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# THE INFLUENCE OF OXYGEN AND NITRATE ON THE FORMATION OF THE CYTOCHROME PIGMENTS OF THE AEROBIC AND ANAEROBIC RESPIRATORY CHAIN OF *MICROCOCCUS DENITRIFICANS*

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SUMMARY

1. The effects of varying the concentrations of terminal electron acceptors on the synthesis of cytochromes by *Micrococcus denitrificans* were studied.

2. Bacteria grown with strong aeration (± nitrate) synthesise cytochrome *b*, *c*, $a$–$a_3$ and *o*. Those grown with low aeration showed oxygen-limited growth even in the presence of nitrate as alternate electron acceptor; they synthesised all the cytochromes listed above *plus* a soluble pigment combining *c*- and *d*-type cytochromes. Anaerobically grown cells did not synthesise cytochromes $a + a_3$, but had a higher content of the soluble *c*–*d* cytochrome.

3. Quantitative differences were observed in the levels of different cytochromes in cells grown in the different conditions, those in cells grown with low aeration being intermediate between aerobically and anaerobically grown bacteria.

4. The results indicated that $O_2$ and not $NO_3^-$ was initially important in determining the type (and total amount) of cytochromes present in anaerobic and aerobic cells, although there was some indication that $NO_2^-$ and $NO_3^-$ might be important in the control of cytochromes concerned with nitrite reduction.

## INTRODUCTION

*Micrococcus denitrificans* can obtain energy both aerobically and anaerobically (using nitrate and nitrite as alternative electron acceptors to oxygen) by the process of electron transport linked respiration. The respiratory chain is reported, by Scholes and Smith[1], to be situated in the cytoplasmic membrane; it resembles the mammalian mitochondrial system in that it includes the cytochrome $a + a_3$ complex, two *c* type and at least one *b*-type cytochrome and ubiquinone, and has NADH and succinate dehydrogenase activities. The total amounts of cytochromes *b* and *c* were found to be higher in cells grown anaerobically whilst the reverse was true for the $a + a_3$ complex.

Newton[2] has described a two-haem cytochrome, with a *c*-type and *d*-type haem, which appears only in the soluble fraction of anaerobically grown cells. It has both nitrite reductase and cytochrome oxidase activity, although Lam and Nicholas[3] report the $K_m$ for oxygen to be very high (27 $\mu$M) compared with a membrane-bound constitutive cytochrome oxidase with a $K_m$ of 0.1 $\mu$M for oxygen.

The *d*-type haem reacts with CO to form a compound with a maximum absorbance at 415 nm in (dithionite reduced *plus* CO) *minus* (dithionite reduced) difference spectra[2,3]. In addition, similar CO difference spectra of cytoplasmic membrane preparations from aerobically grown cells show absorption peaks at 415 and 430 nm[1]. The latter was attributed to the CO compound of cytochrome $a_3$ and the former to that of cytochrome *o*. The cytochrome *o* did not appear to be reduced anaerobically.

Thus differences have been shown in both the total amounts of cytochromes and the type of cytochromes present in aerobic and anaerobically grown cells of *M. denitrificans*, but what is the biochemical explanation for these differences?

Oxygen represses the concentrations of cytochromes *b* and *c* and probably induces the cytochrome $a + a_3$ complex[1]. However, the role of nitrate on the formation of the cytochrome components of the anaerobic electron transport chain has never clearly been established. In this paper we have aimed to distinguish between the effects of the two alternative electron acceptors, oxygen and nitrate by growing the organism with high or low aeration with ammonia as a nitrogen source and comparing the results with cells grown anaerobically and with high or low aeration using nitrate as nitrogen source and as terminal electron acceptor during anaerobic growth. Clearly growth in the presence of both oxygen and nitrate presents a choice of electron acceptors for the organism: investigations of changes in the electron transport carriers under these various conditions may help us understand how the cell responds to such alternatives.

