *M. flavum* relative to mammalian mitochondrial systems is striking. There is far less cytochrome *a* relative to the other cytochromes. However, the mitochondrial systems from *Neurospora crassa*[15] and *Dictyostelium discoideum*[16] show lower ratios than mammalian mitochondria as do some Gram-positive bacteria possessing mammalian type cytochromes, for example, *Sarcina lutea*[17], although none so low as *M. flavum*.

Since there has been some doubt as to the classification of *M. flavum* as a *Mycobacterium* (J. R. POSTGATE, personal communication) it is interesting to note the important differences between the respiratory systems of *M. flavum* and *Mycobacterium phlei*. *M. phlei* contains menaquinone as its sole quinone and does not show sensitivity of its respiratory chain to rotenone or Antimycin A[18]. The properties of the electron transport system of *M. flavum* would therefore tend to support a different taxonomic classification.

The only apparent differences in the respiratory system from bacteria grown on $N_2$ or ammonia are in the cytochrome *c* content which increases in cells grown on ammonia (also noticed by D. R. BIGGINS, personal communication); the succinate oxidase activity in ammonia-grown cells is also higher. It would be interesting to see

whether growth on a carbon source other than succinate would have the same effect.

The aerobic steady states obtained for cytochromes *b* and *c* particularly with succinate as substrate are too similar to say with certainty that only a $b \rightarrow c$ sequence is involved. The effects of antimycin A on aerobic steady states indicate more work is needed on this problem.

The marked similarities of the *M. flavum* respiratory system to mitochondrial systems in its composition and sensitivity to inhibitors place this bacterium in a potentially interesting position evolutionarily and taxonomically.

Future work is directed towards investigations to further elucidate the positions of components in the respiratory chain and on the oxidative phosphorylation associated with this system.

ACKNOWLEDGEMENTS

We wish to thank Dr. D. R. Biggins, Unit of Nitrogen Fixation, Agricultural Research Council, The Chemical Laboratory, University of Sussex, Brighton, Great Britain, for sending us a culture of *M. flavum* 301, Dr. Derek Bendall, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain, for the low-temperature spectra, and Drs. B. A. C. Ackrell and C. W. Jones for discussion.

REFERENCES

1 M. V. Fedorov and T. O. Kalininskaya, *Mikrobioliya*, 30 (1961) 9.
2 D. R. Biggins and J. R. Postgate, *J. Gen. Microbiol.*, 56 (1969) 181.
3 W. A. Bulen, R. C. Burns and J. R. LeComte, *Biochem. Biophys. Res. Commun.*, 17 (1964) 256.
4 A. E. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, 177 (1949) 751.
5 B. F. van Gelder, *Biochim. Biophys. Acta*, 118 (1966) 36.
6 B. Chance and G. R. Williams, in F. F. Nord, *Advances in Enzymology*, Vol. 17, Interscience, New York, 1956, p. 65.
7 B. F. van Gelder and E. C. Slater, *Biochim. Biophys. Acta*, 58 (1962) 593.
8 J. K. Lanyi, *Arch. Biochem. Biophys.*, 128 (1968) 716.
9 C. A. Appleby, *Biochim. Biophys. Acta*, 172 (1969) 88.
10 K. Egger and H. Kleinig, *Z. Anal. Chem.*, 211 (1965) 187.
11 R. A. Morton, in R. A. Morton, *Biochemistry of Quinones*, Academic Press, London and New York, 1965, p. 40.
12 P. B. Scholes and L. Smith, *Biochim. Biophys. Acta*, 153 (1968) 363.
13 D. H. L. Bishop, K. P. Pandya and H. K. King, *Biochem. J.*, 83 (1962) 606.
14 W. D. Bonner, Jr., in J. E. Falk, R. Lemberg and R. K. Morton, *Haematin Enzymes*, Pergamon Press, London, 1961, p. 479.
15 H. Weiss, G. Van Jagow, M. Klingenberg and T. Bucher, *Eur. J. Biochem.*, 14 (1970) 75.
16 S. K. Erickson and J. M. Ashworth, *Biochem. J.*, 113 (1969) 567.
17 S. K. Erickson and G. L. Parker, *Biochim. Biophys. Acta*, 180 (1969) 56.
18 O. Asano and A. F. Brodie, *J. Biol. Chem.*, 239 (1964) 4280.