Expression of the *ompATb* operon accelerates ammonia secretion and adaptation of *Mycobacterium tuberculosis* to acidic environments

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SUMMARY

Homeostasis of intracellular pH is a trait critical for survival of *Mycobacterium tuberculosis* (*Mtb*) in macrophages. However, mechanisms by which *Mtb* adapts to acidic environments are poorly understood. In this study, we analyzed the physiological functions of OmpATb, a surface-accessible protein of *Mtb*. OmpATb did not complement the permeability defects of an *M. smegmatis* porin mutant to glucose, serine and glycerol, in contrast to the porin MspA. Uptake rates of these solutes were unchanged in an *ompATb* operon mutant of *Mtb* indicating that OmpATb is not a general porin. Chemical analysis of low pH culture filtrates showed that the proteins encoded by the *ompATb* operon are involved in generating a rapid ammonia burst, which neutralized medium pH and preceded exponential growth of *Mtb*. Addition of ammonia accelerated growth of the *ompATb* operon mutant demonstrating that ammonia secretion is indeed a mechanism by which *Mtb* neutralizes acidic environments. Infection experiments revealed that the *ompATb* operon was not required for full virulence in mice suggesting that *Mtb* has multiple mechanisms of resisting phagosomal acidification. Taken together, these results show that the *ompATb* operon is necessary for rapid ammonia secretion and adaptation of *Mtb* to acidic environments *in vitro* but not in mice.

Keywords

outer membrane; permeability; porin; virulence; cell wall

INTRODUCTION

Intracellular pH homeostasis is critical for unicellular microorganisms when they face rapid and drastic changes in the environmental pH (Slonczewski *et al.*, 2009). For instance, ingested bacteria encounter the strong acid barrier in the human stomach which has a

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median pH of 1.4 (Teyssen *et al.*, 1995). Bacteria which enter human hosts by other routes often encounter professional phagocytes such as macrophages which engulf bacteria in a membrane vesicle that ultimately fuses with acidic lysosomes. This process delivers not only bactericidal compounds such as reactive nitrogen and oxygen intermediates and defensins (Rohde *et al.*, 2007), but also reduces the pH inside the bacteria-containing phagosome to less than 5.5 (Lukacs *et al.*, 1991). Not surprisingly, pH adaptation is an evolutionary trait of pathogenic bacteria (Foster, 1999).

Mycobacterium tuberculosis is an intracellular pathogen which inhibits the fusion of phagosomes with lysosomes and resides in a mildly acidic environment of pH 6.2. However, macrophages can overcome this fusion block by activation with interferon-γ and acidify the *M. tuberculosis*-containing phagosome to pH 4.5 - 5.0 (MacMicking *et al.*, 2003). Increased expression of acid-responsive genes during macrophage infection (Rohde *et al.*, 2007) and attenuation of acid-sensitive mutants *in vivo* further indicate that *M. tuberculosis* encounters and responds to acidity in the host (Vandal *et al.*, 2009a). Mycobacteria including *M. tuberculosis* maintain a neutral internal pH in an acidic environment (Zhang *et al.*, 1999, Rao *et al.*, 2001, Vandal *et al.*, 2008), but the mechanisms utilized by *M. tuberculosis* in adaptation to acidic environments are poorly understood.

An *M. tuberculosis* mutant lacking Rv3671c, a membrane-associated protease, was sensitive to acid and failed to maintain intrabacterial pH *in vitro* and in activated macrophages (Vandal *et al.*, 2008). Growth of the *rv3671c* deletion mutant was severely attenuated in mice. It was concluded that the ability of *M. tuberculosis* to resist acid is in large part due to Rv3671c, and that this resistance is essential for virulence. Recently, four additional acid-sensitive mutants of *M. tuberculosis* lacking proteins implicated in cell wall biosynthesis were shown to be hypersensitive to lipophilic antibiotics and SDS, underlining the importance of an intact cell wall for protection against acid stress (Vandal *et al.*, 2009b).