## METHODS AND MATERIALS

### *Organisms and culture conditions*

*M. denitrificans* was maintained on Dorset Egg Medium slopes (Oxoid). The basic medium, used in each experiment, was that of Chang and Morris[4], with either 0.1 M $KNO_3$ or 0.03 M $NH_4Cl$, as the nitrogen source and 0.4% glucose as the carbon source. Table I shows the five different growth conditions used and the flow rates of nitrogen and air, for cultures grown in 10-l quantities, using a 15-l New Brunswick Microferm bench top fermentor. The culture was constantly stirred, the temperature maintained at 30 °C and the initial pH was 6.8. The inoculum was prepared by subculturing the organism onto a slope of appropriate growth medium solidified with 2%

TABLE I

THE DIFFERENT CONDITIONS USED FOR THE GROWTH OF *M. denitrificans*

The basic medium used and the concentrations of the nitrogen sources are described in the text of Methods and Materials.

| *Nitrogen source* | *Gas* | *Flow rate of gas* | *Type of growth* | *Growth condition* |
|---|---|---|---|---|
| $NO_3^-$ | $N_2$ | 1 l/min | Exponential | Anaerobic |
| $NH_4^+$ | Air | 40–60 ml/min | Linear with time Oxygen limited | Low $O_2$ + $NH_4^+$ |
| $NO_3^-$ | Air | 40–60 ml/min | Linear with time, Oxygen limited | Low $O_2$ + $NO_3^-$ |
| $NH_4^+$ | Air | 10 l/min | Exponential | Aerobic + $NH_4^+$ |
| $NO_3^-$ | Air | 10 l/min | Exponential | Aerobic + $NO_3^-$ |

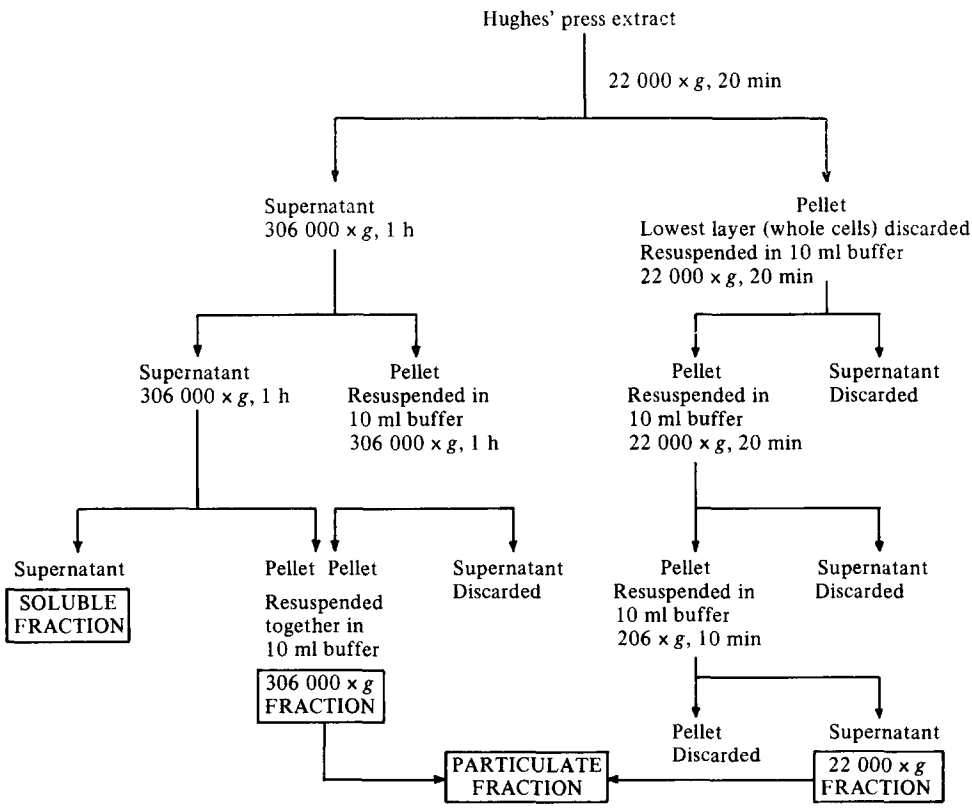
agar, and incubating for 24 h at 30 °C. The slope was then washed off into 500 ml of appropriate growth medium in a 1-l flask and incubated at 30 °C, standing for 24 h, shaken fast for 12 h or slowly shaken for 18 h, for anaerobic, aerobic or low-$O_2$ growths, respectively. Sufficient inoculum was added to the Microferm to give an initial absorbance at 550 nm of about 0.02; growth was then measured optically at this wavelength using a Unicam spectrophotometer.

