INTRODUCTION

Mycobacterium avium Complex (MAC) Epidemiology

Mycobacteria are rod-shaped, non-motile, acid-fast bacteria which have high lipid content in their cell wall (Barksdale and Kim, 1977). *Mycobacterium avium* and *Mycobacterium intracellulare* (members of *M. avium* complex) are slow growing (1 generation / day), opportunistic pathogens of humans and animals (Wolinsky, 1979; Falkinham, 1996) that have been isolated from a diversity of environments (Brooks et al., 1984; Falkinham et al., 1980).

M. avium and *M. intracellulare* are normal inhabitants of natural waters and drinking waters (Falkinham et al., 1980; 2001), soils (Brooks et al., 1984), and water droplets ejected into air (Wendt et al., 1980). One source of human infection is water (von Reyn et al., 1994). By DNA fingerprinting method, it has been shown that isolates of *M. avium* obtained from AIDS patients were the same to those obtained from water consumed by the patients (von Reyn et al., 1994).

M. avium and *M. intracellulare* are animal and human opportunistic pathogens (Wolinsky, 1979; Falkinham, 1996) responsible for pulmonary disease similar to tuberculosis in elderly patients and in immunocompetent individuals with predisposing lung diseases such as silicosis and black lung (Wolinsky, 1979), cervical lymphadenitis in children (Wolinsky, 1995), and disseminated infection in AIDS and immunosuppressed patients (Zakowski et al., 1982; Kiehn et al., 1985).

M. avium Complex Ecology

M. avium numbers are highest in waters with low oxygen content (Brooks et al., 1984) and low oxidation-reduction potential (Havelaar et al., 1985). These organisms are also found to be present in very high numbers in biofilms (600 colony forming units per cm^2) in drinking water

distribution systems (Falkinham et al., 2001). Biofilms have been shown to contain microaerobic and anaerobic zones due to the layers formed by microbial cells and extracellular products (de Beers et al., 1994). *M. avium* and *M. intracellulare* grew at 6% and 12% oxygen in laboratory medium and survive rapid transfer to anaerobic conditions (Lewis, unpublished).

Mycobacterial Adaptation

Reactive oxygen intermediate and reactive nitrogen intermediate production are host defense mechanisms to mycobacterial infection (Chan et al., 2001). For example, nitric oxide is produced by macrophages of mice when the cell-mediated immune system is activated due to infection (Sohaskey, 2005). Nitric oxide production by mononuclear phagocytes has an essential role in killing mycobacteria (Chan et al., 2001). Nitric oxide gets converted to nitrate and nitrite causing increased levels of nitrate and nitrite (Sohaskey, 2005). Thus, nitrate is an important nutrient within a living organism and is readily available inside damaged host tissues (Weber, et al., 2000). *M. avium* and other mycobacteria can adapt to environmental changes in the infected host by enzyme induction and metabolic activity alteration (Barclay and Wheeler, 1989). Although mycobacteria are considered to be obligate aerobes (Ratledge, 1982), they can survive in the anaerobic conditions present inside host tissue i.e. abscesses and necrotic granulomas and become strongly adapted to the changes in environment within the host tissues (Weber et al., 2000). For example, nitrate reductase activity increases in *M. tuberculosis* undergoing hypoxic shift, nitrate gets converted to nitrite at a faster rate than that of aerobically growing cultures and it might play an important role in the persistence of this organism in the oxygen-depleted areas of damaged host tissue (Wayne and Hayes, 1998).

Bacterial Nitrate- and Nitrite-reductases

Aggressive use of fertilizers, atmospheric deposition, and municipal waste treatments has caused increases in the concentrations of nitrate in river basins throughout the USA (Smith, et al. 1987). Nitrate and nitrite can be used by microorganisms as terminal electron acceptors under microaerobic or anaerobic conditions (Moreno-Vivián et al., 1999). Nitrate reduction can serve three different functions. (1) Nitrate can be utilized as a nitrogen source for growth (nitrate assimilation). (2) Nitrate can be utilized to as a terminal electron acceptor in the absence of oxygen (nitrate respiration). (3) Nitrate can be utilized to diminish excess reducing power for redox balance; nitrate dissimilation (Moreno-Vivián et al., 1999). In *Escherichia coli*, each function is carried out by a distinct nitrate reductase; namely assimilatory, respiratory, and dissimilatory nitrate reductases (Moreno-Vivián et al., 1999). The assimilatory nitrate- and nitrite-reductases are located in the cytoplasm, the respiratory nitrate reductase is membrane-bound, the respiratory nitrite reductase is present in periplasm, the dissimilatory nitrate reductase is present in the periplasm, and the dissimilatory nitrite reductase is present in cytoplasm in *E. coli* (Moreno-Vivián et al., 1999). In *E. coli,* the assimilatory nitrate reductase is induced by nitrate and repressed by ammonia, but is not affected by oxygen (Moreno-Vivián et al., 1999). The respiratory nitrate reductase is induced by nitrate and repressed by oxygen, but is not affected by ammonia (Moreno-Vivián et al., 1999). The dissimilatory nitrate reductase is induced by nitrate, but basal activity is present even in the absence of nitrate and neither oxygen nor ammonia has any effect on its activity (Moreno-Vivián et al., 1999).

The assimilatory nitrate- and nitrite-reductases make the organism capable of utilizing nitrate and nitrite as nitrogen sources for biosynthesis of nitrogen compounds (Moreno-Vivián et al., 1999). The respiratory nitrate- and nitrite-reductases allow the bacteria to synthesize ATP

and to generate proton motive force by using nitrate and nitrite as alternative electron acceptor under anaerobic conditions (Moreno-Vivián et al., 1999). The dissimilatory nitrate- and nitrite-reductases help to maintain redox balance and nitrite detoxification (Moreno-Vivián et al., 1999). Thus, the organisms gain the metabolic capability to survive in changing nitrogen and/or oxygen conditions in nature because of the physiological roles played by assimilatory, respiratory, and dissimilatory enzyme activities (Moreno-Vivián et al., 1999).

***M. avium* and Nitrate- and Nitrite-reductases**

The nitrate reduction test is used to distinguish between environmental opportunistic mycobacteria and *M. tuberculosis* (Virtanen, 1960). It has been documented that *M. avium* yields a negative result in the standard test for detection of nitrate reductase activity, i.e., appearance of nitrite (Vestal, 1975; Wayne, 1985). In a study of mycobacterial nitrate reduction carried out by Virtanen (1960), it was found that *M. avium* strains include strongly nitrate reducing, weakly nitrate reducing, and strains lacking nitrate reductase activity (Virtanen, 1960). In his study, Virtanen (1960) measured the nitrate reductase activity as intensity of color formed by production nitrite from nitrate. Nitrate can be reduced to different reduction products, e.g. nitrite, nitrogen oxide and nitrogen gas depending on the bacterial species and growth conditions (Virtanen, 1960). So it is quite possible in *M. avium* which is considered as nitrate reductase negative may be really slowly reducing the nitrate or it might have the nitrite reductase enzyme activity which further reduces nitrite to other reduction products as mentioned above. Because *M. avium* can use nitrate or nitrite as sole nitrogen source for growth (McCarthy, 1987), it must have assimilatory nitrate- and nitrite-reductases. The explanation for the above discrepancy is that nitrate reduction occurs at a slow rate and nitrite reduction occur rapidly, so that nitrite does not accumulate and is not detected by the standard nitrate reduction test and strains incorrectly

recorded as nitrate reductase negative (Virtanen, 1960; McCarthy, 1987). Experiments should be carried out to determine whether *M. avium* has the nitrate- and nitrite-reductase activities, whether nitrite reductase activity is more than nitrate reductase activity, and whether ammonia, oxygen, and nitrate have any regulatory effect on their induction.

Nitrate and Nitrite Reductases in *M. avium* Genome

M. avium genome has been sequenced and is being annotated (Marcel Beher, personal communication). Comparison of the *M. avium* genome sequence with that of *M. paratuberculosis* strain K10, *M. tuberculosis* strain H37Rv ORF genes with known function shows similar genes in the *M. avium* genome. The genes identified in the *M. avium* genome that share sequence similarity to genes for respiratory nitrate reductase, genes coding for dissimilatory nitrite reductase, and also other genes coding for nitrate or nitrite transporters are shown in Table 1 (Alexander, personal communication). We have initiated this study to determine whether *M. avium* has any respiratory and/or dissimilatory nitrate-and nitrite-reductase activities in addition to assimilatory activities because of (1) presence of genes in *M. avium* genome similar to those involved in nitrate- and nitrite-metabolism, (2) evidence for nitrate- and nitrite-assimilation (McCarthy, 1987), (3) the ability of *M. avium* to survive rapid transfer to anaerobic conditions (Lewis, unpublished), and (4) the ability of mycobacteria to adapt to environmental changes in the infected host by enzyme induction and metabolic activity alteration (Barclay and Wheeler, 1989).

***M. avium* Structural Features**

Mycobacteria are neither Gram-positive nor Gram-negative. They have a thin, Gram-negative-like peptidoglycan and have a thick, impermeable outer membrane composed of long chain fatty acids (Faller et al., 2004). Because of the presence of an outer membrane, it is

possible that mycobacteria have a periplasmic space. However, there have been no reports describing mycobacterial periplasm. Because of the method of breakage of cells to be employed here, proteins that are present in periplasm will be released into the cytoplasmic or soluble fraction.

M. avium has been grown in a minimal defined medium aerobically in the presence of one nitrogen source or two nitrogen sources in combination to study the growth response and patterns of nitrate- and nitrite-reductase activities. For enzyme assays, *M. avium* cultures growing aerobically as well as undergoing an anaerobic shift in the presence of either single nitrogen source or two nitrogen sources in combination were utilized. Nitrite- and nitrite-reductase activities were measured. The different types of enzyme activities and their regulation by ammonia, nitrate and oxygen were analyzed. Finally the use of NADH or NADPH as an electron donor for nitrite- and nitrite- reductases was measured.