The primary permeability barrier of mycobacteria is established by an unusual outer membrane (Hoffmann *et al.*, 2007, Hoffmann *et al.*, 2008). Channel-forming proteins such as the porin MspA enable transport of small and hydrophilic solutes across the outer membrane of *M. smegmatis* (Niederweis *et al.*, 1999, Stahl *et al.*, 2001, Faller *et al.*, 2004, Stephan *et al.*, 2005). MspA-like porins do not exist in *M. tuberculosis* (Niederweis, 2008a); however, Rv0899 (OmpATb) of *M. tuberculosis* shares some similarity to the channel-forming OmpA proteins of Gram-negative bacteria. OmpA of *E. coli* appears to exist in two distinct conformations, only one of which forms pores (Sugawara & Nikaido, 1994). However, the closed conformation seems to be predominant, a finding consistent with the low channel activity of purified OmpA (Sugawara & Nikaido, 1992) and the lack of a permeability defect of *ompA* mutants (Nikaido *et al.*, 1977). Homologues of OmpATb are only present in pathogenic mycobacteria, but not in saprophytic mycobacteria such as *M. smegmatis.* Similar to OmpA proteins from Gram-negative bacteria, OmpATb of *M. tuberculosis* forms channels *in vitro* (Senaratne *et al.*, 1998, Alahari *et al.*, 2007, Molle *et al.*, 2006). Uptake experiments indicated that OmpATb may have porin function in *M. tuberculosis* (Raynaud *et al.*, 2002). Growth experiments *in vitro* and mouse infection experiments indicated that OmpATb plays a role in acid resistance and virulence (Raynaud *et al.*, 2002). Consistent with the role of OmpATb in acid adaptation of *M. tuberculosis* was the observation that *ompATb* transcription is strongly induced at acidic pH and in macrophages (Raynaud *et al.*, 2002). However, these two functions appear to be antagonistic because a general response by bacteria to acid stress is to reduce porin expression and thereby decrease the permeability of their outer membrane to protons (Begic & Worobec, 2006, Samartzidou *et al.*, 2003, Thomas & Booth, 1992). Experiments with truncated OmpATb proteins revealed that the N-terminal domain (residues 1-73) is required for export in mycobacteria (Alahari *et al.*, 2007), while the central domain (residues 73-200) is

sufficient to form channels in lipid bilayers (Molle *et al.*, 2006). However, the NMR structure showed that the central OmpATb domain does not form any transmembrane or pore structure (Teriete *et al.*, 2010).

In the light of these contradictory findings, we examined whether OmpATb has porin function in both *M. smegmatis* and *M. tuberculosis* by uptake experiments. We did not detect any porin activity of OmpATb in mycobacteria. However, we provide evidence that *ompATb* is part of an operon of three genes, which is required for fast ammonia secretion, rapid pH neutralization and growth of *M. tuberculosis* in acidic environments.

RESULTS

Expression and localization of OmpATb in the outer membrane of *M. smegmatis*

To examine whether OmpATb of *M. tuberculosis* has porin function, we exploited the pronounced permeability defects of the porin mutant *M. smegmatis* ML16 (Table 1) which lacks three out of four known porins (MspA, MspC, MspD). For example, the permeability of ML16 for glucose is 50-fold lower than that of wt *M. smegmatis* (Stephan *et al.*, 2005), enabling the detection of low-activity porins. To examine whether OmpATb has porin function in mycobacteria, we expressed *ompATb* in *M. smegmatis* ML16 to determine if the permeability defects of this strain could be complemented. OmpATb levels were higher when expressed under the control of the p_{smyc} promoter of *M. smegmatis* in the plasmid pML003 (Table 2) in comparison to the p_{imyc} promoter construct in pML588 (Fig. 1A, lanes 2, 3). By contrast, no background was detected in *M. smegmatis* containing the empty plasmid pMS2 (Fig. 1A, lane 1).