*Harvesting*

The cells from all growth conditions were harvested at 0–4 °C, when the absorbance at 550 nm was 0.8; they were washed once in 10 mM Tris–HCl buffer (pH 7.2).

*Preparation of extracts*

The cells were crushed in a Hughes' press[5]. The extract was homogenised, in a Kontes glass homogenizer, with deoxyribonuclease (Sigma) and 10 mM Tris–HCl buffer (pH 7.2). This homogenate was fractionated according to Scheme 1 at 0–4 °C. The object of fractionation was merely to separate the soluble and particulate fractions so that they were as free as possible of contamination by each other. To check the distribution of protein, the fractions marked 'discarded' were collected together and the protein in this fraction and the protein content of the soluble and particulate fractions was measured.




Scheme 1. Fractionation scheme for the differential centrifugation of Hughes' press extracts of *M. denitrificans*. Buffer: 10 mM Tris–HCl (pH 7.2).

*Spectra and the determination of total amounts of cytochromes*

All spectra were recorded using a Cary 14 Recording Spectrophotometer, with the 0–0.1 and 0.1–0.2 A slide wire attachment and using 1-cm light path cuvettes. (Dithionite reduced) *minus* (ferricyanide oxidized) difference spectra were recorded between 650 nm and 520 nm. (Dithionite reduced *plus* CO) *minus* (dithionite reduced) difference spectra, were recorded between 500 and 400 nm; the dithionite reduced *minus* CO preparations were obtained by bubbling CO through a dithionite reduced preparation for 2 min.

The total amounts of cytochromes *b* and *c* were determined from (reduced) *minus* (oxidized) difference spectra of both particulate fractions and crude extracts by measuring the change in absorbance ($\Delta A$) between the peak at 553 nm and the shoulder at 560 nm, respectively, and a line joining the 575-nm and 356-nm troughs. Cytochrome *c* in the soluble fraction was measured as $\Delta A$ between the peak at 551 nm and a line connecting the trough at 536 nm with the trough at 565 nm or 575 nm depending upon the growth condition. The total amount of cytochrome $a_3$ and the 418-nm CO-binding pigments of the particulate fractions and crude extracts were measured from the CO spectra, as $\Delta A$ between the shoulder at 435 nm and the peak at 418 nm, respectively, and a line joining the trough at 445 nm to the shoulder at 405 nm. The 418-nm CO-binding pigment of the soluble fraction was measured in the same way but for some growth conditions the trough occurred at 432 nm not 445 nm.

The actual quantity of cytochromes present could only be determined for the particulate fraction, since the extinction coefficients for the soluble cytochrome *c–d* were not known, and the crude extracts contained a mixture of soluble and particulate cytochromes. The extinction coefficients used were $\varepsilon = 20.7 \cdot 10^3$ for cytochrome *b* (Falk[6]), $\varepsilon = 26.8 \cdot 10^3$ for *c* (Scholes *et al.*[7]). $\varepsilon = 80 \cdot 10^3$ for cytochrome *o* (Taber and Morrison[8]) and $\varepsilon = 100 \cdot 10^3$ for cytochrome $a_3$ (Smith[9]). The corrections given by Sinclair and White[10] to allow for the absorption increment of *b* at the *c* peak and *vice versa* were made and then the nmoles of cytochrome per mg of protein were calculated from the corrected figures. Since cytochrome *o*-like cytochrome *b* has a protoheme prosthetic group this cytochrome contributes to the absorbance at the $\alpha$ band of *b*. Thus the following correction was used to calculate the quantity of *b* present.

| Amount of *b* (nmoles/mg protein) | = | nmoles/mg protein *b* calculated and corrected from the peak | − | nmoles/mg protein of cytochrome *o* |
|---|---|---|---|---|

*Protein*

This was measured by the method of Lowry *et al.*[6], using Bovine Serum Albumin Powder (Armour Pharmaceutical Company Ltd) as the standard.