Table 1. Data obtained from *Mycobacterium avium* genome sequence listing genes involved in nitrate- and nitrite-reduction.

GENE	PREDICTED FUNCTION	ENYME ACTIVITY
NarG	Nitrate reductase alpha subunit	Respiratory
NarY	Nitrate reductase beta subunit	Respiratory
Nitrate_red_del	Nitrate reductase delta subunit	Respiratory
NarI	Nitrate reductase gamma subunit	Respiratory
NirA	Ferredoxin dependent nitrite reductase	Dissimilatory
NirD	Ferredoxin subunits of nitrite reductase	Dissimilatory
NirB	NAD(P)H-nitrite reductase	Dissimilatory
NarK	Nitrate/nitrite transporter	Transport
FocA	Formate/nitrite family of transporters	Transport

Hypothesis

In addition to assimilatory nitrate- and nitrite-reductase activities, *M. avium* produces respiratory or dissimilatory nitrate- and nitrite-reductase activities and these activities are induced by nitrate or nitrite and are regulated by ammonia and oxygen.

Rationale of the Experimental Approach

1. Glutamine, ammonia, nitrate, or nitrite can be used as sole nitrogen source in a minimal defined medium to support the growth of *M. avium* cells (McCarthy, 1987). Therefore cultures can be grown in a defined minimal medium containing nitrate, nitrite, glutamine or ammonia alone or in combination.

2. *M. avium* cells can survive a rapid shift to anaerobic conditions (Lewis, unpublished).

3. Mycobacteria can adapt to anaerobic conditions by altering metabolism. For example, nitrate reductase activity is induced in *M. tuberculosis* following a shift from aerobic to anaerobic conditions (Wayne & Hayes, 1998).

4. *M. avium* cultures can be grown aerobically as well as following an anaerobic shift. The approach for the growth experiment using different nitrogen sources is shown in Table 2. The regulation of assimilatory, dissimilatory and respiratory nitrate- and nitrite-reductase activities by ammonia, nitrate, nitrite and oxygen as described by Moreno-Vivián et al., (1999) is already mentioned in the introduction. The assumptions for the presence of possible enzyme activities in the *M. avium* A5 cells are based on the same. These assumptions are mentioned in Table 3.

5. *M. avium* cells can be fractionated into soluble and membrane fractions by centrifugation following sonication (George and Falkinham, 1986) that will allow separation of the enzyme present in those fractions.

Table 2. Growth Conditions

	Nitrate	Nitrate+ Ammonia	Ammonia
Air (21% oxygen)	**I** Assimilatory + Respiratory – Dissimilatory +	**II** Assimilatory – Respiratory – Dissimilatory +	**III** Assimilatory – Respiratory – Dissimilatory + (basal activity)
Cultures undergoing anaerobic shift	**IV** Assimilatory + Respiratory + Dissimilatory +	**V** Assimilatory - Respiratory + Dissimilatory +	**VI** Assimilatory - Respiratory + Dissimilatory +(basal activity)

Table 3. Assumptions for the enzyme activities

1. The assimilatory nitrate- and nitrite-reductase activities will be present in *M. avium* cells grown in the presence of nitrate and nitrite as sole nitrogen sources in air.
2. The assimilatory nitrate- and nitrite-reductase activities will be absent in cells grown in the presence of ammonia, even if either nitrate or nitrite is present as well.
3. The respiratory nitrate- and nitrite-reductase activities will only be present in *M. avium* cells shifted to anaerobic conditions in the presence of nitrate or nitrite.
4. The respiratory nitrate- and nitrite-reductase activities should not be reduced by the presence of ammonia as long as cells are maintained under anaerobic conditions in the presence of nitrate or nitrite.
5. The dissimilatory nitrate- and nitrite-reductase activities will be present in cells grown in the presence of nitrate or nitrite and the activities will not be reduced by the presence of oxygen or ammonia.
6. The assimilatory nitrate- and nitrite-reductase activities will be found in the soluble fraction, the possible respiratory nitrate- and nitrite-reductase activities will be found in the membrane fraction, and the dissimilative nitrate- and nitrite-reductase activities will be found in the soluble fraction.

Research Objectives

1. Confirm that *M. avium* can grow on glutamine, ammonia, nitrate or nitrite as sole nitrogen source in a defined minimal medium.

2. Measure nitrate- and nitrite-reductase activities of *M. avium* cells grown in air in the presence of (a) nitrate, (b) nitrite, (c) ammonia, (d) ammonia and nitrate in combination, and (e) ammonia and nitrite in combination.

3. Measure nitrate and nitrite reductase activities of *M. avium* cells shifted to anaerobic conditions in the presence of (a) nitrate, (b) nitrite, (c) ammonia, (d) ammonia and nitrate in combination, and (e) ammonia and nitrite in combination.

4. Determine the sub-cellular location (i.e. soluble or membrane) of the nitrate and nitrite reductases produced by *M. avium* cells from the above mentioned growth conditions.

5. Identify whether NADH, NADPH, or both can be used as electron donor by nitrate- and nitrite- reductase enzymes.

MATERIALS AND METHODS

Mycobacterial Strain

M. avium strain A5 which is a plasmid free AIDS patient isolate was used for this study (Beggs *et al.* 1995).

Growth Medium

The nitrogen growth medium (NT medium) of McCarthy (1987) was used. It contained 60 µm $FeCl_3$, B-2 salts, glycerol (5 ml/liter), 10 % tween 80 (10 ml/liter), and palmitic acid 25 mg (melted in 10 ml of 0.05 N NaOH) and 1 ml of trace element solution per liter. Those components were added to Milli-Q water in above mentioned order. Because nitrate and nitrite reductases require molybdenum for activity (Nicolas and Nason, 1954), 2 ml /liter of 4 mM sodium molybdate was added to the medium. The final concentrations of components of B-2 salts were: 11 mM KH_2PO_4, 10.6 mM Na_2HPO_4, 1.4 mM sodium citrate, and 0.1 mM $MgSO_4$ (McCarthy, 1987). The trace element solution contained: 3.4 mM $CaCl_2$, 3.5 mM $ZnSO_4$ $7H_2O$, and 4 mM $CuSO_4$ $5H_2O$ (McCarthy, 1987). Individual medium components were prepared at 10-fold concentration. The components were sterilized by pressure filtration using a 0.2 µm pore size filter and stored in sterile containers at 4^o C. Stock solution of each nitrogen source i.e. glutamine, ammonium sulfate, potassium nitrate and sodium nitrite were prepared at 100 mM, sterilized by autoclaving, and stored at 4^o C. The final concentration of each nitrogen source in the final NT growth medium was 2 mM.

Growth of *M. avium* A5 in Different Nitrogen sources

M. avium A5 cells from a stock culture in our lab were used. These cells were grown on Middlebrook 7H9 broth medium (BBL Microbiology System, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin (Pethel and Falkinham, 1989). The

cells were washed with sterile distilled water before use to remove nitrogenous compounds and albumin present in that broth and those washed cells were used as inocula for the minimal defined NT growth medium. 750 µl of washed *M. avium* A5 cells were inoculated in 15 ml of NT medium containing a single nitrogen source at 2 mM concentration in a 250 ml side arm flask. These cultures were incubated in a water bath maintained at 37^{o}C at sixty rpm for at least 10 days. After twelve days of incubation at 37^{0} C, 5 ml aliquots of the culture were transferred aseptically in 16 x 125 mm sterile screw capped tubes and stored at 4^{o}C. A 50 ml culture grown in a similar manner was harvested aseptically and 5 ml aliquots were distributed into sterile 16 x 125 mm screw capped tubes and stored at – 70^{o}C.

The purity of the culture was confirmed by streaking the culture on Middlebrook 7H10 agar medium (BBL Microbiology System, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid- albumin (Pethel and Falkinham, 1989) and incubating for at least 10 days. Contamination was checked by streaking the cultures on Plate Count Agar (Difco Laboratories, Becton, Dickinson and Company, Sparks, MD) and incubating for 48 hours.

M. avium A5 cells were grown in the presence of 2 mM glutamine, 2 mM ammonia, 2 mM nitrate or 2 mM nitrite as sole nitrogen source in a similar manner as mentioned above. Growth was measured as increase in turbidity (absorbance) at 580 nm. The absorbance at 580 nm was plotted against time (in days) on four cycle semi log paper. Thus, growth curve was obtained representing growth of *M. avium* A5 on individual nitrogen source. A best fitting straight line was drawn. The generation time was deduced as the time required for doubling of the turbidity reading at 580 nm. The growth curves were compared with each other based on turbidity readings, presence or absence of lag phase and generation time.

Growth of the cultures for enzyme assays

First, 15 ml cultures were grown aerobically in the presence of nitrate and ammonia alone and in combination using 250 ml flasks in an identical manner as described above. Five ml of inoculum from these cultures were used as to incubate 50 ml of NT medium in the presence of nitrate and ammonia and in combination using 500 ml flasks. These 50 ml cultures were used in a similar manner to inoculate 500 ml NT medium containing nitrate or ammonia alone or in combination using 1000 ml flask. Bugstoppers (Whatman Inc., Clifton, NJ) were used to seal the flask and avoid contamination. The anaerobic shift was carried out as mentioned below. For all these growth experiments a shaker with a speed of 60 rpm kept inside a walk in 37^{o}C incubator room was used. Cultures growing aerobically, as well as undergoing anaerobic shift were utilized for enzyme assays.

Anaerobic Shift

The anaerobic shift of the aerobically growing cultures was based on the model used by Veerraghavan et al. (2002). Specifically, after 7 days of aerobic growth the cultures were shifted to the anaerobic phase by replacing the Bugstoppers (Whatman Inc., Clifton, NJ) with sterile non porous rubber stoppers. The cultures were incubated for additional 7 days with continuous shaking. To demonstrate anaerobiosis methylene blue indicator strip was kept inside the flask.

