Nitrate and Nitrite Reductase Activities of Mycobacterium avium

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Abstract

Background: In spite of the fact that the standard test for nitrate reductase activity is negative for Mycobacterium avium, it can grow in a defined minimal medium with either nitrate (NO₃⁻) or nitrite (NO₂⁻) as sole nitrogen sources. Methods: NO₃⁻ and NO₂⁻-reductase activities were measured in soluble and membrane fractions of aerobically grown cells of M. avium and those grown aerobically and shifted to anaerobiosis. Results: NO₃⁻ and NO₂⁻-reductase activities were only detected in the membrane fractions and the two enzyme activities were significantly reduced if cells were grown aerobically in the presence of ammonia (NH₃). The NO₂⁻-reductase activity of membrane fractions was 2-fold higher than that of NO₃⁻-reductase consistent with the fact that NO₂⁻-reductase activity of M. avium cannot be detected if measured by nitrite formation. Membrane fractions of M. avium cells grown 1 week aerobically and then 2 weeks under anaerobic conditions had NO₂⁻-reductase activities. Conclusion: The results are consistent with the presence of assimilatory NO₂⁻ and NO₂⁻-reductase activities in cells of M. avium grown under aerobic conditions. Further, the data suggest that a shift to anaerobic conditions results in the appearance of ammonium-insensitive NO₃⁻ and NO₂⁻-reductase activities; quite possibly that function in a dissimilatory role (redox balancing).

Keywords: Ammonium, Mycobacterium avium, nitrate, nitrate reductase, nitrite, nitrite reductase

Introduction

Mycobacterium avium is an environmental opportunistic pathogen whose infections have been linked to drinking water. As M. avium is a natural inhabitant of natural and engineered water systems, including household plumbing, the bacterium ought to show signs of adaptation to that habitat, for example, to nitrate and nitrite that are present in natural and engineered water systems. The observation that M. avium numbers in water samples of six different drinking water systems across the United States were modestly correlated ($r^2 = 0.2974$) with nitrate concentrations is consistent with demonstration that either nitrate or nitrite can be utilized as sole nitrogen sources by M. avium. The concentration of nitrate is increased in water resources in the United States, maybe responsible, in part, for the increased frequency of isolation of M. avium. Two other habitats occupied by M. avium are human macrophages and the lungs of cystic fibrosis (CF) patients. Studies have shown that the infection of human macrophages with mycobacteria leads to significantly increased levels of nitrate and nitrite and that CF patient lung tissue also has high levels of both nitrate and nitrite, and that evidence suggests that M. avium nitrate reductase and nitrite reductase may contribute to survival, growth, and virulence in macrophages and lungs of CF patients.

The genome of M. avium has multiple genes with sequence similarities to nitrate and nitrite reductases (link to M. avium genome). In addition to assimilatory nitrate or nitrite reductases required for M. avium growth on nitrate or nitrite, nitrate reductase might also contribute to the survival of M. avium under anaerobic conditions by maintaining redox balance through the regeneration of oxidized nicotinamide adenine dinucleotide (NAD). Redox balancing by nitrate reductase could be responsible for the ability of M. avium to survive rapid exposure to anaerobic conditions, unlike Mycobacterium tuberculosis. Curiously, although nitrate and nitrite can be

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utilized as sole nitrogen sources by \textit{M. avium};\textsuperscript{[5]} \textit{M. avium} is described as being nitrate reductase negative.\textsuperscript{[13,14]} This inconsistency can be explained if nitrite reductase is present, so that nitrite does not accumulate and is not detected by using the standard nitrate reductase test.\textsuperscript{[15]} Further, a negative nitrate reductase test might result if \textit{M. avium} nitrate reductase activity was prevented by growth in the presence of ammonium or glutamate; both constituents of media used for mycobacterial cultivation (e.g., Lowenstein–Jensen and Middlebrook 7H9 broth and 7H10 agar). Consequently, if either proves to be the case, \textit{M. avium} isolates would be incorrectly recorded as nitrate reductase negative.\textsuperscript{[15]}

Herein, we confirm the utilization of nitrate and nitrite as sole nitrogen sources for \textit{M. avium} growth, and the activity of nitrate and nitrite reductases under aerobic and anaerobic conditions in the presence and absence of ammonia. The long-term goal of the studies is to describe the role of nitrate and nitrite reductases in the physiology, ecology, and virulence of \textit{M. avium}.