OmpATb is accessible at the cell surface of *M. tuberculosis* and *M. bovis* BCG (Song *et al.*, 2008). However, it was unknown whether heterologous *ompATb* expression in *M. smegmatis* would result in correctly localized protein, which may require other proteins of *M. tuberculosis*. To determine whether OmpATb correctly inserted in the outer membrane of the triple porin mutant *M. smegmatis* ML16, an ELISA with whole cells was employed as recently described (Huff *et al.*, 2009). OmpATb was detected on the cell surface of *M. smegmatis* ML16 by OmpATb antibodies (Fig. 2). Truncated OmpATb$_{73-326}$, which lacks the N-terminal domain required for export (Alahari *et al.*, 2007), was expressed in ML16 (Fig. 1A, lane 5), but was not detected on the surface of whole cells (Fig. 2), demonstrating that cells in the ELISA were intact. It is concluded that full-length OmpATb is surface-accessible in *M. smegmatis*. Further, surface detection of OmpATb (Fig. 2) mirrored expression levels determined in Western blots of cell extracts (Fig. 1A). More OmpATb was detected on the surface of whole cells when *ompATb* was expressed under the control of the p_{smyc} promoter (pML003) in comparison to the p_{imyc} promoter (pML588), indicating that higher expression levels equate to higher levels of correctly localized, surface-accessible OmpATb in *M. smegmatis*. For this reason, further experiments in *M. smegmatis* were performed using the p_{smyc} promoter to drive expression of *ompATb*.

OmpATb does not enhance growth or nutrient uptake of *M. smegmatis* ML16

Lack of the porins MspA, MspC and MspD and the concomitantly much slower uptake of hydrophilic nutrients results in slower growth of *M. smegmatis* ML16 on agar plates and in liquid culture (Stephan *et al.*, 2005). This phenotype is complemented by expression of *mspA* (Fig. S1) and can be used to detect porin activity *in vivo*. In contrast, expression of *ompATb* did not complement the slow growth defect of *M. smegmatis* ML16 on agar plates (Fig. S1).

Porin activity can be quantified by measuring the intracellular accumulation of small, hydrophilic solutes if diffusion across the outer membrane constitutes the rate-limiting step

in uptake of a particular solute. Glucose has been used as a model solute for these purposes in mycobacteria (Stahl *et al.*, 2001, Mailaender *et al.*, 2004, Yuan *et al.*, 1998, Stephan *et al.*, 2005). As the OmpATb pore closes at low pH (Molle *et al.*, 2006), uptake experiments were performed near a neutral pH. Uptake of glucose by the porin mutant ML16 was reduced 25-fold compared to wt *M. smegmatis* (Fig. 3A and Table 3), in good agreement with previous results (Stephan *et al.*, 2005, Huff *et al.*, 2009). Expression of *mspA* in ML16 increased the rate of glucose uptake to wt levels, confirming that reduced uptake was a result of a reduced number of pores in the outer membrane (Stephan *et al.*, 2005). Thus, measuring uptake of radiolabelled glucose by *M. smegmatis* ML16 is a sensitive method to quantify porin activity *in vivo*. However, in contrast to *mspA*, expression of *ompATb* did not increase the rate of glucose uptake in the ML16 porin mutant.

While these experiments indicated that OmpATb is not permeable to glucose in *M. smegmatis*, it cannot be excluded that OmpATb is a solute-specific pore. For instance, LamB of *E. coli* enables transport of maltodextrines but not of other sugars or solutes (Charbit, 2003). Previously, it was reported that OmpATb enhanced uptake of serine but not of the smaller amino acid glycine (Raynaud *et al.*, 2002). Thus, we sought to determine serine permeability of OmpATb in the porin mutant ML16. As shown in Fig. 3B, the porin MspA complemented uptake of serine whereas OmpATb did not increase serine uptake above the background observed in ML16 carrying the empty vector.