Anaerobic Shift using Anaerobic Chamber

As the above mentioned approach was not found to achieve complete anaerobiosis, a different technique was used as described below. The initial 7 days of aerobic growth was carried out in the same manner as previous experiments and on 8th day the cultures were transferred to an anaerobic chamber and were incubated for next 7 days. Anaerobiosis was achieved after 3 hours.

Isolation of Crude Extract, Membrane, and Soluble Fractions

The crude extract, membrane, and soluble fractions of cells were isolated as described by George and Falkinham (1989) from approximately 500 ml culture grown as described above. The cells were concentrated in 10 ml in the following manner. One-fourth of the culture was transferred to each of four centrifuge bottles of 250 ml capacity. Those bottles were centrifuged at 5,000 x g for twenty minutes at 4^0C. The pellet was washed three times in 40 mM potassium phosphate buffer (pH 6.5) containing 1.0 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), and 2.0 mM $MgCl_2$. One mg each of DNase I and RNase were added to the final 10 ml of cell suspension in buffer. Cells were broken open by sonication. Sonication was carried out on 5 ml aliquots of washed cell suspension by sonic dismembrator model no. 550 (Fisher Scientific Company, Pittsburgh, PA) using microtip. The centrifuge tube containing this sample was kept cool by placing it inside an ice-water slurry throughout the sonication period. The sonication was carried out for at least 15 minutes using the pulse mode. Breakage was estimated microscopically. Sonicated samples were centrifuged at 15,000 x g for 15 minutes at 4^0C and the supernatant was collected without contamination by the cells or debris in the pellet. The crude extract was centrifuged at 100,000 x g for 60 minutes at 4^0 C to separate the cytoplasmic membrane (pellet) and soluble (supernatant) fractions. The pellet was washed two times in 10 ml of 40 mM potassium phosphate buffer (pH 6.5) containing 1.0 mM EDTA, 0.3 mM PMSF, and 2.0 mM $MgCl_2$ and collected by centrifugation as above and suspended in a final volume of 2.5 ml of the same buffer.

Lowry's Assay and Protein Content of the Cell Fractions

Total protein was measured using the method of Lowry *et al* (1951) with bovine serum albumin (BSA) fraction V (Sigma-Aldrich, St. Louis, MO) as a standard.

Enzyme Assays

Nitrate- and nitrite-reductase activities of the membrane and soluble fraction were measured as described by Bastarrachea and Goldman (1961). Each enzyme assay (total volume 1.0 ml) contained 20 umoles of phosphate buffer (pH 6.5), 20 umoles of KNO_3 or $NaNO_2$, 5.0 umoles of NADH or NADPH and 0.2-0.3 mg of either the soluble or membrane fraction and Milli-Q water to make up the total volume to 1ml. In the control assays, everything except the cell fraction was added to the cuvette and absorbance values were monitored. Reduction of nitrate or nitrite was monitored by the measuring the decrease in absorption at 340 nm.

Measurement of Nitrate- and Nitrite-reductase Activity

Nitrate is reduced to nitrite as shown in equation 1. From the stoichiometry of the reaction as shown in equation 2 and 3, for every micromole of NAD(P)H oxidized, one micromole of nitrate or nitrite is reduced.

(1) $NO_3^- + 2\ e^- \rightarrow\ NO_2^-$

(2) $NO_3^- + NAD(P)H + H+ \text{--> } NO_2^- + NAD(P)^+ + H_2O$

(3) $NO_2^- + NAD(P)H + H+ \text{--> } NO^- + NAD(P)^+ + H_2O$

The enzyme activity was calculated by using equation 4.

(4) Enzyme activity = A/E^o x assay volume/1000 x 1/amount of protein used for assay x 10^{-6}

Where A = Δ Absorbance/Δ Time = Average change in absorbance per minute

E^o = Extinction coefficient of NAD(P)H = 6220 $moles^{-1}/cm^{-1}$.

Assay volume = 1ml

Amount of protein used = varies from 0.2 to 0.3 mg

The final values of enzyme activities were reported in micromoles of nitrate or nitrite reduced/min/mg of protein.

Measurement of ammonia, nitrate and nitrite concentrations

The ammonia concentration was measured indophenol blue assay (Keeney and Nelson, 1982). Using samples containing 0, 2, 4, 6, 8, 10 and 12 µg of ammonia-nitrogen, standard curves were derived. The concentrations of the ammonia nitrogen ranged from 0.001 mM to 0.006 mM in these samples. A linear relationship was obtained between concentration of ammonia-nitrogen and absorbance at 636 nm. In a similar manner, standard curves were derived in the presence of 0.014 mM nitrate, 0.0003 mM nitrite and 0.04 mM glutamine. This linear relationship was not interfered due to presence of those concentrations of nitrate, nitrite and glutamine.

The nitrate concentration was measured by the brucine method (Jenkins and Medsker, 1964). Using samples containing 0, 0.5, 1.5, 3.5, 5.5.7.5 and 8 µg of nitrate-nitrogen, standard curves were derived. The concentration of nitrate-nitrogen ranged from 0.035 mM to 0.56 mM in these samples. A linear relationship was obtained between concentration of nitrate-nitrogen and absorbance at 410 nm. In a similar manner, standard curves were derived in the presence of 0.00072 mM nitrite, 0.003 mM ammonia and 0.09 mM glutamine. The linear relationship was not interfered due to presence of those concentrations of nitrite, ammonia and glutamine.

The nitrite concentration was measured by the method of Hanson and Philips (1981). Using samples containing 0, 5, 10, 15, 20, and 25 µg microgram of nitrite-nitrogen, standard curves were derived. The concentration of nitrite- nitrogen ranged from 0.0004 mM to 0.002 mM. A linear relationship was obtained between concentration of nitrate-nitrogen and absorbance at 543 nm. In a similar manner, standard curves were derived in the presence of 0.014 mM nitrate, 0.001 mM ammonia and 0.04 mM glutamine. The linear relationship was not interfered due to presence of those concentrations of nitrate, ammonia and glutamine.

RESULTS

Growth of *M. avium* A5 on Different Nitrogen Sources

It has been documented that *M. avium* can utilize nitrate or nitrite as sole nitrogen source in a minimal defined medium (McCarthy, 1987). Similar experiments were performed to confirm that finding with *M. avium* strain A5. The growth response of *M. avium* A5 to glutamine, ammonium sulfate, potassium nitrate and sodium nitrite was measured in the nitrogen test medium (NT Medium), which is a defined minimal medium containing palmitic acid and lacking albumin (McCarthy, 1987).

M. avium A5 cells from a stock culture grown on Middlebrook 7H9 broth medium (BBL Microbiology System, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid- albumin (Pethel and Falkinham, 1989) were washed with sterile distilled water before use to remove nitrogenous compounds and albumin present in that broth. Seven hundred and fifty µl of the washed *M. avium* A5 cells were inoculated in 15 ml of NT medium in a 250-ml flask with a single nitrogen source at 2 mM concentration. A flask containing 15 ml of NT medium lacking any nitrogen source was incubated as negative control in each growth experiment. The cultures were incubated at least for 10 days. After 12 days the cultures were streaked on M7H10 agar medium containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid- albumin (Pethel and Falkinham, 1989) to confirm purity. Mycobacteria grew very slowly and very minute colonies were not visible until the 7^{th} day of incubation. Contamination was monitored by streaking the cultures on Plate Count Agar (Difco). In the experiments reported here, such pure cultures were stored in refrigerator and used to inoculate the media for cultures to be utilized for enzyme assays.

The characteristic cell aggregate formation (Virtanen, 1960) was observed after initial few days. Because these aggregates dispersed easily after swirling the liquid in the flask, they did not appear to interfere with measurement of turbidity. It has been documented that *M. avium* can utilize 0.25 mM to 2 mM of ammonia, nitrate, and nitrite as sole nitrogen source (McCarthy, 1987). In our study, *M. avium* A5 grew very well on all the nitrogen sources except nitrite as shown in Figures 1- 5. Representative growth curves are shown in Figures 1-5 and a brief summary of the growth experiments is given in Table 4. *M. avium* A5 cells were grown in the presence of 2 mM glutamine, 2 mM ammonia, 2 mM nitrate or 2 mM nitrite as sole nitrogen source in a similar manner as mentioned above. Growth was measured as increase in turbidity (absorbance) at 580 nm. The absorbance at 580 nm was plotted against time (in days) on four cycle semi log paper. Thus, a growth curve was obtained representing growth of *M. avium* A5 on each individual nitrogen source. A best fitting straight line was drawn. The generation time was deduced as the time required for doubling of the turbidity reading at 580 nm.The growth curves were compared with each other based on turbidity readings, presence or absence of lag phase and generation time.

The cultures growing on glutamine, ammonium sulfate and nitrate did not show any lag phase. A two-fold rapid increase in the turbidity reading after 24 hrs of incubation was noted in all cultures except those growing on nitrite as nitrogen source. The initial turbidity readings upon inoculation are almost same for all cultures except those grown on nitrite. The turbidity readings on day 10 were comparable for cultures grown on glutamine, ammonium sulfate, and nitrate. The turbidity reading on day 10 for the culture growing on nitrite was much less compared with the other cultures. The turbidity reading values on day 10 were less than those obtained by McCarthy (1987) on day 6 of her experiment. The generation time for cultures

grown on ammonium sulfate and glutamine was 4 days and that for nitrate grown culture was 5 days. The generation time for cultures grown on nitrite was 6 days.