## RESULTS

All data are an average of at least two separate experiments, at each of the five different growth conditions. The $22\,000 \times g$ pellet and $306\,000 \times g$ pellet were assayed separately, but the results for both these fractions were added together, after correcting for total volume, total $\Delta A$ and total protein, and expressed as one value for the 'particulate fraction'. Protein recoveries from fractionation were between 93 and 100%; 20–30% of the protein was particulate, 25–35% soluble and 40–50% was discarded.

*Type of growth under the different growth conditions*

Growth with vigorous aeration and anaerobically was exponential (Fig. 1a).

At the low aeration rate of 40–60 ml of air per min growth was linear with time, indicating that growth was oxygen limited (Fig. 1b).

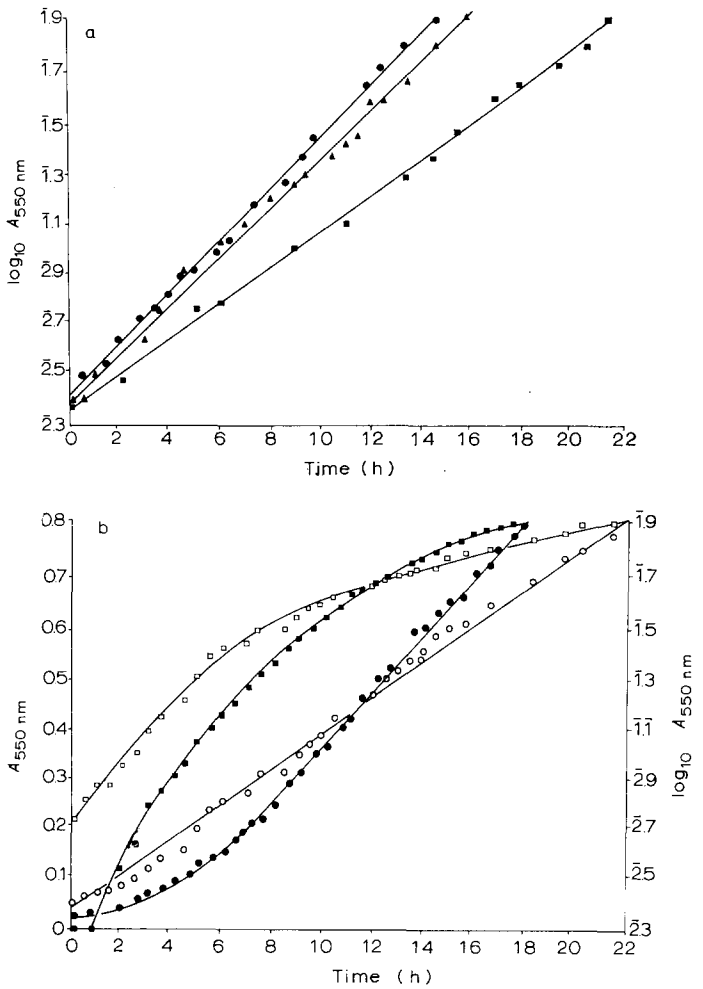



Fig. 1. (a) Exponential growth of *M. denitrificans*. ●—●, aerobic + $NO_3^-$; ▲—▲, aerobic + $NH_4^+$; and ■—■, Anaerobic. (Full details of each growth condition are given in Methods and Materials and Table I.) (b) Oxygen-limited growth of *M. dentrificans*. Linear growth (●—●) and the logarithmic curve (■—■) for cells grown under the low $O_2$ + $NO_3^-$ growth condition. Linear growth (○—○) and the logarithmic curve (□—□) for cells grown under the low $O_2$ + $NH_4^+$ growth condition.

*Qualitative differences in the cytochrome components of cells grown under different conditions*

*Observations with unfractionated extracts.* The α bands of cytochromes $a + a_3$ at 506–610 nm and of the haem of cytochrome *c–d* at 620–630 nm are in the region of the (oxidised) *minus* (reduced) difference spectra where most changes are observed.

