Uptake of sulfate but not phosphate by *Mycobacterium tuberculosis* is slower compared to *Mycobacterium smegmatis*

Houhui Song$ and Michael Niederweis*

Department of Microbiology, University of Alabama at Birmingham, 609 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294, U.S.A.

$ Current address: College of Animal Science and Technology, Zhejiang A&F University, 88 HuanBei Rd., Lin'an, Zhejiang Province 311300, P.R.China

**Running title:** Uptake of inorganic anions by mycobacteria

**Key words:** permeability, nutrient, growth rate, phosphate, nitrate, sulfate

**Abbreviations:** OM: outer membrane; CCCP: carbonyl cyanide 3-chlorophenylhydrazone, DCC: N,N'-dicyclohexylcarbodiimide; wt: wild-type

* For correspondence:

Phone: +1-205-996-2711
Fax: +1-205-934-9256
E-Mail: mnieder@uab.edu
SUMMARY

Knowledge of the metabolic pathways used by *Mycobacterium tuberculosis* during infection is important for understanding its nutrient requirements and host adaptation. However, uptake, the first step in utilization of nutrients, is poorly understood for many essential nutrients such as inorganic anions. Here, we show that *M. tuberculosis* utilizes nitrate as the sole nitrogen source albeit at lower efficiency than asparagine, glutamate and arginine. Growth of the porin triple mutant *M. smegmatis* ML16 in media with limiting amounts of nitrate and sulfate as sole nitrogen and sulfur sources, respectively, was delayed compared to the wild-type strain. Uptake of sulfate was 40-fold slower compared to the wild-type strain indicating that efficient uptake of these anions is dependent on porins. Uptake by *M. tuberculosis* of sulfate and phosphate was approximately 40- and 10-fold compared to *M. smegmatis*, respectively, consistent with the slower growth of *M. tuberculosis*. However, uptake of these anions by *M. tuberculosis* is orders of magnitude faster than diffusion through lipid membranes indicating that unknown outer membrane proteins are required to facilitate this process.
INTRODUCTION

In 2009, *Mycobacterium tuberculosis* caused 9.4 million cases of tuberculosis resulting in the death of approximately 1.7 million people (53). After inhalation, *M. tuberculosis* is phagocyted by alveolar macrophages and resides in nutrient-limited phagosomes. *M. tuberculosis* can prevent phagosome acidification and influx of many toxic compounds into the phagosome by blocking fusion with late endosomes and lysosomes. This mechanism is critical for survival of *M. tuberculosis* in macrophages and a key feature of its virulence in the host environment (34). However, it is obvious that acquisition of essential nutrients is also required for replication of *M. tuberculosis* in macrophages. Recently, carbon metabolism has received increased attention due to its importance for virulence of *M. tuberculosis* (5, 23), but knowledge about the metabolism of other essential nutrients is scarce (25).

On a molecular level, uptake of nutrients precedes any intracellular metabolism and is often the target of regulatory mechanisms. In this study, we examined the uptake of inorganic anions, in particular nitrate, sulfate and phosphate, by *M. tuberculosis* and *M. smegmatis*. A possible source of nitrate for *M. tuberculosis* in vivo is the oxidation of nitric oxide, which is generated in large amounts within macrophages and restricts growth of *M. tuberculosis* (13). The activity of nitrate reductase drastically increases upon entry of *M. tuberculosis* into the dormant state (49, 50) indicating that *M. tuberculosis* uses nitrate as an alternative terminal electron acceptor under anaerobic conditions. Sohaskey demonstrated that nitrate enhances survival of *M. tuberculosis* during a sudden shift from aerobic to anaerobic respiration (39). NarK2 is a putative nitrate/nitrite transporter of *M. tuberculosis*, which is required to reduce nitrate anaerobically (41). In macrophages, *M. tuberculosis* is exposed to reactive nitrogen and oxygen intermediates which, among other reactions, oxidize and nitrosylate cysteines. Genes involved in cysteine biosynthesis are upregulated in dormancy models for *M. tuberculosis* consistent with the need for replacement of these damaged proteins (37, 48). Cysteine synthesis requires the availability of sulfur, e.g. by uptake of sulfate by the ABC transporter composed of CysW, CysT and CysA (52). Genes encoding inner membrane phosphate transport systems such as Pst are essential for the survival of *M. tuberculosis* in macrophages and mice (33, 36), indicating that phosphate inside phagosomes of macrophages is indeed limited.
In this study, we examined the utilization and uptake of these inorganic anions by *M. tuberculosis* and compared these processes with *M. smegmatis*. For a long time, the focus has been on inner membrane transporters of *M. tuberculosis* because they can be easily recognized by similarities to known transporters of other bacteria. However, it has recently been established that mycobacteria have an outer membrane (12), which constitutes the first and primary permeability barrier which has to be overcome for transport of any nutrient molecule. To this end, the outer membrane porin MspA and its Msp paralogues are required for phosphate uptake by *M. smegmatis* (14, 51). It has been proposed that *M. tuberculosis* also uses outer membrane pore proteins for uptake of inorganic anions (25). Indeed, porin activity has been demonstrated in both *M. tuberculosis* (16) and *M. bovis* BCG (19), but the identity of these proteins is unknown and experimental evidence for their physiological function is lacking. We used Msp porin mutants to show that these outer membrane channel proteins are required for nitrate utilization and sulfate uptake by *M. smegmatis*. Quantitative analysis of the permeability for inorganic anions indicates that *M. tuberculosis* uses porins to enable transport of inorganic anions across its outer membrane.
MATERIAL AND METHODS

Chemicals and Enzymes.