As shown in Figure 4 and 5, *M. avium* A5 grew initially slowly on nitrite with a lag phase of about 48 hrs and 72 hrs respectively. Also, the cultures grown on nitrite were not viable after 3-4 weeks. This was confirmed by streaking these cultures on Middlebrook 7H10 agar where no colonies were observed after even after 14 days of incubation. That is why these cells could not be used as inocula for the next set of growth experiments. *M. avium* A5 grown on all the other nitrogen sources were viable even after 12 weeks. We used them successfully to inoculate the next set of cultures. These cultures grew in the same manner as before.

Anaerobic Shift

The anaerobic shift was carried out as mentioned before. The methylene blue indicator strip was found to be blue even after 7 days. Complete anaerobiosis was not obtained by seven days. Repeat experiments carried out using anaerobic chamber achieved complete anaerobiosis.

Sonication and Isolation of Cell Fractions

The cells were broken by sonication. *M. avium* A5 cells were found to be very difficult to break open. The sonicated sample appeared less dense as compared with nonsonicated sample. For all cultures, non-sonicated sample and sonicated sample were compared under the microscope. *M. avium* A5 appeared as intact rods. For those samples which were sonicated for 15 minutes, approximately 30 % breakage was found and the breakage was estimated to be 40 % for the samples which were sonicated for 20 minutes. The membrane fraction appeared as thick dark brown colored pellet which was difficult to disperse into solution during the washing.

Lowry's Assay and Protein Content of the Cell Fractions

Total protein was measured using the method of Lowry *et al.* (1951). Two hundred ul of

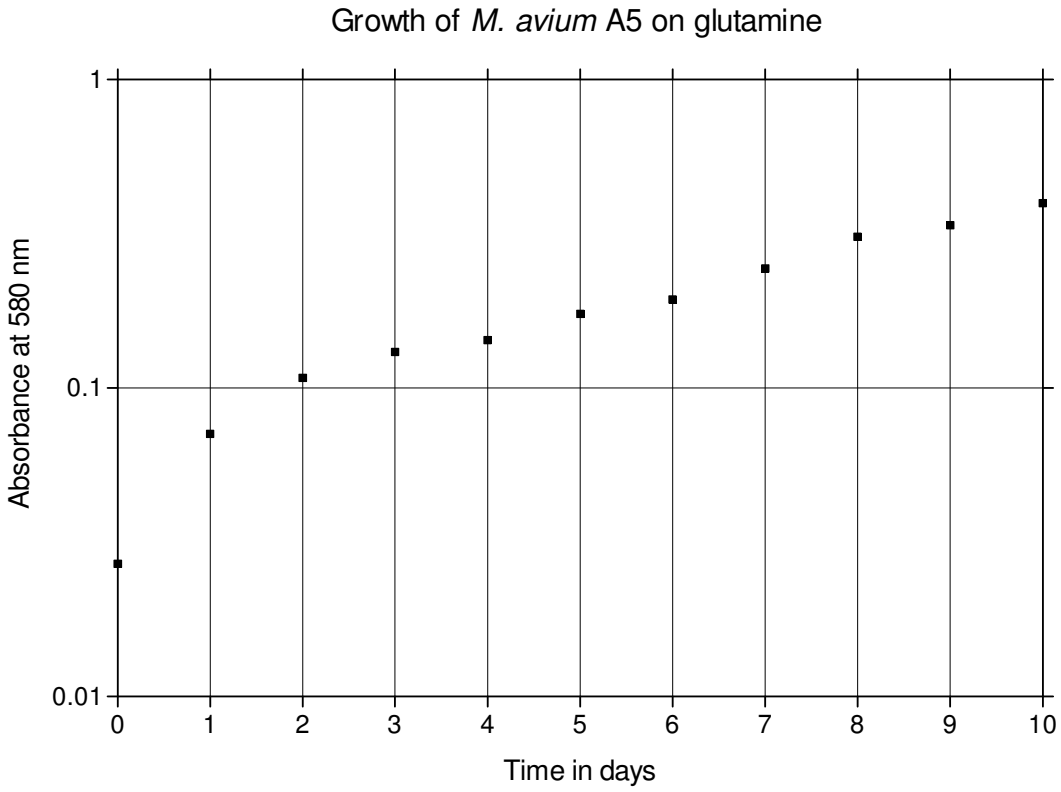

Figure 1. *M.avium A5* was incubated in 15 ml of NT medium in a 250 ml flask with 2 mM glutamine nitrogen source. The turbidity readings at 580 nm for the first 10 days are shown. No lag phase present. Generation time was 4 days.

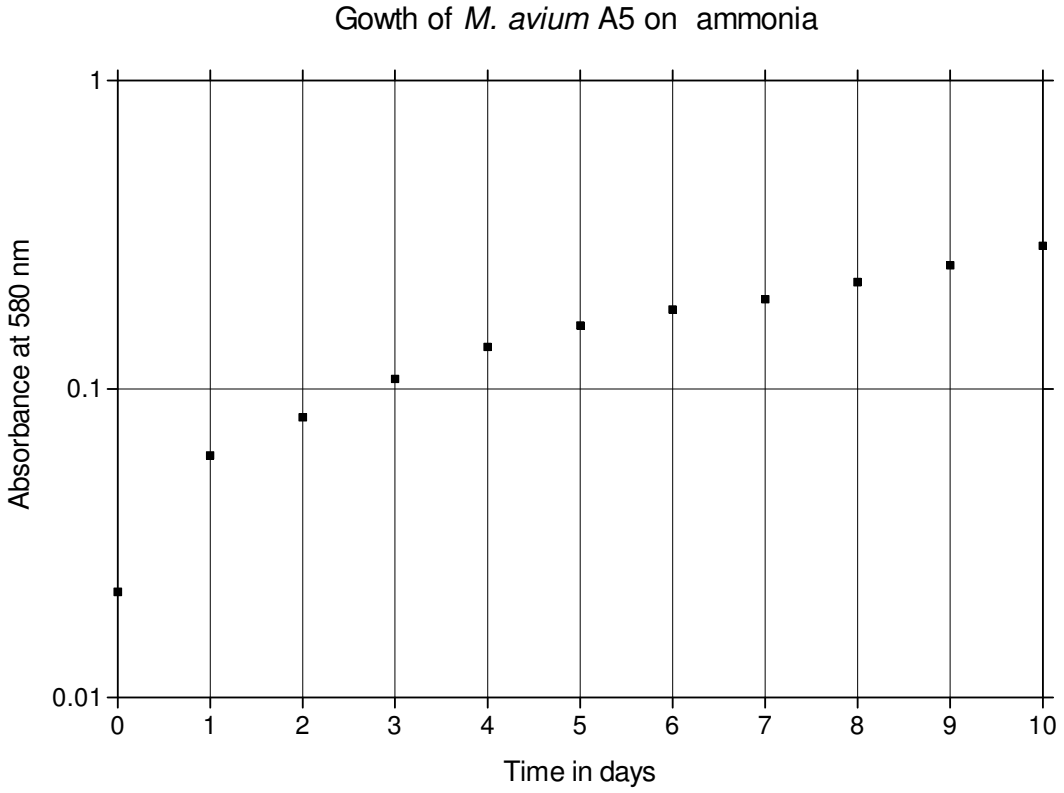

Figure 2. *M.avium A5* was incubated in 15 ml of NT medium in a 250 ml flask with 2 mM $(NH_4)_2SO_4$ as nitrogen source. The turbidity readings at 580 nm for the first 10 days are shown. No lag phase present. Generation time was 4 days.

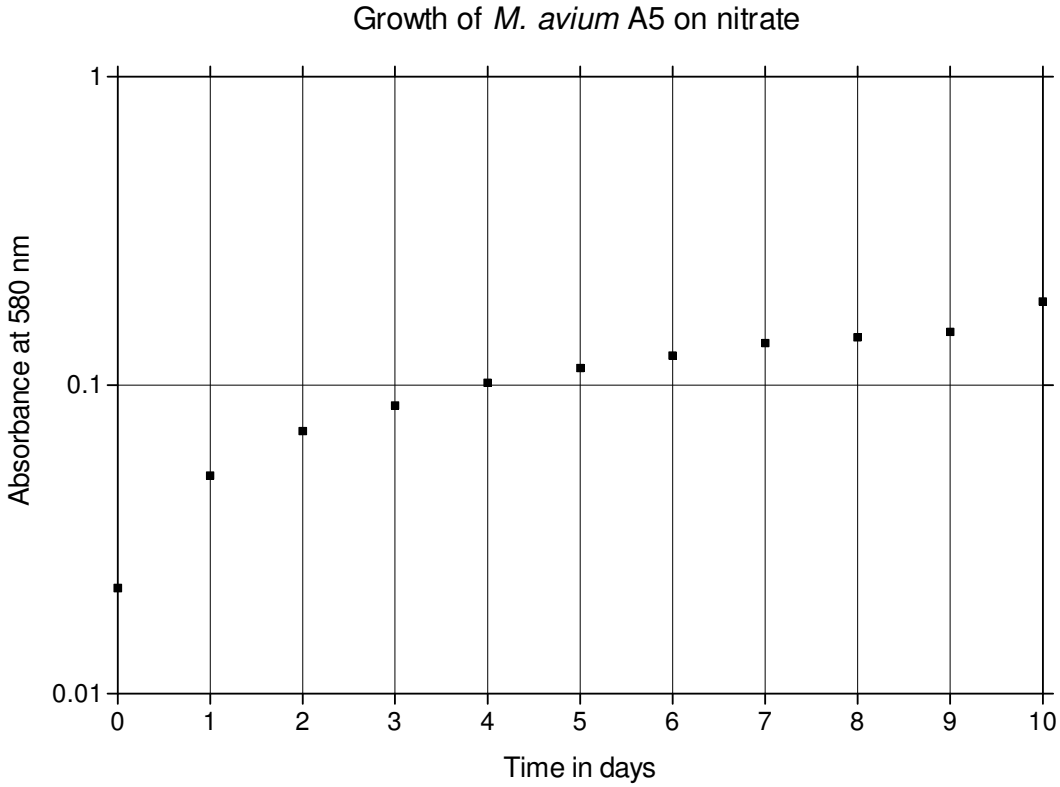

Figure 3. *M.avium A5* was incubated in 15 ml of NT medium in a 250 ml flask with 2 mM KNO_3 as nitrogen source. The turbidity readings at 580 nm for the first 10 days are shown. No lag phase present. Generation time was 5 days.