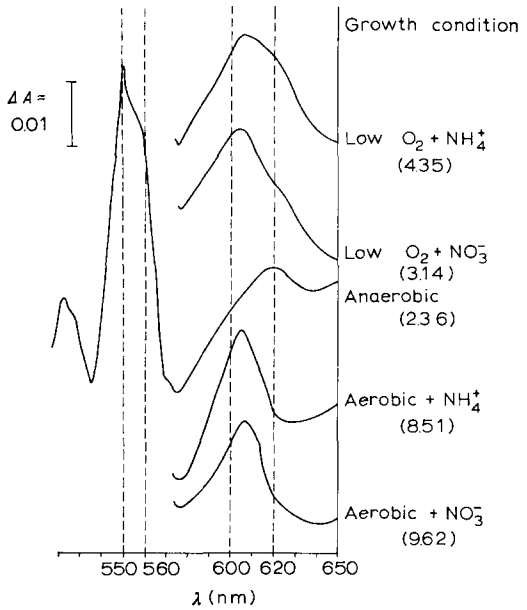



Fig. 2. Qualitative differences in (dithionite reduced) *minus* (ferricyanide oxidized) difference spectra, of unfractionated Hughes' press extracts of *M. denitrificans*. The figure in parentheses (mg/ml) represents the quantity of protein in the preparation.

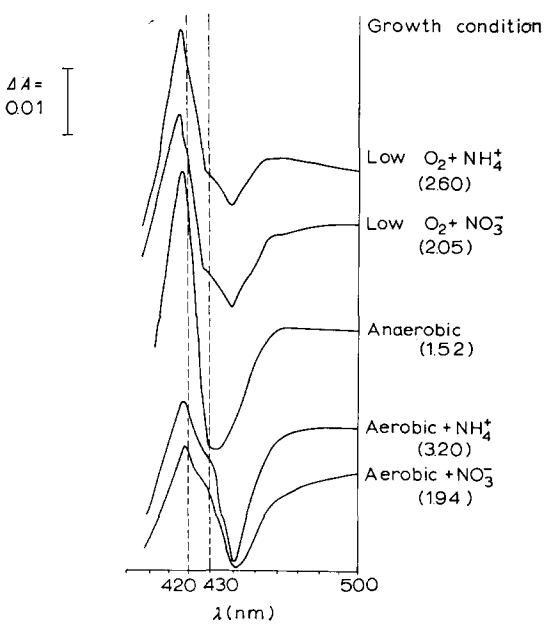



Fig. 3. Qualitative difference in the CO spectrum of unfractionated Hughes' press extracts of *M. denitrificans* from five growth conditions. The figure in parentheses (mg/ml) represents the quantity of protein in the preparation.

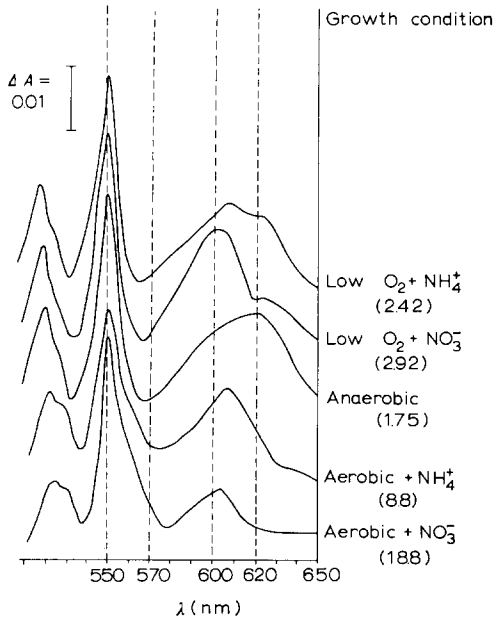



Fig. 4. Qualitative differences in the (dithionite reduced) *minus* (ferricyanide oxidized) difference spectra of the soluble fraction of *M. denitrificans* from five growth conditions. The figure in parentheses (mg/ml) represents the quantity of protein in the preparation.