It is conceivable that OmpATb may permeate smaller compounds or substrates unrelated to carbohydrates and amino acids. Thus, we measured the uptake of glycerol, which can be used as a carbon source by both *M. smegmatis* and *M. tuberculosis* (Winder & Brennan, 1966). Here, we show that MspA complemented the uptake defect of ML16 for this substrate to wt levels (Fig. 3C). However, expression of *ompATb* did not complement the uptake rate of the small glycerol molecule. Taken together, it is concluded that OmpATb alone does not enable the diffusion of the solutes glycerol, serine and glucose across the outer membrane of *M. smegmatis* and does not function as a general porin in this organism.

Construction of an *ompATb* gene deletion mutant in *M. tuberculosis*

The results obtained with a previously published *ompATb* mutant of *M. tuberculosis* (Raynaud *et al.*, 2002) were difficult to interpret due to lack of a complemented strain, as noted before (Smith, 2003). To resolve this issue and to further examine the physiological functions of OmpATb, an *M. tuberculosis* deletion mutant lacking the *ompATb* gene was constructed using a two-step allelic exchange approach in combination with sequence-specific recombination to unmark the mutant as recently described (Song *et al.*, 2008). First, the single cross over strain ML157 (Table 1) was obtained after incubation at 41°C, a temperature non-permissive for replication of the plasmid pML564 (Table 2). In the second step, the double cross over strain ML160 (Δ*ompATb::loxP-hyg-loxP*) was obtained after counter-selection of ML157 against the *sacB*-containing pML564 deletion vector on 2% sucrose plates. Then, expression of the Cre recombinase from the plasmid pCreSacB1 was used to excise the *hyg* gene from the chromosome to generate the out-of-frame, marker-free *ompATb* deletion mutant ML163 (Δ*ompATb::loxP*). Southern blot analysis of the *ompATb* loci of these strains demonstrated that the allelic exchange in the strain ML160 and excision of the *hyg* gene in ML163 were specific (Fig. S2). The replacement of a 689 bp DNA fragment of the *ompATb* gene by a *loxP* site in the chromosome of *M. tuberculosis* ML163 was confirmed by DNA sequencing. For complementation experiments, the *ompATb* expression vector pML759 was integrated at the mycobacteriophage L5 attachment site in the chromosome of the *ompATb* deletion mutant *M. tuberculosis* ML163. This complemented strain was named *M. tuberculosis* ML168 (Δ*ompATb::loxP; attB::ompATb*).

Extraction of membrane proteins with SDS and detection with an OmpATb antiserum showed that wt *M. tuberculosis* produced OmpATb, while no protein was detected in the Δ*ompATb* mutant *M. tuberculosis* ML163 (Fig. 1B). Expression of *ompATb* was fully restored in the complemented strain *M. tuberculosis* ML168 (Fig. 1B).

OmpATb has no detectable porin activity in *M. tuberculosis*

As the lack of porin activity by OmpATb in *M. smegmatis* may have been due to the absence of other *M. tuberculosis* proteins required for function, the effect of *ompATb* deletion on outer membrane permeability was examined in *M. tuberculosis*. No differences in colony morphology or size were noted for the Δ*ompATb* mutant ML163 either on 7H10/ OADC or Dubos agar plates (not shown). Permeability defects of the Δ*ompATb* mutant ML163 were examined by uptake of glucose, serine and glycerol as performed for *M. smegmatis*. Uptake rates for these solutes were similar for both wt *M. tuberculosis* H37Rv and the Δ*ompATb* mutant at pH 6.9 (Fig. 4). It is noteworthy that uptake of glucose by *M. tuberculosis* was very slow compared to *M. smegmatis* (Table 3). These findings are consistent with the results obtained in *M. smegmatis* and demonstrate that OmpATb does not play any role in outer membrane permeability of *M. tuberculosis* for these solutes under the conditions investigated.