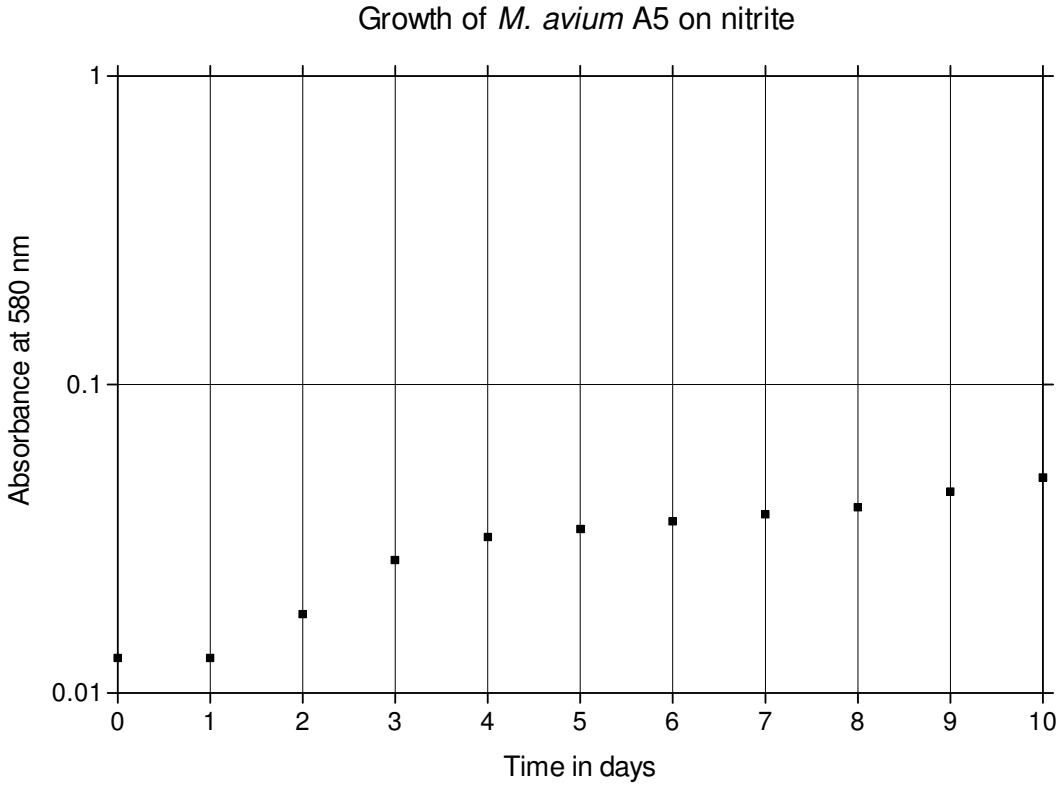

Figure 4. *M.avium A5* was incubated in 15 ml of NT medium in a 250 ml flask with 2 mM $NaNO_2$ as nitrogen source. The turbidity readings at 580 nm for the first 14 days are shown. Lag phase present. Generation time was 6 days.

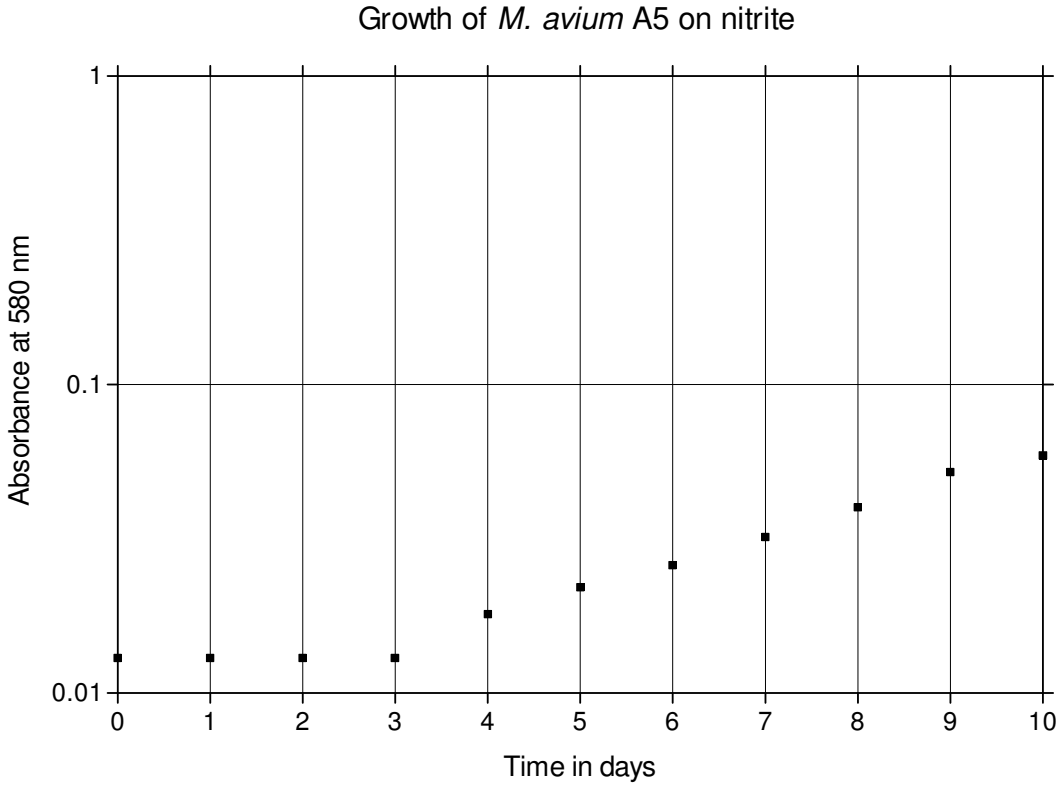

Figure 5. *M.avium A5* grown on nitrite was incubated in 15 ml of NT medium in a 250 ml flask with 2 mM $NaNO_2$ as nitrogen source. The turbidity readings at 580 nm for the first 10 days are shown. Lag phase present. Generation time was 5 days.

Table 4. Growth of *M. avium* A5 in different nitrogen sources

Figure Number	Nitrogen Source	Lag phase	Generation Time	Turbidity reading on day 0 at 580 nm	Turbidity reading on day 1 at 580 nm	Turbidity reading on day 10 at 580 nm
1	Glutamine	None	4 days	0.027	0.071	0.398
2	Ammonium sulfate	None	4 days	0.022	0.061	0.292
3	Nitrate	None	5 days	0.022	0.051	0.190
4	Nitrite	48 hrs	6 days	0.013	0.013	0.050

M. avium A5 strain was inoculated in 15 ml of NT medium in the presence of single nitrogen source at a 2 mM concentration. Cultures were grown aerobically in a water bath maintained at 37^0C with a speed of 60 rpm. Each culture was grown at least for 10 days.

the different cell fractions were used in Lowry's assay. The protein content of different fractions is shown in Table 5. The protein content of the soluble fraction was always greater than that of the membrane fraction. As the protein content of the membrane fraction was less, we had to use more of that fraction in the enzyme assay as compared with the soluble fraction.

Nitrate- and Nitrite-reductase Assays

Nitrite and nitrite reductase activities were measured on the soluble and membrane fractions. Reduction of nitrate or nitrite was monitored by the measuring the decrease in absorption at 340 nm in duplicate assays. Decrease in absorbance in the absence of nitrate or nitrite indicated the presence of NADH oxidase activity. Because NADH oxidase activity was found to be present in both fractions during the initial assays, the assays were carried out in a specific order as shown in Figure 6. All components except nitrate or nitrate were added to the cuvette and the absorbance recorded for first minute. The slope (Δ absorbance/Δ time) of the line (slope 1) going through those points represents the NADH oxidase activity. After the first minute nitrate or nitrite was added to the cuvette and the absorbance values recorded every minute for 10 minutes.

At this point, addition of 40 µl of nitrate or nitrite to the 960 µl of reaction mixture was equivalent to diluting the mixture by a factor of 4 %. This dilution should have given an instant decrease in absorbance at the same moment when nitrate or nitrite was added. Since we did not use a continuous spectrophotometer, we could not record this value and that is why we took into consideration this dilution factor. All components except nitrate or nitrate were added to the cuvette and the absorbance value observed after first minute was noted down. Four percent of this absorbance value was calculated (the dilution factor) and it was subtracted from the absorbance value at one minute. This value represented the expected instant decrease in reading

Table 5. Protein concentrations of *M. avium* A5 cell fractions as derived by Lowry's Assay

Nitrogen source	Growth condition	Cell Fraction	Protein Concentration in ug/ml
KNO_3	Aerobic	Membrane	300
		Soluble	2050
KNO_3+$(NH_4)_2SO_4$	Aerobic	Membrane	375
		Soluble	1700
$(NH_4)_2SO_4$	Aerobic	Membrane	970
		Soluble	2500
KNO_3	Anaerobic Shift	Membrane	730
		Soluble	1800
KNO_3+$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	1000
		Soluble	2080
$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	350
		Soluble	2100

at that same moment because of addition of nitrate or nitrate. This value was plotted on the graph along with those absorbance values which were obtained every minute after the addition of nitrate or nitrite. The slope (Δ absorbance/Δ time) of the line going through those points (Slope 2) represented the combined activity of NADH oxidase and nitrate- or nitrite-reductase enzymes. Subtraction of slope 1 from slope 2 gave the change in absorbance which corresponded to the actual nitrite- or nitrite-reductase activities.