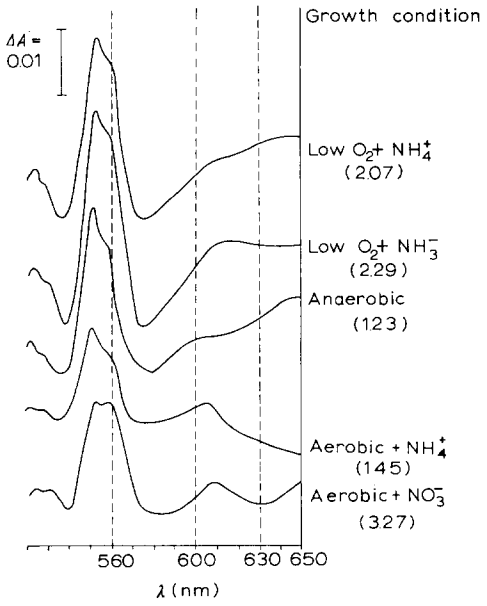



Fig. 5. Qualitative differences in the (dithionite reduced) *minus* (ferricyanide oxidized) difference spectra of the 22000 × *g* pellet from Hughes' press extracts of *M. denitrificans*. The figure in parentheses (mg/ml) represents the quantity of protein in the preparation.

Anaerobically cytochrome $a + a_3$ cannot be detected while the $\alpha$ band of the *d* haem is prominent: aerobically the converse is true. Under limiting oxygen conditions both cytochromes are present. No changes are observed in the position of the $\alpha$ band of cytochrome *c* at 553 nm, although under oxygen-limited or anaerobic conditions the cytochrome *b* peak may shift nearer to 558 nm (Fig. 2).

The CO spectrum of cells from all five growth conditions (Fig. 3) showed a peak at 418 nm, not 415 nm as reported by other workers, but in both aerobic growths there was a shoulder at 430 nm, which was typical of cytochrome $a_3$. This shoulder was also present in oxygen-limited growths but not detected in anaerobically grown cells.

*Changes in the soluble and particulate fraction.* Cytochrome *b* seems to be almost entirely bound to particulate constituents (Fig. 5) although a small amount is present in the soluble fraction of aerobic cells. Cytochrome $a + a_3$ peaks at 605–610 nm can be detected in the soluble fraction of all but the anaerobic preparation. The $\alpha$ band of the *d* haem (620–630 nm) is almost entirely soluble and occurs when oxygen is low or absent (Fig. 4).

*Quantitative differences in cytochromes of M. denitrificans from different growth conditions (Table II)*

*Unfractionated extracts.* Anaerobiosis or restricted oxygen led to an increase in cytochromes *b* and *c*, whilst the reverse was true for cytochrome $a + a_3$ which could not be detected anaerobically and was reduced to a third of the aerobic level when oxygen was limiting. The 418 nm CO-binding pigments were three times higher anaerobically than under any of the four other growth conditions.

TABLE II

THE QUANTITY OF CYTOCHROMES, EXPRESSED AS $\Delta A$/g PROTEIN, IN FRACTIONATED HUGHES' PRESS EXTRACTS OF *M. denitrificans*

N.D., not detected; N.M., present but not measurable. The figures in parentheses refer to the nmoles/mg protein of cytochromes present using the corrections and calculations given in Methods and Materials.

| *Fraction* | *Cytochrome* | *Growth condition* | | | | |
|---|---|---|---|---|---|---|
| | | *Anaerobic* | *Low* $O_2$+ $NO_3^-$ | *Low* $O_2$+ $NH_4^+$ | *Aerobic*+ $NO_3^-$ | *Aerobic*+ $NH_4^+$ |
| Unfractionated Extracts | $b + o$ | 13.4 | 12.7 | 13.9 | 3.5 | 5.3 |
| | $c$ | 17.4 | 15.9 | 17.6 | 2.9 | 5.4 |
| | $a_3$ | N.D. | 2.1 | 2.1 | 6.7 | 6.0 |
| | 418 nm CO-binding pigments | 29 | 10.7 | 10.8 | 9.8 | 7.8 |
| Soluble | $b + o$ | N.D. | N.D. | N.D. | N.M. | N.M. |
| | $c$ | 18.3 | 11.3 | 13.3 | 1.6 | 2.5 |
| | $a_3$ | N.D. | 1.6 | 0.8 | 0.5 | 0.7 |
| | 418-nm CO-binding pigment | 31.4 | 6.6 | 9.8 | 3.0 | 2.5 |
| Particulate | $b + o$ | 17.9 (0.47) | 10.5 (0.29) | 12.7 (0.35) | 6.2 (0.10) | 6.8 (0.20) |
| | $c$ | 17.0 (0.45) | 13.1 (0.35) | 11.6 (0.30) | 5.5 (0.17) | 7.6 (0.23) |
| | $a_3$ | N.D. | 1.5 (0.015) | 1.3 (0.013) | 8.6 (0.086) | 8.2 (0.082) |
| | 418-nm CO-binding pigment | 26.3 (0.33) | 13.1 (0.16) | 18 (0.25) | 14.4 (0.18) | 8.0 (0.10) |