Hygromycin B was purchased from Calbiochem. All other chemicals were obtained from Merck, Amersham, Roche or Sigma at the highest purity available.

Bacterial strains and growth conditions.

*M. smegmatis* and *M. tuberculosis* (*Table 1*) were grown at 37°C in Middlebrook 7H9 (Difco) or Dubos medium (7.4 mM KH₂PO₄, 17.7 mM Na₂HPO₄, 4.3 mM sodium citrate, 2.4 mM MgSO₄, 15 mM asparagine, 0.2% casamino acids (w/v), 0.1% Tween 80, 0.2% glycerol (v/v) and 4% Dubos medium albumin (Becton Dickinson, pH 7.2) or Hartmans-de Bont (HdB) medium (30 μM EDTA, 500 μM MgCl₂, 7 μM CaCl₂, 0.8 μM NaMoO₄, 1.68 μM CoCl₂, 5.49 μM MnCl₂, 6.95 μM ZnSO₄, 20 μM FeSO₄, 0.8 μM CuSO₄, 6 μM K₂HPO₄, 6 μM NaH₂PO₄, 15 mM (NH₄)₂SO₄, pH 6.9) supplemented with 0.2% glycerol and 0.05% Tween 80 (38). To control the concentration of the nitrogen source, (NH₄)₂SO₄ in the HdB medium was replaced by Na₂SO₄. Then, NaNO₃, NaNO₂, asparagine, glutamic acid, or arginine was added as sole nitrogen sources as indicated. Similarly, to control the concentration of the sulfur source, HdB medium was modified by replacing all sulfate salts by chloride salts. Then, Na₂SO₄ was added as the sole sulfur source as indicated. Hygromycin was used when required at a concentration of 50 μg/ml.

Growth experiments in liquid media.

*M. smegmatis* SMR5/pMS2, ML16/pMS2 and ML16/pMN016 were incubated on HdB agar plates at 37 °C until the colonies showed a smooth appearance. Then, 5 ml HdB medium containing 0.2% glycerol and 0.05% Tween 80 were inoculated with cells from these plates and incubated for 12 - 20 h at 37 °C with shaking (200 rpm). This procedure significantly reduced the occurrence of clumps, which may otherwise differentially affect the growth rates of the strains. Cells were harvested by centrifugation (3,000 x g at 4 °C for 10 min), washed three times and then resuspended in 5 ml of the HdB media supplemented with 30 mM or 0.1 mM NaNO₃ as the sole nitrogen source, or in 0.01 mM Na₂SO₄ as the sole sulfur source. Pre-cultures was used to inoculate 100 ml HdB medium
supplemented with 0.1 mM NaNO₃ as the sole nitrogen source or with 0.01 mM Na₂SO₄ as the sole sulfur source to a final OD₆₀₀ of 0.01. Growth in these cultures was followed by measuring the OD₆₀₀ every 12 h.

*M. tuberculosis* H37Rv was inoculated into 20 ml 7H9 medium containing 10% OADC supplement (Becton Dickinson), 0.2% glycerol and 0.05% Tween 80 and incubated at 37 °C to an OD₆₀₀ of 3.0. Cells were harvested by centrifugation (3,000 x g for 10 min), washed three times and then resuspended in 5 ml of sterile Millipore water, and inoculated into 200 ml HdB media containing 10 mM (NH₄)₂SO₄, 30 mM or 10 mM NaNO₃, 10 mM NaNO₂, 10 mM asparagine, 10 mM glutamic acid, or 10 mM arginine as the sole nitrogen source to a final OD₆₀₀ of 0.01. Growth of the strains in the cultures was followed by measuring the OD₆₀₀ in triplicate every two days. The exponential growth phase was fitted to the equation 

\[ N = N₀ \times e^{kt} \]  

In this formula, the number of bacteria \( N \) (measured by the optical density of the culture) is an argument of the function of the growth time \( t \). The parameters \( N₀ \) and \( k \) are the initial number of bacteria and the specific growth rate, respectively. The generation time \( t_g \) was calculated using the equation \( t_g = \ln(2)/k \).

Nitrate- and sulfate-dependent growth of *M. smegmatis* on plates.

Cultures of 5 ml HdB medium were inoculated with *M. smegmatis* SMR5/pMS2, ML16/pMS2, ML16/pMN016 and grown overnight at 37°C. The cultures were centrifuged at 3,000 x g, washed 3 times with sterile Millipore-Q water, and filtrated through 5 μm pore size filters to remove clumps of bacterial cells and to improve dispersed growth. The optical density (OD) of the filtrate was measured at 600 nm. The filtrates were initially diluted to an OD₆₀₀ of 0.05 using sterile Millipore-Q water. Then 10⁻⁴, 10⁻⁵ and 10⁻⁶-fold dilutions were made. For each dilution, 100 μl were plated on HdB agar plates containing different concentrations of NaNO₃ or Na₂SO₄ (ranging from 0 to 100 mM) as the sole nitrogen or sulfur sources, respectively. The plates were wrapped with parafilm and incubated at 37°C. Pictures of single colonies were taken at 16-fold magnifications over 4 or 5 days using a Stemi 2000-C stereomicroscope (Zeiss) equipped with a digital camera (Zeiss AxioCam MRc). For drop assays, 5 μl cells at the dilutions of an OD₆₀₀ ranging from 10⁻² - 10⁻⁶ were dropped on appropriate agar plates.
Measurement of sulfate and phosphate uptake by *M. smegmatis* and *M. tuberculosis*.