The three different plots that could be theoretically obtained while performing these assays are shown in Figure 6. If the NADH oxidase is very rapid, a plot similar to plot A will be obtained. If the NADH oxidase activity is present but at slower rate, a plot similar to plot B will be obtained. Plot C represents the ideal plot which will be obtained if NADH oxidase activity is absent. In our study, we found that the NADH oxidase of the soluble fractions was less than that of membrane fractions. The plots obtained for both the fractions were similar to plot B.

The data obtained from enzyme assays is shown in Table 6. Cultures I, II, and III were grown aerobically in the presence of nitrate alone, nitrate and ammonia in combination, or ammonia alone respectively. Culture IV, V, and VI were subjected to oxygen depletion in the presence of nitrate alone, nitrate and ammonia in combination, or ammonia alone, respectively. The results are as follows,

(1) *M. avium* A5 cells showed presence of nitrate- as well as nitrite-reductase activities.

(2) The nitrate- and nitrite-reductase activities were predominantly found in membrane fractions.

(3) The activity was almost negligible in the soluble fraction. The lowest level of nitrate- or nitrite-reductase activity able to be measured was 0.001 micromoles /min/ mg of protein.

(4) The nitrite reductase activity of *M. avium* A5 cells was higher than their nitrate reductase activity, except for the culture grown on ammonium sulfate under aeration.

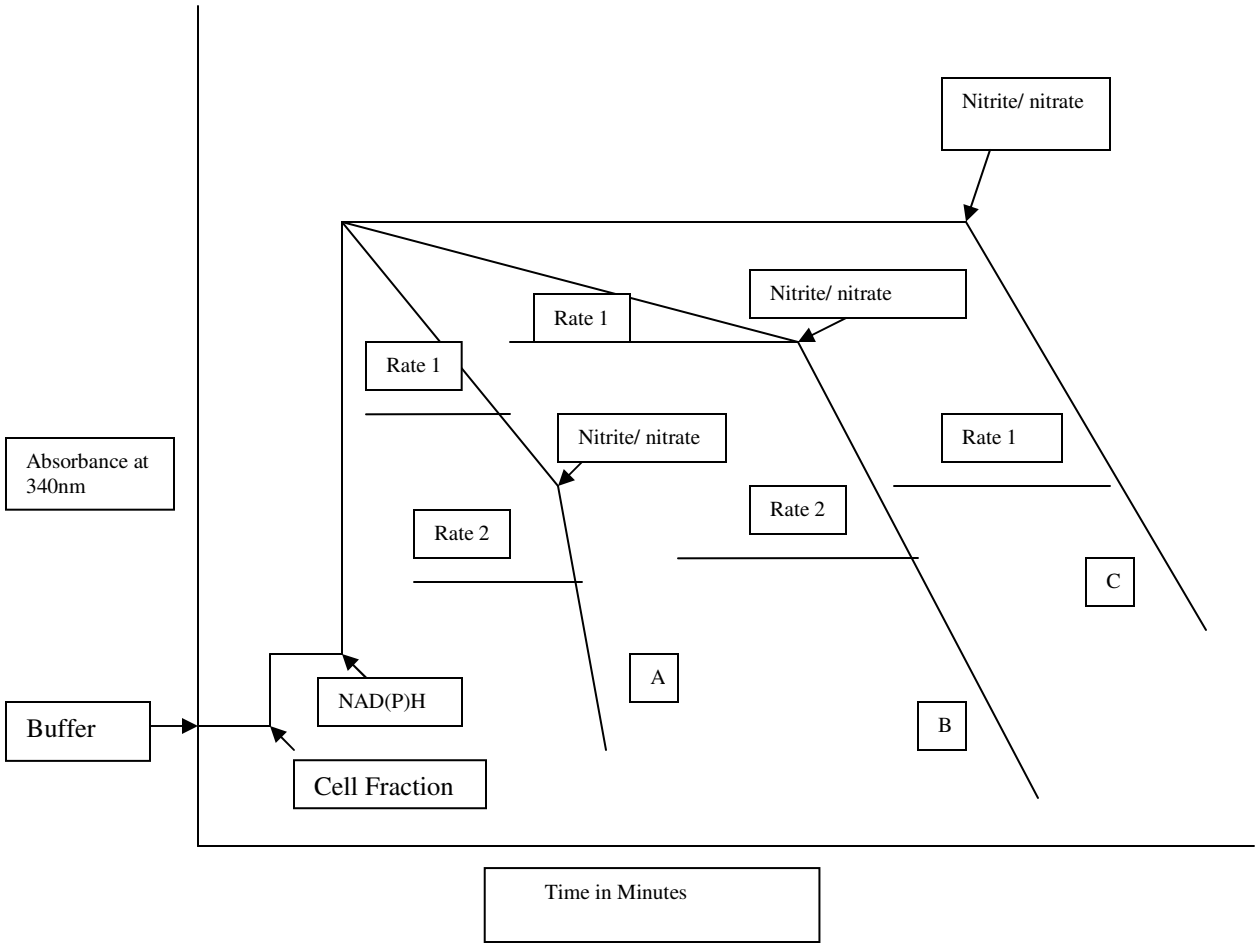

Figure 6. Analysis of Enzyme Activity Measurements.

Everything except nitrate or nitrate was added to the cuvette and the readings were noted down for first one minute. These readings were plotted on the graph paper. The slope of the line (slope 1) going through those points represents the NADH oxidase activity. Then nitrate or nitrite was added to the cuvette and the readings were noted down every minute. These readings were plotted on the same graph paper. The slope of the line going through those points (Slope 2) represented the combined activity of NADH oxidase and nitrate- or nitrite-reductase enzymes. The subtraction of slope 1 from slope 2 gave the change in absorbance (A) which corresponded to the actual nitrite or nitrite reductase activity. Figure 6 also mentions the three different plots one can obtain while performing these assays.

(5) The nitrate- and nitrite-reductase activities for culture I were 0.010 and 0.020 $\pm$ 0.005 micromoles / min / mg of protein, respectively. The nitrate- and nitrite-reductase activities for culture IV (incomplete anaerobiosis) were 0.0045 $\pm$ 0.0035 and 0.0095 $\pm$ 0.0005 micromoles / min / mg of protein, respectively. Thus both activities were found to be induced in the cells grown in the presence of nitrate (Culture I and IV).

(6) The nitrate- and nitrite-reductase activities for culture II were 0.0035 $\pm$ 0.0005 and <0.001 micromoles / min / mg of protein, respectively. The nitrate- and nitrite reductase activity for culture III were 0.003 $\pm$ 0.001 and 0.003 $\pm$ 0.001 micromoles / min / mg of protein, respectively. Thus both activities were decreased in the cells grown in the presence of ammonia alone or ammonia in combination with nitrate under aerobic conditions (Cultures II and III).

(7) The nitrate- and nitrite-reductase activities for culture IV were 0.0045 $\pm$ 0.0035 and 0.0095 $\pm$ 0.0035 micromoles / min / mg of protein, respectively. Thus, culture IV undergoing incomplete oxygen depletion in the presence of nitrate alone showed decreased activities (~ 50 %) as compared with that of culture I grown in the presence of nitrate alone aerobically.

(8) The nitrate- and nitrite-reductase activities for culture II were 0.0035 $\pm$ 0.0005 and <0.001 micromoles / min / mg of protein, respectively. The nitrate- and nitrite-reductase activities for culture V (incomplete anaerobiosis) were 0.003 $\pm$ 0.001 and 0.004 micromoles / min / mg of protein, respectively. Incubation for 7 days of the cultures undergoing oxygen depletion did not induce these activities as compared to that grown in the presence of nitrate alone (Culture IV) or nitrate in combination with ammonium sulfate in oxygen (CultureII).

(9) Surprisingly, the nitrate- and nitrite reductase activities for culture VI were 0.007 and 0.010 $\pm$ 0.005 micromoles / min / mg of protein, respectively. Thus both the activities were present in the

cultures undergoing incomplete oxygen depletion in the presence of ammonium sulfate alone (Culture VI).

Anaerobic Shift using Anaerobic Chamber

The data obtained from this experiment is shown in Table 8. Culture VII, VIII, and IX were shifted to anaerobiosis in the presence of nitrate alone, ammonia and nitrate in combination or ammonia alone respectively. The details are as follows,

(1) *M. avium* A5 cells showed presence of nitrate- as well as nitrite-reductase activities.

(2) The nitrate- and nitrite-reductase activities were predominantly found in membrane fractions.

(3) The activity was almost negligible in the soluble fraction. The lowest level of nitrate- or nitrite-reductase activity able to be measured was 0.001 micromoles / min / mg of protein.

(4) The nitrite reductase activity of *M. avium* A5 cells was higher than their nitrate reductase activity.

(5) The nitrate- and nitrite-reductase activities for culture undergoing anaerobic shift in the presence of nitrate alone (Culture VII) were 0.005 and 0.008 micromoles / min / mg of protein, respectively. Thus, culture VII undergoing anaerobic shift in the presence of nitrate alone showed decreased activities (~ 50 %) as compared with that of culture I (refer to data in Table 6) grown in the presence of nitrate alone aerobically.

(6) The nitrate- and nitrite-reductase activities for culture undergoing anaerobic shift in the presence of nitrate and ammonia in combination (Culture VIII) were 0.006 $\pm$ 0.001 and 0.009 micromoles / min / mg of protein, respectively. Thus, culture VII undergoing anaerobic shift in the presence of nitrate and ammonia in combination showed increased activities compared with that of culture II (refer to data in Table 6) grown in the presence of nitrate and ammonia in combination aerobically.

(7) The nitrate- and nitrite reductase activities for culture undergoing anaerobic shift in the presence of ammonia alone (Culture IX) were 0.008 and 0.012 $\pm$ 0.004 micromoles / min / mg of protein, respectively. Thus both the activities seem to be induced in the cultures undergoing anaerobic shift in the presence of ammonia alone compared to the aerobic culture (Culture III).