The *ompATb* gene is encoded in an operon

The *ompATb* mutant CK69 exhibited delayed growth compared to wt *M. tuberculosis* at pH 5.5 but not at pH 7.2 (Raynaud *et al.*, 2002). Exponential growth of our *ompATb* mutant ML163 at pH 5.5 was also delayed by three days in Dubos medium (not shown) and by approximately ten days in Hartmans de Bont (HdB) minimal medium (Fig. 5A). However, expression of an integrated *ompATb* gene from three different promoters including the native promoter (not shown) and overexpression of *ompATb* using the plasmid pML588 (Fig. 1B, lane 4) did not complement the growth defect of the Δ*ompATb* mutant ML163 at pH 5.5 (Fig. 5A). This finding suggested that deletion of *ompATb* may have impaired expression of the downstream genes *rv0900* and *rv0901* (Fig. S3A). Reverse transcription PCR showed that *ompATb, rv0900* and *rv0901* are indeed transcribed in an operon (Fig. S3B). No amplification was obtained with the same primer pairs in samples without reverse transcriptase, demonstrating the absence of DNA contamination in RNA preparations. Therefore, the entire operon was cloned into expression plasmids (Table 2) and used to repeat the uptake experiments in *M. smegmatis* and for further characterization of the functions of OmpATb in *M. tuberculosis*.

OmpATb, Rv0900 and Rv0901 do not have porin activity in *M. smegmatis*

It is conceivable that channel activity was not detected in the *M. smegmatis* porin mutant ML16 containing the *ompATb* expression vector because the entire operon might be required for putative pore formation. Thus, the growth and uptake experiments described above were repeated in *M. smegmatis* using the p_{smyc}-driven *ompATb* operon expression plasmid pML1450. Importantly, expression of *ompATb* from this construct was higher (Fig. 1A) and subsequently resulted in more surface-exposed OmpATb, as determined by whole cell ELISA (Fig. 2). This indicated that Rv0900 and/or Rv0901 contribute to functional expression of *ompATb* by increasing expression, localization, and/or stability of OmpATb. However, even expression of the *ompATb* operon did not complement the growth defect of the triple porin mutant *M. smegmatis* ML16 on agar plates (Fig. S1) nor the uptake defects of ML16 for glucose, serine and glycerol (Fig. 3). Thus, it can be concluded that neither OmpATb alone nor in concert with Rv0900 and Rv0901 has any detectable porin activity in *M. smegmatis*.

The *ompATb* operon is required for neutralization of acidic pH in culture

The OmpATb channel closed more frequently *in vitro* at pH 6 and lower (Molle *et al.*, 2006), yet it was found that transcription of *ompATb* was upregulated 30-fold at pH 5.5 compared to pH 7.2 (Raynaud *et al.*, 2002). Fifteen-fold higher OmpATb protein levels were indeed obtained when *M. tuberculosis* was grown at pH 5.5 compared to pH 7.2 (Fig. S4). Based on these results and the finding that overall outer membrane permeability of *M. tuberculosis* was reduced at pH 5.5 (Raynaud *et al.*, 2002), it was suggested that OmpATb functions not as a porin but rather in acid adaptation (Niederweis, 2003). The lack of identifiable porin activity along with the growth defect of the *ompATb* mutant at low pH indicates this assumption may be true. However, the molecular mechanisms underlying the contribution of OmpATb, Rv0900 and Rv0901 to adaptation of *M. tuberculosis* to acidic environments remain unknown. Considering that *M. tuberculosis* does not grow at pH ≤ 5.5 (Vandal *et al.*, 2009a), we assumed that acidic medium must be neutralized prior to logarithmic phase growth. Thus, growth experiments were performed in which the medium pH was monitored along with the optical density of the cultures. Prior to the onset of and throughout exponential phase growth of wt *M. tuberculosis* (Fig. 5A), the pH of the medium increased from pH 5.5 to approximately 7.5 (Fig. 5B). The delay in the onset of exponential phase growth of the *ompATb* mutant ML163 also coincided with a delay in pH neutralization. Expression of the *ompATb* operon from the native *ompATb* promoter in pML763 produced high levels of OmpATb (Fig. 1B) and fully complemented both phenotypes (Fig. 5A, B) suggesting a mechanism by which proteins encoded by the *ompATb* operon are involved in pH neutralization by *M. tuberculosis*. This result also showed that the *M. tuberculosis* ML163 strain is an *ompATb* operon mutant.