*M. smegmatis* and *M. tuberculosis* H37Rv cells were grown in 7H9 and Dubos medium, respectively. Cells were harvested at an OD$_{600}$ of 0.6 by centrifugation (3,000 x g at 4°C for 10 min), washed twice in uptake buffer (2 mM PIPES, 0.05 mM MgCl$_2$, 0.05% Tween 80, pH 6.8) and resuspended in the same buffer. Radioactively labelled Na$_2^{35}$SO$_4$ and KH$_2^{32}$PO$_4$ were mixed with unlabelled Na$_2$SO$_4$ or KH$_2$PO$_4$ and added to cell suspensions of *M. smegmatis* (kept on ice) and *M. tuberculosis* (kept at room temperature) to obtain final sulfate or phosphate concentrations of 1, 2.5, 5, 10 and 20 μM, respectively. The cells were incubated with their substrates at 37°C for 5 min. Samples of 150 μl were taken at the indicated times and mixed with 300 μl killing buffer (0.1 M LiCl in 10% formalin). Cells were filtered by centrifugation through a 0.45 μm pore size Spin-X tube (Costar) at 12,000 x g for 1 min. The Spin-X tube filters were washed once with 600 μl killing buffer by centrifugation and their radioactivity was measured in a liquid scintillation counter (Beckman). All experiments were performed in triplicate. The amount of accumulated solutes is expressed in nmol per mg cells (dry weight).

Uptake rates were determined by fitting data obtained for the first two or three time points (up to 2 or 5 min). The Michaelis-Menten constant $K_m$, the maximal uptake velocity $v_{max}$ for the overall transport, and a minimal estimate of the permeability coefficient were determined as described previously (44).

As a control, uptake of inorganic ions was inhibited by the following procedure. *M. smegmatis* SMR5 and *M. tuberculosis* H37Rv were harvested at an OD$_{600}$ of 0.6 by centrifugation, washed twice and resuspended in uptake buffer as described above. The cells were incubated at 37°C as described (52), followed by incubation for 1 h with 0.01 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (1) or 0.15 mM N,N'-dicyclohexylcarbodiimide (DCC) (52). Samples were taken at 0.5, 1, 2, 4, 8 and 16 min after addition of the inhibitors as described above to determine the uptake of sulfate and phosphate at a solute concentration of 20 μM.

Growth of *M. smegmatis* on plates in the presence of inhibitors

*M. smegmatis* SMR5 (wt) and *M. tuberculosis* H37Rv were plated on HdB or 7H10/OADC agar plates with or without 0.01 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and 0.15 mM N,N'-dicyclohexylcarbodiimide (DCC). The plates were incubated at 37°C for 3-4 days (for *M. smegmatis*).
or 3 weeks (for *M. tuberculosis*). Pictures of single colonies were taken at 16-fold magnification using a Zeiss stereomicroscope Stemi 2000-C. Plates were scanned using a digital scanner.

**Computer models of the MspA pore with solutes**

To examine whether the size and charge of glucose, glycerol, serine, NO$_3^-$, SO$_4^{2-}$ and PO$_4^{3-}$ fit the MspA pore, the 3-D structure of MspA (1UUN) and the structures of the chemical compounds of glucose (CID5793), glycerol (CID753), serine (CID617), NO$_3^-$ (CID943), SO$_4^{2-}$ (CID1117) and PO$_4^{3-}$ (CID1061) were obtained from [http://ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov). The fit of these solutes into the constriction zone of the MspA pore was evaluated using the UCSF Chimera software.
RESULTS

*M. tuberculosis* utilizes nitrate as the sole nitrogen source.

Nitrogen is often acquired by bacteria in the form of nitrate. While it was shown a long time ago that *M. tuberculosis* assimilates nitrate (6, 11), it was also reported that nitrate is not used as the sole nitrogen source by *M. tuberculosis* possibly due to inhibitory effects of nitrite and other intermediates (21, 47). Another study showed that the avirulent *M. tuberculosis* H37Ra strain utilizes nitrate efficiently in contrast to the virulent strain H37Rv (11). Thus, to clarify the role of nitrate in nitrogen utilization by *M. tuberculosis*, we first examined whether nitrate can be utilized as a sole nitrogen source by *M. tuberculosis* H37Rv. To this end, wild-type *M. tuberculosis* H37Rv and *M. smegmatis* SMR5, as a positive control (17), were grown in modified Hartmans-de Bont (mHdB) minimal medium with 30 mM nitrate as the sole nitrogen source. These experiments showed that both *M. smegmatis* SMR5 (Fig. 1A) and *M. tuberculosis* H37Rv (Fig. 1B) utilized nitrate as the sole nitrogen source, whereas no growth was observed in medium without a nitrogen source.

Asparagine is generally regarded as the preferred nitrogen source for growth of *M. tuberculosis* (22). In addition, glutamic acid is frequently used in rich media such as in Middlebrook media (24). Growth experiments in HdB minimal medium showed that asparagine and glutamate are indeed preferably utilized by *M. tuberculosis* as nitrogen sources (Fig. 1C). *M. tuberculosis* also grows on other amino acids such as arginine as well as small compounds such as ammonia (NH₄⁺) as sole nitrogen sources. However, these compounds do not support growth of *M. tuberculosis* to the same extent as glutamic acid and asparagine. No growth was observed in the presence of 10 mM sodium nitrite (NaNO₂) (Fig. 1C), presumably due to its toxic effect as observed earlier (11). The growth rate of *M. tuberculosis* in HdB minimal medium was the highest for glutamate (generation time 1.2 d) as the sole nitrogen source and decreased for asparagine (1.3 d), ammonia (1.8 d), arginine (2.1 d), nitrate (2.8 d). These results indicate that *M. tuberculosis* preferentially uses glutamate and asparagine as nitrogen sources.
Porins are required for efficient growth of *M. smegmatis* on nitrate as the sole nitrogen source.