Enzyme Assays using NADPH as Cofactor

The enzyme assays were carried out in a similar manner as mentioned before to check whether nitrate- and nitrite-reductase use NADPH as an electron donor. Decrease in absorbance was observed even without addition of nitrate or nitrite. Enzyme activities were measured as mentioned before. These assays could not be done in duplicate especially on the membrane fractions because the membrane fraction got over due to less amount of protein content. The data obtained is shown in Table 7 and Table 9. It demonstrated that nitrate- and nitrite-reductases used NADPH as electron donor.

Table 6. Nitrate- and nitrite-reductase activities of the membrane fractions of *M. avium* A5 cells grown under different conditions using NADH as electron donor.

No.	Nitrogen source	Growth condition	Cell Fraction	Nitrite Reductase activity in micromoles of $NaNO_2$ reduced / min / mg of protein	Nitrate Reductase activity in micromoles of KNO_3 reduced / min / mg of protein
I	KNO_3	Aerobic	Membrane	0.020 ± 0.005	0.010
II	KNO_3+$(NH_4)_2SO_4$	Aerobic	Membrane	<0.001	0.0035 ± 0.0005
III	$(NH_4)_2SO_4$	Aerobic	Membrane	0.003 ± 0.001	0.003 ± 0.001
IV	KNO_3	Anaerobic Shift	Membrane	0.010 ± 0.0005	0.0045 ± 0.0035
V	KNO_3+$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	0.004	0.003 ± 0.001
VI	$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	0.010 ± 0.005	0.007

Nitrite- and nitrate-reductase activities of all the soluble fractions were always < 0.001 µM /min/ mg of protein

Table 7. Nitrate- and nitrite-reductase activities of the membrane fractions of *M. avium* A5 cells grown under different conditions using NADPH as electron donor.

No.	Nitrogen source	Growth condition	Cell Fraction	Nitrite Reductase activity in micromoles of $NaNO_2$ reduced / min / mg of protein	Nitrate Reductase activity in micromoles of KNO_3 reduced / min / mg of protein
I	KNO_3	Aerobic	Membrane	-	-
II	KNO_3+$(NH_4)_2SO_4$	Aerobic	Membrane	-	0.0025
III	$(NH_4)_2SO_4$	Aerobic	Membrane	0.002	0.002
IV	KNO_3	Anaerobic Shift	Membrane	0.002	0.001
V	KNO_3+$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	0.002	< 0.001
VI	$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	-	-

Nitrite- and nitrate-reductase activities of all the soluble fractions were always < 0.001 µM /min/ mg of protein.

- Enzyme assay could not be carried out as the fraction was already utilized for previous assays using NADH as electron donor.

Table 8. Nitrate- and nitrite-reductase activities of the membrane fractions of *M. avium* A5 cells undergoing anaerobic shift in anaerobic chamber using NADH as electron donor.

No.	Nitrogen source	Growth condition	Cell Fraction	Nitrite Reductase activity in micromoles of $NaNO_2$ reduced / min / mg of protein	Nitrate Reductase activity in micromoles of KNO_3 reduced / min / mg of protein
VII	KNO_3	Anaerobic Shift	Membrane	0.008	0.005
VIII	KNO_3+$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	0.009	0.006 $\pm$ 0.001
IX	$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	0.012 $\pm$ 0.004	0.008

Nitrite- and nitrate-reductase activities of all the soluble fractions were always < 0.001 µM /min/ mg of protein

Table 9. Nitrate- and nitrite-reductase activities of membrane fractions of *M. avium* A5 undergoing anaerobic shift in anaerobic chamber using NADPH as electron donor.

No.	Nitrogen source	Growth condition	Cell Fraction	Nitrite Reductase activity in micromoles of $NaNO_2$ reduced / min / mg of protein	Nitrate Reductase activity in micromoles of KNO_3 reduced / min / mg of protein
VII	KNO_3	Anaerobic Shift	Membrane	–	–
VIII	KNO_3+$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	0.008	0.006
IX	$(NH_4)_2SO_4$	Anaerobic Shift	Membrane	0.009	0.009

Nitrite- and nitrate-reductase activities of all the soluble fractions were always < 0.001 µM /min/ mg of protein.

- Enzyme assay could not be carried out as the fraction was already utilized for previous assays using NADH as electron donor.

DISCUSSION

Introduction

The nitrate reduction test is a standard method used to distinguish between mycobacterial species (Virtanen, 1960). Virtanen (1960) carried out a large scale study on nitrate reduction in mycobacteria. *Mycobacterium tuberculosis* is strongly positive for this test (Virtanen, 1960). *M. avium* had some strains strongly positive, some weakly positive and a few were negative for this test (Virtanen, 1960). However, currently used clinical tests consider *M. avium* negative for nitrate reduction (Vestal, 1975; Wayne, 1985). Based on Dr.McCarthy's work (1987), we carried out this study to investigate the enzyme activities in order to find out the answers to following questions. Does *M. avium* grow in a defined minimal media in the presence of single nitrogen source? Does *M. avium* have nitrate reductase activity? Does *M. avium* have nitrite reductase activity? Is nitrite reductase activity greater than nitrate reductase activity? Is there any respiratory and /or dissimilatory activity in addition to assimilatory activity? Is there any regulatory role of ammonia, nitrate/ nitrite and oxygen on these activities?

M. avium Growth on Nitrogen Sources

We confirmed that *M. avium* can grow in a defined minimal media utilizing a single nitrogen source. Theses data agree with Dr.McCarthy's work (1987). The major problem for the growth experiments was contamination. Mycobacteria being slow growing organisms need at least 10 days of incubation. It makes them very much prone to contamination while growing in laboratory medium. We used Bugstoppers (Whatman Inc., Clifton, NJ) instead of cotton plugs to seal the flasks. Bugstoppers proved to be helpful. Outside air might have effect on the temperature of the water bath so it is always better to carry out the incubation in a walk in incubator. Tween 80 helps to prevent mycobacteria from forming clumps in liquid medium (Stermann et al., 2003). We have used 10 ml of 10 % Tween 80 per liter of the NT medium. The

characteristic clump formation was observed within 4 days of incubation but the clumps could be easily dispersed and did not affect the turbidity measurements.

It has been documented that *M. avium* can utilize 0.25 mM to 2 mM of ammonia, nitrate, and nitrite as single nitrogen source (McCarthy, 1987). In our study, *M. avium* A5 grew very well on all the nitrogen sources except nitrite. The concentration of each nitrogen source was 2 mM. It has been documented that mycobacteria can grow in a culture medium even in the absence of nitrogen source by utilizing its own nitrogen in the presence of suitable carbon source (Marschak and Schaefer, 1952). We used *M. avium* A5 cells from a stock culture grown in the Middlebrook 7H9 broth medium (BBL Microbiology System, Cockeysville, MD) containing 0.5 % (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin. Although we washed the cells, it is quite possible that we could not remove all the nitrogenous compounds and albumin present in that broth. These compounds might have been the source of nitrogen for some days and that is why the culture still showed some growth when grown on 2 mM nitrite. The cultures grown on nitrite were not viable after 3-4 week. It has already documented that *M. avium* growth is inhibited by 3 mg of sodium nitrite per ml and this susceptibility is can be used as an indicator to clinical diagnosis of *M. avium* (Sato et al., 1992). Nitrite is also reported to be toxic for mycobacteria (Ratledge, 1982).

Mycobacterial Adaptation

After entering the host tissues, mycobacteria adapt themselves to that environment by induction of enzymes and changing metabolism (Barclay and Wheeler, 1989). For example, mycobacteria adapt to the reduced concentrations of oxygen in necrotic and dead tissues of the host (Weber et al., 2000) by inducing nitrate reductase activities. We carried out the anaerobic shift in order to find out whether *M. avium* has any dissimilatory or respiratory nitrate- or nitrite-

reductase activities. Unfortunately, total anaerobiosis was never achieved. The methylene blue indicator strip remained blue even after 7 days. One possible reason can be the very slow generation time of the culture which might have lead to slow consumption of available oxygen. In a study carried out by Wayne and Hayes (1996), to obtain slow and continuous depletion of oxygen a head space ratio (ratio of length of column of air above the surface of culture medium to the length of column of culture medium) of 0.5 was maintained. However, as we used 1000 ml flask for these growth experiments, the available air column above the surface of liquid medium was quite big and the head space ration was more than 1. Repeat experiments were carried out using anaerobic chamber and complete anaerobiosis was achieved and enzyme assays were repeated as mentioned before. The data is analyzed in detail in following sections.

Mycobacterial Breakage

Mycobacteria are difficult to break open. Even after 15 minutes of sonication, the protein concentration for the membrane fraction was much lower compared to the soluble fraction. Sonication for 20 minutes yielded increased amount of proteins in the membrane fraction. It is questionable though to increase the sonication time in order to get more amount of protein as it might affect the enzyme activity because of excessive heat formation. Instead of continuous sonication, we used a pulse mode which prevents excessive heat formation and loss of enzyme activity and throughout the process the sample containing tube was kept cool by placing it inside an ice-water slurry. The membrane fraction appeared as thick dark brown colored pellet which was difficult to disperse into solution during the washing. Different detergents like Tween 20, Triton X-100 and CHAPS can be used to solubilize the membrane fraction. But again there is risk of loss of enzyme activity.

Enzyme Assays

Oxidation of NADH even in the absence of nitrate or nitrite has already documented by Bastarrachea and Goldman (1961). Presence of NAD(P)H oxidase was major problem, while performing the enzyme assays. The protocol which we followed helped to discriminate the nitrate and nitrite reductase activities. Since we did not use a continuous spectrophotometer, we have taken into consideration the dilution factor which is explained in detail in the results section. The use of continuous spectrophotometer can give more precise pattern of decrease in absorbance. As a control, we added everything except the cell fraction to the cuvette and absorbance was monitored. Samples from boiled cell fractions can be used to carry out control assays. If no activity is found, we can confirm that the change in absorbance during the assays is due to the presence of enzyme activity which gets denatured because of boiling.