*The soluble fraction.* Although cytochrome *b* was detected in aerobic cells it could not be measured accurately. There was more soluble cytochrome *c* (presumably from cytochrome *c–d*) under truly anaerobic conditions than in cells from both $O_2$-limited growths, and these in turn showed an increase in total amount compared with aerobically grown cells. There was a similar pattern for the 418-nm CO-binding pigment (heam *d*), anaerobic cells having 3 times as much as oxygen-restricted organisms.

*The particulate fractions.* The 418-nm CO-binding pigment in this fraction was probably the cytochrome *o* reported by Scholes and Smith[1] and not haem *d*, as in the soluble fraction, since the $\alpha$ peak of the latter was absent in the (reduced) *minus* (oxidised) difference spectra. Comparing the values expressed as nmoles/mg of protein, anaerobic cells contained quantitatively more of cytochromes *b*, *c* and *o* than in any other growth condition. Similarly there was more cytochrome *b* and *c*, under oxygen limitation than aerobically; cytochrome *o*, however, dit not present such a clear cut picture. Cytochrome $a_3$ was not detected anaerobically and was lower under oxygen limitation than aerobically.

## DISCUSSION

It seems clear from these experiments that oxygen rather than nitrate has the most significant regulatory effect on cytochrome levels in *M. denitrificans*. Anaerobic and oxygen-limited cells with or without nitrate have certain common features: (a) An increase, in the soluble fraction, of haem *d* and of heam *c*, which are associated with nitrite reductase activity and make up cytochrome *c-d*. (b) Higher levels of the particulate cytochromes *b* and *c*. (c) A reduction in total amount of cytochrome $a_3$.

When oxygen is limiting and nitrate is present the cell has a choice of electron acceptors. However, in these circumstances little difference is observed between nitrate- or ammonium grown cells. It seems that oxygen concentration has the dominant effect. Anaerobic cells have more of cytochromes *b*, *c* and *c-d* than either oxygen limited growth. It may be that the small amount of oxygen present represses cytochrome synthesis or that some other factor is responsible for the elevated anaerobic levels. When nitrate is present nitrite will only appear in the medium in the absence of oxygen. It has been reported by Lam and Nicholas[12,13] that nitrite induces nitrite reductase (cytochrome *c-d*) in *M. denitrificans*. This observation may explain why haems *c* and *d* are higher anaerobically, *i.e.* they are partially oxygen repressed and partially nitrite induced. Kodama[14] has reported similar behaviour with *Pseudomonas stuzerii* although he states that nitrate itself might counteract the effect of nitrite.

It is not known if the cytochromes present anaerobically are the same as their aerobic counterparts. A slight shift in the position of the cytochrome *b* shoulder could indicate that it is a different cytochrome; alternatively it could be a reflection of a change in organisation of the respiratory chain or interference from an increase in the cytochrome *c* peak. Further light on the organisation of the electron transport pathways will be reported in a later communication.

It seems that oxygen-limited growth leads to adaptation of the electron transport system to a point which may be midway between the aerobic and anaerobic states. The major qualitative difference is the disappearance of cytochrome $a_3$ anaerobically. Otherwise the anaerobic respiratory chain appears to be little more than a proliferation of the aerobic one, although this would have to be established functionally

in future experiments. There is no obvious stoichiometric relationship between the component cytochromes and this seems to be true for most bacteria investigated in batch culture[1].

Finally the role of cytochrome *o*, which appears in high concentrations in anaerobic cells is obscure. It is generally accepted that cytochrome *o* is a terminal cytochrome oxidase in the pathway to oxygen[15].

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