To examine nitrate uptake mechanisms across the outer membrane, we employed the well-characterized porin triple mutant ML16 of *M. smegmatis* (44). Agar dilution experiments on minimal medium agar plates containing NaNO₃ as the sole nitrogen source showed a reduction in the number of colony-forming units by three to four orders of magnitude caused by the loss of porins in *M. smegmatis* ML16 at nitrate concentrations of 0.1 to 100 mM (Fig. S1). One millimolar was the minimal nitrate concentration that did not impair growth of wt *M. smegmatis* on agar plates (Fig. S1).

In liquid minimal medium 1 mM NaNO₃ did not support maximal growth rate of the porin mutant ML16 compared to wt *M. smegmatis* (Fig. 2A). Further, colonies of the porin triple mutant ML16 were significantly smaller than those of wild-type SMR5 for all nitrate concentrations (Fig. S2). These growth defects of the porin mutant under nitrate limiting conditions were completely reversed by expression of the porin gene *mspA* (Figs. 2A, S1, S2), underlining the importance of porins for nitrate utilization by *M. smegmatis*.

Background growth on unknown nitrogen-containing contaminants was observed for all three strains. However, growth of the porin mutant ML16 was more affected by the lack of nitrogen sources than that of wild-type *M. smegmatis* and of the complemented mutant (Fig. S2). Furthermore, optimal growth rate was achieved by ML16 between 10 to 100 mM nitrate, while this concentration was approximately 10-fold lower for wild-type *M. smegmatis* and the complemented porin mutant (Fig. S2).

The nitrogen concentration provided as ammonium sulfate in standard 7H10 Middlebrook medium is 7.5 mM, which is in the same range as the nitrate concentration required for optimal growth indicating that both nitrogen sources can be utilized efficiently by wild-type *M. smegmatis*. These experiments indicate that porins are required for efficient utilization of nitrate by *M. smegmatis*.

Porins are required for efficient growth of *M. smegmatis* with sulfate as the sole sulfur source.

Sulfate is considered the primary source of sulfur for most bacteria and can be taken up by *M. tuberculosis* using the inner membrane transporter composed of CysW, CysT and CysA (52).

However, as for nitrate, it is unknown how sulfate is taken up across mycobacterial outer membranes.

We hypothesized that mycobacteria might take up sulfates through porins in a manner similar to...
phosphates (51). Dilution experiments on HdB minimal medium agar plates containing Na$_2$SO$_4$ as the sole sulfur source showed a reduction in the number of colony-forming units by two to three orders of magnitude caused by the loss of porins in *M. smegmatis* ML16 at sulfate concentrations of 0.01 to 100 mM (Fig. S3). Interestingly, wt *M. smegmatis* grew on sulfur contaminations in the medium containing no added Na$_2$SO$_4$, while the porin mutant ML16 did not (Figs. S3, S4) indicating that utilization of these sulfur contaminants appears highly porin-dependent. The optimal growth rate was achieved by ML16 at 10 mM sulfate, while this concentration was at least 10-fold lower for wild-type *M. smegmatis* and the mspA-complemented porin mutant. A severe growth defect of the porin mutant ML16 was also observed in liquid minimal HdB medium containing 0.01 mM Na$_2$SO$_4$ (Fig. 2B), which was the minimal sulfate concentration that did not impair growth of wt *M. smegmatis* on agar plates (Fig. S3). Further, colonies of the porin triple mutant ML16 were significantly smaller than those of wt SMR5 at all sulfate concentrations (Fig. S4). Very high sulfate concentrations reduced growth of *M. smegmatis* independent of the presence of porins size (Figs. S3, S4) defining an upper threshold of sulfate concentrations for efficient utilization. The growth defects of the porin mutant under sulfate limiting conditions were completely reversed by expression of the porin gene mspA (Figs. 2B, S3, S4), underlining the importance of porins for sulfate utilization by *M. smegmatis*.

It should be noted that the porin mutant *M. smegmatis* ML16 initially grew very slowly in medium with sulfate as the sole sulfur source, but the growth rate increased significantly after ~60 hours (Fig. 2B). This is likely not due to a chromosomal mutation that compensates for the loss of porins because the same growth rate was observed in three independent cultures. The observation that we never observed any compensatory mutation in other growth experiments with porin mutants of *M. smegmatis* further supports this argument (43, 44, 51). However, exponential growth of the porin mutant ML16 was greatly delayed compared to wt *M. smegmatis*. It may be possible that low-level expression of the porin gene mspB in ML16 (44) is sufficient to support and sustain the early onset of growth on nitrate but not on sulfate (Fig. 2). The fact that no growth of ML16 was observed on agar plates without added sulfate in contrast to wt *M. smegmatis* indicates that Msp porins also play a role in utilization of unknown sulfur-containing contaminants (Fig. S4). Taken together these experiments indicated that porins are required for efficient utilization of sulfate by *M. smegmatis*.
Msp porins are required for uptake of sulfate by *M. smegmatis*.