**METHODS**

\textbf{Mycobacterial strain and growth medium}

\textit{M. avium} strain A5, a plasmid-free AIDS patient isolate was used for this study.\textsuperscript{[16]} The nitrogen growth medium (NT) of McCarthy\textsuperscript{[10]} containing 8 \muM sodium molybdate\textsuperscript{[17]} was used for the growth of cells. The medium lacks albumin and contains palmitic acid. Ammonium sulfate, potassium nitrate, or sodium nitrite were added to the medium at a final concentration of 2 mM. Growth, reflected by increases in turbidity (absorbance at 580 nm) on the different nitrogen sources, was measured in 15 ml of NT medium in 250 ml side-arm flask incubated at 37°C with aeration (60 rpm). For enzyme assays, \textit{M. avium} strain A5 was grown in 500 ml of NT medium containing 2 mM KNO$_3$, 2 mM (NH$_4$)$_2$SO$_4$, or both in 1-liter flasks at 37°C with aeration (60 rpm) for 3 weeks. For cultures grown in air, the samples were withdrawn at 0, 1, 2, and 3 weeks for measurement of NO$_3$, NO$_2$, and NH$_4$ concentrations. For aerobically grown cultures, the enzyme activity measurements were made after 3-week incubation. For anaerobic shifts, cultures were incubated in air for 1 week and then transferred to an anaerobic chamber and incubated for an additional 2 weeks at 37°C without agitation.

\textbf{Enzyme assays}

Membrane and soluble cell fractions were isolated\textsuperscript{[18]} and nitrate and nitrite reductase activities were measured as described\textsuperscript{[19]} in the presence of either NAD hydrogen (NADH) or NAD phosphate (NADPH). The protein concentration of fractions was measured using the method of Lowry et al.\textsuperscript{[20]} using bovine serum albumin fraction V (Sigma-Aldrich, St. Louis, MO) as standard.

\textbf{Measurement of ammonia, nitrate, and nitrite}

Ammonium was measured by the indophenol blue assay\textsuperscript{[21]} nitrate by the Brucine method,\textsuperscript{[22]} and nitrite by the method of Hanson and Philips.\textsuperscript{[23]}

**RESULTS**

\textbf{Growth of Mycobacterium avium A5 on ammonium, nitrate, and nitrite}

The growth rates of \textit{M. avium} strain A5 on glutamine, ammonium sulfate, potassium nitrate, and sodium nitrite as sole nitrogen sources are summarized in Table 1. During growth, cells formed aggregates as described by others;\textsuperscript{[13]} however, the aggregates could be dispersed easily by swirling; hence, they did not interfere with the measurement of turbidity. Data confirm that \textit{M. avium} strain A5 can utilize nitrate or nitrite as sole nitrogen sources in a minimal defined medium.\textsuperscript{[5]} Growth on nitrite was poor, and there was a measurable 48 h lag phase before growth occurred [Table 1]. Further, unlike cultures grown on nitrate, ammonia, or glutamine, cultures grown on nitrite were not viable after 3–4 weeks’ incubation. This meant that cultures to be grown on nitrite as sole nitrogen source required inoculation with washed cells grown on nitrate.

\textbf{Nitrate and nitrite reductase activities of aerobically grown cells}

Nitrate and nitrite reductase activities were measured on the soluble and membrane fractions. Due to the presence of NADH oxidase activity in the fractions, the rate of oxidation of NADH was measured for the first 2 min in the absence of nitrate or nitrite and then either one was added, and the rate of NADH oxidation measured the difference in rates due to the reductase activity. NADH-dependent nitrate and nitrite reductase activities were expressed by \textit{M. avium} A5 cells grown in the presence of nitrate as a sole nitrogen source [Table 2]. The rates of both enzymes were lower when measured with NADPH (data not shown). In aerobic cultures grown on nitrate, the nitrite reductase activity was higher than that of nitrate reductase [Table 2]. Higher nitrite reductase activity would lead to a negative nitrate reductase test\textsuperscript{[13–15]} because nitrite would be reduced before it could accumulate.