According to results of this study, *M. avium* showed presence of nitrate as well as nitrite reductase activities. Nitrite reductase activity was more than nitrate reductase activity. It has been documented that *M. avium* utilized nitrate slowly and nitrite rapidly (McCarthy, 1986). Our data further supports Dr.McCarthy's work.

Cofactors

As the enzyme assays were carried out by the method described by Bastarrachea and Goldman (1961), we decided to use NADH and NADPH as cofactor for our study. In the study carried out by Bastarrachea and Goldman (1961), they found that NADPH was not used as an electron donor by nitrate reductase enzyme in *M. tuberculosis* but in our study, nitrate- and nitrite-reductase enzymes of *M. avium* used NADH as well as NADPH as electron donor. The data is shown in Tables 6 and 7 respectively. Using a different cofactor might give more amount of enzyme activity. For example, as shown in Table 1, it is evident that *M. avium* has ferredoxin

dependent nitrite reductase genes. So in future experiments the enzyme assays can be done using ferredoxin ($FADH_2$) as cofactor.

Interpretation of Enzyme Activities

The data obtained from the enzyme assays is shown in Table 6. Cultures I, II, and III were grown aerobically in the presence of nitrate alone, nitrate and ammonia in combination or ammonia alone respectively. Cultures IV, V, and VI were subjected to oxygen depletion in the presence of nitrate alone, nitrate and ammonia in combination, or ammonia alone respectively. As mentioned in Table 8, Cultures VII, VIII, and IX were shifted to anaerobiosis in the presence of nitrate alone, ammonia and nitrate in combination or ammonia alone respectively. As mentioned in Table 10, the comparison of enzyme activities from culture I with II or that of VII with VIII was done to deduce assimilatory enzyme activity. To deduce dissimilatory enzyme activities, the enzyme activities from culture II and III or VIII and IX were taken into consideration. In a similar manner comparison of enzyme activities from culture I with VII or that of II with VIII or III with IX was done to deduce respiratory enzyme activity.

The nitrate- and nitrite-reductase activities for culture I were 0.010 and 0.020 $\pm$ 0.005 μM /min/ mg of protein, respectively. The nitrate- and nitrite-reductase activities for culture II were 0.0035 $\pm$ 0.0005 and <0.001 μM /min/ mg of protein, respectively. Therefore, there appears to be an ammonia repressible nitrate- and nitrite-reductase. The nitrate- and nitrite reductase activity for culture III were 0.003 $\pm$ 0.001 and 0.003 $\pm$ 0.001 μM /min/ mg of protein, respectively. This is likely the basal level of reductase activity; perhaps another, non-nitrate- or nitrite-reductase using another substrate.

The nitrate- and nitrite reductase activities for culture IV were 0.0045 $\pm$ 0.0035 and 0.0095 $\pm$ 0.0005 μM /min/ mg of protein respectively, approximately half the activity of cultures

Table 10. Interpretation of enzyme activities

Type of Enzyme Activity	Cultures to be taken into consideration for Comparison of enzyme activities
Assimilatory	I & II and VII & VIII
Dissimilatory	II & III and VIII & IX
Respiratory	I & VII, II & VIII, and III & IX

The comparison of enzyme activities from culture I with II or that of VII with VIII was done to deduce assimilatory enzyme activity. To deduce dissimilatory enzyme activities, the enzyme activities from culture II and III or VIII and IX were taken into consideration. In a similar manner comparison of enzyme activities from culture I with VII or that of II with VIII or III with IX was done to deduce respiratory enzyme activity.

grown aerobically. The nitrate- and nitrite-reductase activities for culture V were 0.003 $\pm$ 0.001 and 0.004 µM /min/ mg of protein respectively, approximately equal to those of oxygenated culture II. The nitrate- and nitrite reductase activities for culture VI were 0.007 and 0.010 $\pm$ 0.005 µM /min/ mg of protein respectively, approximately equal to those of oxygenated cultures. Because complete oxygen depletion was not achieved the data for partially anaerobic cultures cannot be used to determine whether *M. avium* has a dissimilatory or respiratory reductases.

By comparing the activities of culture I with II or that of IV with V, it is evident that *M. avium* A5 cells produce an assimilatory nitrate - and nitrite-reductase activities because these activities got induced in the presence of nitrate, repressed in the presence of ammonia. This regulation is very much similar to that in *E. coli*.

By comparing the enzyme activities from culture I with IV or that of II with V, it is evident that respiratory activities were not induced due to 7 days of anaerobiosis. In fact the activities are less. But the comparison of enzyme activities from culture III with that of VI shows some enzyme activity induction. But we can not designate it as respiratory because complete anaerobiosis was never achieved in any of the cultures undergoing anaerobic shift. Respiratory activity should be repressed by presence of oxygen and in the absence of the inducer i.e. nitrate or nitrite. Virtanen (1960) has reported in his study that there is no correlation between nitrate reduction and oxygen consumption of mycobacteria. But Weber et al (2000) have reported expression of nitrate reductase activity under anaerobic conditions in *M. bovis* BCG. Wayne and Hayes (1998) reported similar findings in *M. tuberculosis*. So this issue needs to be further explored in case of *M. avium* A5.

The nitrate- and nitrite-reductase activities for culture VII were 0.005 and 0.008 micromoles / min / mg of protein, respectively. The nitrate- and nitrite-reductase activities for

culture VIII were 0.006 $\pm$ 0.001 and 0.009 micromoles / min / mg of protein, respectively. The nitrate- and nitrite reductase activities for culture IX were 0.008 and 0.012 $\pm$ 0.004 micromoles / min / mg of protein, respectively.

There is not much difference between the activities for culture VII and that of culture VIII. By comparing the activities of culture I (refer to Table 6) to those of culture VII (refer to Table 8), it can be deduced that the activities were not greatly induced in culture VII which was undergoing anaerobic shift in the presence of nitrate alone. By comparing the activities of culture II (refer to Table 6) to those of culture VIII (refer to Table 8), it can be deduced that the activities were induced in culture VIII which was undergoing anaerobic shift in the presence of ammonia and nitrate in combination. Similarly, by comparing the activities of culture III (refer to Table 6) to those of culture IX (refer to Table 8), it can be deduced that the activities were induced in culture IX which was undergoing anaerobic shift in the presence of ammonia alone.

Based on these results, the enzyme activities were present in the absence of nitrate (Culture IX) and these activities were not affected by the presence of ammonia and oxygen. This pattern is very much similar to that of dissimilatory enzyme activity in *E. coli* (Moreno-Vivián et al., 1999). The nitrate- and nitrite-reductase activities were induced in the culture VIII which was undergoing anaerobic shift in the presence of nitrate and ammonia in combination. But these activities were not greatly induced in the culture VII which was undergoing anaerobic shift in the presence of nitrate alone. So it will be questionable to designate these activities as respiratory because respiratory activity should be induced in the presence of nitrate and absence of oxygen (Moreno-Vivián et al., 1999). This issue can be solved by repeating the same experiment and redoing the enzyme assays.

Nitrate Reductase Test

Virtanen (1960), Bastarrachea and Goldman (1961) mentioned the absence of nitrite reductase activity in *M. tuberculosis* and determined the nitrate reductase activity by measurement of nitrite. In contrast, this work shows that *M. avium* has both nitrate- and nitrite-reductase activities. This implies that *M. avium* cells further reduced nitrite produced by nitrate reductase to other reduction products. These products cannot be detected by standard nitrate reductase test as it looks only for the presence of nitrite. So *M. avium* should not be considered nitrate reductase test negative. Similar experiment should be carried out in other mycobacteria which are designated as nitrate reductase negative by the standard method to check for presence of nitrite reductase activity. Even if nitrate reductase test is a rapid, simple and inexpensive method, more studies should be carried out to determine the accuracy before it to be considered as standard method for clinical use (Agneby et al., 2002). Our data definitely support this issue.

Future Experiments

Similar experiments can be done for other mycobacteria which are designated as nitrate reductase test negative by the standard test.

Experiments can be done to study the pattern of utilization of nitrogen sources in *Mycobacterium avium*. The ammonia concentration was measured indophenol blue assay (Keeney and Nelson, 1982). The nitrate concentration was measured by the brucine method (Jenkins and Medsker, 1964). The nitrite concentration was measured by the method of Hanson and Philips (1981). From the standard curves obtained, there was no interference in the linear relationship between the concentration of nitrogen source and absorbance even in the presence of other nitrogen sources.

Samples can be taken from *M. avium* A5 culture growing on 2 mM ammonium sulfate and concentration of ammonia can be measured to find out utilization. Samples taken from the cultures growing in the presence of 2 mM nitrate can be used to find out how much nitrate is utilized and also to find out how much nitrite is formed?

In case of the culture growing in the presence of ammonia and nitrate in combination, samples can be taken to find out the concentrations of ammonia and nitrate. We think that mycobacteria will utilize available ammonia first then they will switch to metabolize nitrate. So there will be decline in the ammonia concentration proportional with growth with nitrate concentration remaining constant. Once available ammonia is utilized, there will be induction of nitrate reductase and nitrate concentration will start to fall down. The growth curve of this culture will be biphasic. The first one will represent growth on ammonia (4 days per generation) and second will represent the growth due to nitrate (5 days per generation). The determination of rates of utilization can further help us to understand the nitrogen metabolism in *M. avium.*

Future research should be done to correlate enzyme activities with genes, to examine expression of different nitrate- and nitrite reductases in biofilms and tissue and to identify genetic basis for multivalent regulation of nitrate and nitrite reductases.

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