The limited growth of the porin mutant ML16 on NaNO₃ and Na₂SO₄ as sole nitrogen and sulfur sources (Figs. 2, S1, S2, S3, S4), respectively, indicated that Msp porins are used by *M. smegmatis* to enable diffusion of nitrates and sulfates across the outer membrane. To provide direct evidence for this assumption, we wanted to directly examine the role of porins in uptake of these solutes by employing radio-labelled substrates. However, the longest-lived radioactive nitrogen isotope $^{13}$N has a half-life of less than 10 minutes and is not suitable for these experiments, whereas the $^{35}$S sulfur isotope has a half-life of 87 days and can be effectively used to measure sulfate uptake kinetics. To this end, wt *M. smegmatis* and the porin mutant ML16 were grown in Middlebrook 7H9 medium containing 4 mM Na₂SO₄. Uptake rates were measured at Na₂SO₄ concentrations ranging from 1 to 20 µM. Figure 3A shows the kinetics of sulfate uptake at 20 µM sodium sulfate as an example. The apparent uptake rates of 1.22 and 0.02 nmol x min⁻¹ x mg⁻¹ cells for *M. smegmatis* SMR5 (wt) and ML16, respectively, were obtained by fitting the first three time points (Fig. 3A). The uptake rates at different sulfate concentrations followed Michaelis-Menten kinetics (Fig. 3B) and yielded $v_{max}$ values of 1.3 and 0.13 nmol x min⁻¹ x mg⁻¹ and $K_m$ values of 2.1 and 36.4 µM for wt *M. smegmatis* and the mutant ML16, respectively. Thus, apparent permeability coefficients were 7.7 x 10⁻⁶ and 1.9 x 10⁻⁷ cm / sec for wt *M. smegmatis* and the triple porin mutant ML16, respectively (Table S1). The 40-fold reduced permeability of the porin mutant ML16 strongly indicates that inorganic sulfate diffuses through Msp porins in *M. smegmatis*.

To examine whether increased detection of cell-associated radiolabeled sulfate over time was the result of uptake rather than adsorption to the cell surface, uptake experiments were performed in the presence of 150 µM N,N'-dicyclohexylcarbodiimide (DCC), an inhibitor of the proton-translocating F₁F₀-ATPase, or 10 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a proton-gradient uncoupler. It should be noted that DCC and CCCP do not directly inhibit diffusion through outer membrane porins but inhibit uptake across the inner membrane by transporters that utilize ATP or a proton gradient, respectively, as energy sources. In the absence of uptake across the inner membrane, solutes accumulate rapidly in the periplasm, which has a small volume compared to the cytoplasm. Rapid solute equilibration across the outer membrane halts net porin-mediated diffusion.
These principles were established by Hiroshi Nikaido and co-workers for *E. coli* (28, 29). The fact that porin mutants take up many solutes more slowly shows that porin-mediated diffusion across the outer membrane is also the rate-limiting step in *M. smegmatis* under the conditions of these experiments (43, 44, 51). Inhibitor concentrations of 150 µM DCC and 10 µM CCCP were selected as they did not cause non-specific growth defects in *M. smegmatis* on agar plates (Fig. S5A and B). Addition of DCC completely eliminated uptake of sulfate in contrast to only mild effects of CCCP (Fig. 3C), indicating that sulfate was indeed taken up rather than absorbed on the cell surface and is predominantly transported across the inner membrane by an ATP-transporter in mycobacteria. This interpretation is consistent with the DCC-sensitivity of the *M. tuberculosis* sulfate transporter composed of CysA, CysW and CysT (52). Genome analysis revealed that a similar transporter exists in *M. smegmatis*.

### Uptake of sulfate and phosphate by *M. tuberculosis*

A quantitative transport analysis is necessary to understand acquisition of nutrient molecules by *M. tuberculosis*. Therefore, we sought to determine sulfate and phosphate uptake kinetics for *M. tuberculosis*. To this end, transport experiments with sulfate and phosphate at concentrations ranging from 1 to 20 µM were performed with *M. tuberculosis* H37Rv. Uptake of sulfate was much slower by *M. tuberculosis* compared to *M. smegmatis* by factors ranging from 5- to 30-fold (Figs. 3D, 3E; Table S1). For example, in *M. tuberculosis* the uptake rates were 0.01 and 0.25 nmol x mg⁻¹ x min⁻¹ cells at sulfate concentrations of 1 µM and 20 µM, respectively. While uptake rates at higher sulfate concentrations approached an asymptotic value in *M. smegmatis* (Fig. 3B), they were still in a linear range for *M. tuberculosis* (Fig. 3E). This made it impossible to determine Michaelis-Menten parameters for sulfate uptake in *M. tuberculosis*. Similar to *M. smegmatis*, 150 µM DCC and 10 µM CCCP did not inhibit growth of *M. tuberculosis* on agar plates (Fig. S5C). However, DCC completely inhibited uptake of sulfate by *M. tuberculosis*, compared to partial inhibition by CCCP (Fig. 3F). Taken together these experiments indicated that sulfate was actively taken up by *M. tuberculosis* and was not adsorbed on the cell surface.

Similar experiments with ³²P-labelled phosphate showed that phosphate was taken up by *M. tuberculosis* with rates of 0.01 and 0.33 nmol x min⁻¹ x mg⁻¹ cells at concentrations of 1 µM and
20 μM, respectively (Fig. 4A). Phosphate uptake rates increased linearly with the solute concentration for *M. tuberculosis* similar to the observations for sulfate (Fig. 4B). As was the case for sulfate, addition of inner membrane transport inhibitors inhibited phosphate uptake by *M. tuberculosis* (Fig. 4C), indicating that *M. tuberculosis* actively uptakes phosphate. Unlike sulfate, however, CCCP completely inhibited uptake whereas DCC only had mild effects, indicating that phosphate uptake is driven by a symporter which couples phosphate and proton transport across the inner membrane rather than an ABC transporter as is the case for the sulfate uptake system composed of CysA, CysW and CysT (52).
DISCUSSION

*M. tuberculosis* can utilize nitrate but prefers amino acids as nitrogen sources.