The *ompATb* operon is required for efficient efflux of ammonia under acidic conditions

Considering the channel activity of OmpATb *in vitro* (Senaratne *et al.*, 1998, Alahari *et al.*, 2007), its surface accessibility (Fig 2) (Song *et al.*, 2008), cell wall-association (Rezwan *et al.*, 2007), and its requirement for acid adaptation (Fig. 5) (Raynaud *et al.*, 2002), we hypothesized that OmpATb might be involved in secretion of a basic compound that neutralizes protons in an acidic medium. To test this hypothesis, we chemically analyzed the supernatants of a culture of wt *M. tuberculosis* and of the *ompATb* operon mutant ML163 in acidified Dubos medium (pH 5.5) after 14 days of incubation at 37°C. Gas chromatography combined with mass spectroscopy (GS/MS) demonstrated the presence of ammonia while aliphatic and aromatic amines were not detected (not shown). The concentration of ammonia in the culture supernatant of wt *M. tuberculosis* was 5.2 ± 0.1 mM, which was determined photometrically using Nessler's reagent, a colorimetric reagent used for detection of ammonia (London *et al.*, 1974). This is consistent with previous findings of large amounts of ammonia in cultures of *M. tuberculosis* (Long, 1958, Gordon *et al.*, 1980). Importantly, the amount of ammonia in the supernatant of the *ompATb* operon mutant ML163 was significantly reduced to 4.1 ± 0.2 mM at the same cell density. Some amino acids, amines and hydrazones are known to interfere with the Nessler's reagent. However, similar photometric readings were observed when culture filtrates of both strains were distilled and the trapped liquid was analyzed using Nessler's reagent (not shown). This proves that a volatile species, probably ammonia, is indeed produced by wt *M. tuberculosis* and at a reduced level by the *ompATb* operon mutant ML163.

To examine the role of the *ompATb* operon in ammonia release by *M. tuberculosis*, we measured ammonia production using an enzymatic assay based on the synthesis of glutamate from 2-oxoglutarate and ammonia by glutamate dehydrogenase. While *M. tuberculosis* grew in Hartmans de Bont (HdB) minimal medium with nitrate as a single nitrogen source at pH 7.0, it did not grow at pH 5.5 (will be published elsewhere). However, when the sole nitrogen source was substituted in HdB medium for that of Dubos medium,

asparagine, ammonia was rapidly detected in the supernatant of *M. tuberculosis* cultures. Within the first seven days, the concentration of ammonia in the supernatant of wt *M. tuberculosis* increased to only 1.2 mM (Fig. 5C). This was accompanied by minor increases in pH from 5.5 to 6.0 (Fig. 5B) and in growth (Fig. 5A). Over the next five days, the ammonia concentration increased by more than 10-fold to a final concentration of 25 mM. This "ammonia burst" was associated with a rise in pH from 5.5 to 7.5 and preceded exponential growth of wt *M. tuberculosis*. It should be noted that the pH subsequently dropped dramatically after 12 days of incubation, but this did not initially affect growth of wt *M. tuberculosis* (Fig. 5).

Interestingly, the ammonia burst by the *ompATb* operon mutant ML163 was similar in magnitude as wt *M. tuberculosis*, but was delayed by about ten days. The delayed ammonia burst accompanied a delayed increase in pH, which both preceded exponential growth of the mutant. Thus, the phenotype of the *ompATb* operon mutant consists of severe delays in ammonia secretion, medium pH neutralization, and growth compared to wt *M. tuberculosis*. However, all changes in these parameters after onset were similar in magnitude and kinetics. Expression of the *ompATb* operon but not *ompATb* alone fully reverted the delayed onset of ammonia secretion, pH neutralization and growth to wt levels (Fig. 5).