The nitrate and nitrite reductase activities were predominantly found in membrane fractions with the activity almost negligible in the soluble fraction (data not shown). Nitrate was required for the appearance of both nitrate and nitrite reductase activities; the reductase activities were almost negligible in ammonia-grown cultures [Table 2]. The lower limit of measurement of nitrate or nitrite reductase activity was

| Table 1: Growth characteristics of Mycobacterium avium strain A5 on different nitrogen sources\textsuperscript{a} |
|---------------------------|---------------------|----------------------|
| Nitrogen source           | Lag phase           | Generation time      |
| Glutamine                 | None                | 4 days/generation     |
| Ammonium sulfate          | None                | 4 days/generation     |
| Potassium NO$_3$          | None                | 5 days/generation     |
| Sodium NO$_2$             | 48 h                | 6 days/generation     |

\textsuperscript{a}Mycobacterium avium A5 strain was inoculated in 15 ml of NT medium in the presence of single nitrogen source at a 2 mM concentration. Cytoclines were grown aerobically in a water bath maintained at 37°C at a speed of 60 rpm. Each culture was grown at least for 10 days. NO$_3$; Nitrate, NO$_2$; Nitrite, NT: Nitrogen test.
0.001 micromoles NADH oxidized/min/mg protein. Nitrate and nitrite reductase activities were significantly reduced by aerobic growth of M. avium strain A5 cells in the presence of nitrate and ammonium [Table 2].

### Nitrate and nitrite reductase activities following a shift to anaerobiosis

To explore the possibility that M. avium survival under a rapid shift to anaerobic conditions is due to redox balancing by dissimilatory nitrate and nitrite reductases, cells were grown in the presence of nitrate or nitrate and ammonia under aerobic conditions for 1 week and then shifted to anaerobic conditions for an additional 2 weeks. Nitrate and nitrite reductase activities were present in those shifted cells with nitrate reductase activity higher than that of nitrite reductase [Table 2]. The presence of ammonia in the growth medium reduced but did not abolish the levels of nitrate and nitrite reductase activities in the anaerobic-shifted cells [Table 2].

### Discussion

Measurement of aerobic growth of M. avium strain A5 in a defined minimal medium confirmed that either nitrate or nitrate could serve as sole nitrogen sources [Table 1] as had been shown by McCarthy. Further, the results document the presence of nitrate and nitrite reductase activities in M. avium strain A5 [Table 2]. As the standard enzymatic test for the identification of mycobacteria involves the detection of nitrite, the presence of M. avium nitrite reductase would lead to a false-negative assay for the presence of nitrite reductase. As mycobacterial isolates are routinely grown on ammonium-containing medium, the activity of both reductases would be reduced to undetectable levels [Table 2]. Measurements of soluble and membrane fractions of aerobically grown cells of M. avium demonstrated that the presence of nitrate and nitrite reductase activities only in the membrane fractions. As shown for M. tuberculosis nitrate reductase, NADPH was unable to replace NADH for M. avium nitrate reductase activity.

Membrane fractions of M. avium cells grown 1 week aerobically and then 2 weeks under anaerobic conditions had both nitrate and nitrite reductase activities [Table 2]. Nitrate reductase activity was higher than that of nitrite reductase in such shifted anaerobic cells [Table 2]. Both activities in the anaerobically shifted cells were reduced by growth in the presence of nitrate and ammonium [Table 2]. However, the activity may be sufficient for dissimilatory redox balancing. Proof that the reductases are responsible for redox balancing will require the use of selective inhibitors or construction of M. avium mutants of the different nitrate and nitrite reductase genes to isolate the effects of the assimilatory and dissimilatory enzymes.

The ability of M. avium to utilize nitrate and nitrite as sole nitrogen sources is consistent with the hypothesis that this species is adapted to growth and persistence in natural and human-engineered water systems. Further, although speculative, the increased prevalence of M. avium may be due to the reported increased concentration of nitrate in United States waters. Finally, the presence of nitrite reductase may contribute to the increased survival of M. avium cells following a rapid shift to anaerobiosis compared to M. tuberculosis. In cultures of M. tuberculosis exposed to reduced oxygen, it was speculated that the accumulation of nitrate repressed growth. In contrast, the presence of M. avium nitrite reductase would prevent nitrite accumulation, and there would be no growth limitation. It remains to be seen whether other Mycobacterium species, most of which are environmental opportunists, follow the pattern seen here for M. avium.

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### Conflicts of interest

There are no conflicts of interest.

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