In this study we established that *M. tuberculosis* can utilize nitrate as the sole nitrogen source, albeit at lower efficiency than asparagine, glutamate and arginine. The beneficial effects of asparagine and glutamate for growth of *M. tuberculosis* have been reported a long time ago and have been incorporated as nitrogen sources in standard media for mycobacteria (24). The capacity of *M. tuberculosis* to release ammonia from asparagine and glutamate is crucial for rapid adaptation to acidic conditions *in vitro* (42) and may play a role in survival of *M. tuberculosis in vivo*. These findings also indicate that *M. tuberculosis* has uptake systems for these amino acids which enable their utilization as nitrogen sources. Uptake of these amino acids by *M. tuberculosis* must involve both inner and outer membrane proteins (26); however, the identities of these proteins are unknown.

Nitrate and sulfate uptake depends on porins in *M. smegmatis*.

The growth delay of the porin triple mutant *M. smegmatis* ML16 in media with limiting amounts of nitrate and sulfate as sole nitrogen and sulfur sources indicates that efficient uptake of these solutes is dependent on porins. This was confirmed by sulfate uptake experiments (Fig. 3). In previous experiments, a porin mutant of *M. smegmatis* showed much slower growth on low phosphate concentrations and uptake of phosphate was strongly reduced in this strain indicating that phosphate uptake across the outer membrane of *M. smegmatis* is also dependent on porins (51). This was a surprising finding because the major porin MspA and the other very similar Msp porins have a highly negatively charged constriction zone (7). The observation that uptake of sulfate by *M. smegmatis* at each substrate concentration is faster than that of phosphate (Fig. 5) is also consistent with diffusion of these solutes through Msp pores due to the lower negative charge of sulfate compared with phosphate, resulting in less electrostatic repulsion in the negatively charged constriction zone of the Msp pores. A space-filling model of the MspA channel based on its crystal structure shows that nitrate, sulfate, and phosphate fit into the constriction zone of the pore (Fig. 6). Taken together, these results show that small, inorganic anions use MspA and other Msp porins to enter cells of *M. smegmatis*. 

Uptake of inorganic anions by mycobacteria 14 
DISCUSSION 327 
M. tuberculosis can utilize nitrate but prefers amino acids as nitrogen sources. 

In this study we established that M. tuberculosis can utilize nitrate as the sole nitrogen source, albeit at lower efficiency than asparagine, glutamate and arginine. The beneficial effects of asparagine and glutamate for growth of M. tuberculosis have been reported a long time ago and have been incorporated as nitrogen sources in standard media for mycobacteria (24). The capacity of M. tuberculosis to release ammonia from asparagine and glutamate is crucial for rapid adaptation to acidic conditions in vitro (42) and may play a role in survival of M. tuberculosis in vivo. These findings also indicate that M. tuberculosis has uptake systems for these amino acids which enable their utilization as nitrogen sources. Uptake of these amino acids by M. tuberculosis must involve both inner and outer membrane proteins (26); however, the identities of these proteins are unknown.

Nitrate and sulfate uptake depends on porins in M. smegmatis.

The growth delay of the porin triple mutant M. smegmatis ML16 in media with limiting amounts of nitrate and sulfate as sole nitrogen and sulfur sources indicates that efficient uptake of these solutes is dependent on porins. This was confirmed by sulfate uptake experiments (Fig. 3). In previous experiments, a porin mutant of M. smegmatis showed much slower growth on low phosphate concentrations and uptake of phosphate was strongly reduced in this strain indicating that phosphate uptake across the outer membrane of M. smegmatis is also dependent on porins (51). This was a surprising finding because the major porin MspA and the other very similar Msp porins have a highly negatively charged constriction zone (7). The observation that uptake of sulfate by M. smegmatis at each substrate concentration is faster than that of phosphate (Fig. 5) is also consistent with diffusion of these solutes through Msp pores due to the lower negative charge of sulfate compared with phosphate, resulting in less electrostatic repulsion in the negatively charged constriction zone of the Msp pores. A space-filling model of the MspA channel based on its crystal structure shows that nitrate, sulfate, and phosphate fit into the constriction zone of the pore (Fig. 6). Taken together, these results show that small, inorganic anions use MspA and other Msp porins to enter cells of M. smegmatis.
These results also indicate that *M. smegmatis* does not have an anion-specific porin in contrast to many Gram-negative bacteria (2, 10, 18).

**How are nitrate and sulfate taken up by *M. tuberculosis***?

This study and other studies show that nitrate and sulfate are taken up by *M. tuberculosis*. While the transporters NarK2 (40) and that composed of CysT, CysW and CysA (52) have been identified as inner membrane uptake systems for nitrate and sulfate, respectively, it is unclear how these solutes cross the outer membrane of *M. tuberculosis*. It is obvious that the slow growth of *M. tuberculosis* with a generation time of about 24 hours requires uptake of less nutrients per time than faster growing mycobacteria such as *M. smegmatis* with a generation time of three to four hours. In this study, we found that uptake rates for sulfate by wt *M. tuberculosis* are much slower compared to *M. smegmatis* (Fig. 5A) and are rather similar to the porin triple *M. smegmatis* ML16 (compare Figs. 3B and 3E), which had an overall permeability for sulfate of $1.9 \times 10^{-7}$ cm/s (Table S1). This may indicate that the number of porins and their permeability for sulfate in *M. tuberculosis* rather resembles that of the *M. smegmatis* porin mutant. This is consistent with early findings that the amount of porin proteins which can be extracted from *M. tuberculosis* cells is rather low (16). However, despite this slow sulfate uptake compared to *M. smegmatis* and other bacteria, it is important to note that the permeability of *M. tuberculosis* for sulfate is still orders of magnitude faster than that of other inorganic anions for model lipid membranes. E.g., the permeability coefficient of the smaller and less charged chloride anion for model lipid membranes is $1.5 \times 10^{-11}$ cm/s (46). In addition, the mycobacterial outer membrane has an extremely low fluidity due to the extraordinary length of the mycolic acids (20), their covalent attachment to the arabinogalactan-peptidoglycan network (3) and the unusual outer membrane architecture (12, 26). Since the fluidity of the mycobacterial outer membrane is much lower than that of a model lipid membrane made of lipids with C16 fatty acids, direct diffusion of sulfate across the outer membrane of *M. tuberculosis* should be much slower and, therefore, cannot account for the observed sulfate uptake. A similar argument can be made for nitrate which has a permeability coefficient of $2 \times 10^{-9}$ cm/s for model lipid membranes (32), indicating that uptake of both nitrate and sulfate across the outer membrane requires porin-like channel proteins in *M. tuberculosis*. 
Uptake rates for phosphate in *M. tuberculosis* and *M. smegmatis* are similar.

This study showed that the uptake of phosphate by *M. tuberculosis* is almost as fast as that by *M. smegmatis* (Fig. 5) which has a permeability coefficient of $2 \times 10^{-6}$ cm/s (51). This is a surprising finding because *mspA* is the most highly expressed gene in *M. smegmatis* (30) and provides an efficient diffusion pathway for many hydrophilic solutes (14, 27, 43, 44), while *M. tuberculosis* does not have MspA homologs (43). However, we have shown earlier that phosphate diffusion through the MspA and MspC pores is rather inefficient in contrast to uncharged or zwitterionic solutes such as glucose or serine. For example the apparent permeability coefficient of phosphate for *M. smegmatis* is more than eight-fold lower compared to that of glucose (51). Further, loss of the porins MspA and MspC resulted in only a two-fold decrease of the apparent permeability coefficient of phosphate for *M. smegmatis* in stark contrast to a 75-fold reduced permeation of glucose (44, 51). While these results show that MspA and MspC provide the main pores for diffusion of phosphate across the outer membrane of *M. smegmatis*, diffusion of phosphate through the Msp pores is much less efficient than that of glucose. This finding has been rationalized by the highly negatively charged constriction zone of MspA which is constituted by aspartates 90 and 91 and are thought to impede diffusion of anions more than that of uncharged solutes. By contrast, uptake of glucose at a concentration of 20 μM by *M. tuberculosis* is almost 100-fold slower than that by *M. smegmatis* (42) and is so slow that a permeability coefficient cannot be reliably calculated. In fact, phosphate is taken up by *M. tuberculosis* almost ten times faster than glucose (0.3 versus 0.04 nmol x mg$^{-1}$ x min$^{-1}$) (Fig. 5). Since *M. tuberculosis* contains several copies of the high-affinity Pst phosphate uptake system, transport of phosphate across the inner membrane might be more efficient than that of glucose, for which no transporter is known (25). An alternative explanation is diffusion across the outer membrane is the rate-limiting step. The extremely low permeability of phosphate across model lipid membranes of $10^{-12}$ to $10^{-13}$ cm/s (4) is six orders of magnitude higher than the permeability coefficient for *M. tuberculosis* cells as deduced from comparison with *M. smegmatis* (Fig. 5, Table S1), also indicating that *M. tuberculosis* utilizes outer membrane porins for uptake of phosphate. Indeed, porin activity was observed in detergent extracts of *M. tuberculosis* (16) and *M. bovis* BCG (19), which could account for...
a preferential uptake of phosphate by *M. tuberculosis*. However, these porins have not been identified yet.
Conclusions.

In this study we determined the permeability of *M. tuberculosis* for sulfate and phosphate. Uptake of these solutes by *M. tuberculosis* is several orders of magnitude faster compared with model lipid membranes strongly indicating that it is mediated by proteins. While sulfate and phosphate transporters in the inner membrane of *M. tuberculosis* are known (8, 9), their counterparts in the outer membrane have yet to be identified. It was shown that phosphate uptake is required for virulence of *M. tuberculosis* (31). The finding that exogenous nitrate protects hypoxic mycobacteria from acid stress also suggests an important role of nitrate uptake *in vivo* (45). Taken together, these findings suggest that the proteins which enable diffusion of small, inorganic anions across the outer membrane might be required for survival of *M. tuberculosis* *in vivo*. 
ACKNOWLEDGEMENTS

We thank Mikhail Pavlenok for generating the three-dimensional models of MspA and Jason Huff for critically reading the manuscript. This work was supported by grants AI063432 and AI074805 of the National Institutes of Health to MN.
REFERENCES


Uptake of inorganic anions by mycobacteria 23


FIGURE LEGENDS

Fig. 1. Growth of *M. smegmatis* and *M. tuberculosis* with varying sole nitrogen sources.
The cell density (OD_{600}) of *M. smegmatis* SMR5 (A) and *M. tuberculosis* H37Rv (B) grown in liquid Hartmans-de Bont (HdB) media with 30 mM NaNO_{3} as the sole nitrogen source (filled circles) or without any nitrogen sources (open circles) was determined using a spectrophotometer. (C) Growth of *M. tuberculosis* H37Rv in HdB liquid media containing 10 mM of the following sole nitrogen sources: (NH_{4})_{2}SO_{4} (filled circles), NaNO_{3} (open circles), NaNO_{2} (filled triangles), asparagine (open triangles), glutamic acid (filled squares) or arginine (open squares). The experiments were performed in triplicate.

Fig. 2. Growth of *M. smegmatis* in nitrate- and sulfate-limited medium depends on Msp porins.
(A) Growth in Hartmans-de Bont (HdB) medium supplemented with nitrate as the sole nitrogen source. *M. smegmatis* SMR5/pMS2 (filled circles), ML16/pMS2 (filled triangles) and ML16/pMN016 (open circles) were grown in HdB medium containing 1 mM NaNO_{3} as the sole nitrogen source.
(B) Growth in HdB medium supplemented with sulfate as the sole sulfur source. *M. smegmatis* SMR5/pMS2 (filled circles), ML16/pMS2 (filled triangles) and ML16/pMN016 (open circles) were grown in HdB medium containing 0.01 mM Na_{2}SO_{4} as the sole sulfur source. The experiments were performed in triplicate. Data are shown with their standard deviations.

Fig. 3. Uptake of sulfate by *M. smegmatis* and *M. tuberculosis*.
Accumulation of ^{35}S-labelled sulfate by *M. smegmatis* SMR5 (wild-type), ML16 (ΔmspA ΔmspC ΔmspD) (A, B), and wild-type *M. tuberculosis* H37Rv (D, E) were measured at 37°C at a final concentration of 20 µM. The uptake rates (v) were determined by regression analysis of the first two minutes and calculated to be 1.22, 0.025 and 0.25 nmol x min^{-1} x mg^{-1} cells dry weight for wild-type *M. smegmatis*, the mutant ML16 (A), and *M. tuberculosis* (D), respectively. The dotted lines represent regression lines.
A series of sulfate uptake measurements of *M. smegmatis* SMR5, the mutant ML16 (B), and *M. tuberculosis* (E) were performed at sulfate concentrations ranging from 1 to 20 µM. The dotted line represents the approximation of the data using the Michaelis-Menten equation. Data analysis yielded
Uptake of inorganic anions by mycobacteria

\[ v_{\text{max}} \] values of 1.3 and 0.13 nmol x min\(^{-1}\) x mg\(^{-1}\), \( K_m \) values of 2.1 and 36.4 \( \mu \)M for wild-type \textit{M. smegmatis} and the porin mutant ML16, respectively.

Uptake of sulfate at a final concentration of 20 \( \mu \)M by \textit{M. smegmatis} (C) and \textit{M. tuberculosis} (F) at 37°C in the presence of the inhibitors CCCP (10 \( \mu \)M) or DCC (150 \( \mu \)M).

\textbf{Fig. 4. Uptake of phosphate by \textit{M. tuberculosis}.}

\textbf{(A)} Accumulation of \( ^{32}\)P-labelled phosphate by \textit{M. tuberculosis} H37Rv was measured at 37°C at a final concentration of 20 \( \mu \)M. The uptake rate was determined by regression analysis to be 0.33 nmol x min\(^{-1}\) x mg\(^{-1}\) cells dry weight. The dotted line represents the regression line.

\textbf{(B)} Michaelis-Menten analysis. A series of phosphate uptake measurements of \textit{M. tuberculosis} were performed with phosphate concentrations ranging from 1 to 20 \( \mu \)M. The dotted line represents the approximation of the data using the Michaelis-Menten equation.

\textbf{(C)} Uptake of phosphate at a final concentration of 20 \( \mu \)M by \textit{M. tuberculosis} at 37°C in the presence of the inhibitors CCCP (10 \( \mu \)M) or DCC (150 \( \mu \)M).

\textbf{Fig. 5. Comparison of sulfate and phosphate uptake by \textit{M. smegmatis} and \textit{M. tuberculosis}.}

The sulfate (A) and phosphate (B) uptake measurements of \textit{M. smegmatis} and \textit{M. tuberculosis} were performed with isotope concentrations ranging from 1 to 20 \( \mu \)M. The dotted line represents the approximation of the data using the Michaelis-Menten equation. The phosphate uptake data for \textit{M. smegmatis} was taken from Wolschendorf \textit{et al.} (51).

\textbf{Fig. 6. Structural models of the MspA pore with small, hydrophilic solutes.}

Permeation of chemical compounds through the MspA pore viewed from the top (A) and bottom (B). Glucose is shown inside the MspA constriction zone because it represents the largest molecule.

These models were generated using the UCSF Chimera software. Negatively and positively charged amino acids are shown in red and blue, respectively. Other amino acids are shown in grey.
<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Relevant Genotype or Description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em> SMR5</td>
<td>mc²155 derivative, SmR (rpsL*);</td>
<td>(35)</td>
</tr>
<tr>
<td><em>M. smegmatis</em> ML16</td>
<td>SMR5 derivative ΔmspA, ΔmspC, ΔmspD</td>
<td>(44)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>H37Rv</td>
<td>ATCC 27294</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMS2</td>
<td>PAL5000 origin, CoLE1 origin, hgy</td>
<td>(15)</td>
</tr>
<tr>
<td>pMN016</td>
<td>PAL5000 origin, CoLE1 origin, hgy, p_{smyc-mspA}</td>
<td>(15)</td>
</tr>
</tbody>
</table>

**Table 1**: Bacterial strains and plasmids used in this study