To determine whether secretion of ammonia alone rescued the growth defect of *ompATb* operon mutant, 10 mM exogenous ammonia was added to the ML163 culture on day eight (Fig. 5B). This "artificial ammonia burst" instantaneously increased the medium pH to 6.8 as expected according to a titration curve of HdB medium (Fig. S5). Interestingly, it did not trigger immediate growth of *M. tuberculosis* indicating that another factor in addition to pH increase is required for growth of *M. tuberculosis*. Approximately three more days, a subsequent increase in ammonia, and a further rise in pH were required before growth of the *ompATb* operon mutant was observed.

Asparagine is the ammonia source for *M. tuberculosis* at acidic pH *in vitro*

To identify whether *M. tuberculosis* is also capable of utilizing amino acids other than asparagine for ammonia production and growth under acidic conditions, *M. tuberculosis* was grown in HdB minimal medium at pH 5.5 supplemented with only one of the twenty natural amino acids as the sole nitrogen source. *M. tuberculosis* only grew in the presence of asparagine (Asn), aspartic acid (Asp), glutamine (Gln) or glutamic acid (Glu) in acidic medium (Fig. 6A). Growth of *M. tuberculosis* coincided with an increase in the pH of the medium of these cultures (Fig. 6B). This finding confirmed previous observations that *M. tuberculosis* cannot grow at pH lower than 5.5 (Vandal *et al.*, 2009a) and needs to increase the pH to above 5.5 before growth is initiated. Interestingly, ammonia was detected only in significant amounts in the culture containing asparagine (Fig. 6C). The small spike of ammonia detected with glutamate as the sole nitrogen source occurred after an increase in the medium pH and subsequent growth of *M. tuberculosis* (Fig. 6). This experiment also indicated *M. tuberculosis* releases other alkaline compound(s) accounting for pH neutralization in the presence of aspartic or glutamic acid (Fig. 6B). The pH also increased and growth occurred in acidified *M. tuberculosis* cultures containing glutamine as a sole nitrogen source indicating that either ammonia or another alkaline compound is produced. However, this cannot be distinguished using the enzymatic ammonia detection (Fig. 6C). Taken together, these results suggest that, among all naturally occurring amino acids, *M. tuberculosis* can only utilize asparagine and possibly glutamine for ammonia production and growth under acidic conditions *in vitro*.

The *ompATb* operon is not involved in asparagine uptake

An alternative explanation for the delayed ammonia secretion by *M. tuberculosis* ML163 is that the *ompATb* operon might be involved in uptake of asparagine which is required for ammonia production in our growth experiments. However, uptake experiments using [^{3}H]asparagine did not show any difference between wt *M. tuberculosis* and ML163 (Fig. 4D) demonstrating that the *ompATb* operon is not required for asparagine uptake under these conditions.

The *ompATb* operon is not required for full virulence of *M. tuberculosis* in mice

M. tuberculosis infects alveolar macrophages where it resides in slightly acidified vacuoles (Ehrt & Schnappinger, 2009). Thus, the growth defect of the *ompATb* operon mutant in acidic medium (Fig. 5A) indicates that OmpATb may be required for *in vivo* survival. The finding that the *ompATb* mutant CK69 was attenuated in both macrophages and in mice (Raynaud *et al.*, 2002) appeared to support a critical role of OmpATb in virulence of *M. tuberculosis*. However, screens with high-density transposon libraries did not identify *ompATb* as a gene required for survival in mice (Sassetti & Rubin, 2003), in macrophages (Rengarajan *et al.*, 2005) or for adaptation to low pH (Vandal *et al.*, 2008). Therefore, we sought to examine whether the *ompATb* operon is required for virulence of *M. tuberculosis* using the mutant strain ML163. The wt strain *M. tuberculosis* H37Rv and the *ompATb* operon mutant were administered to BALB/c mice by low-dose aerosol infection and bacterial burden was determined over time. While the infectious dose determined on day 1 was higher for the *ompATb* operon mutant, a minor reduction in CFUs in the lung compared to wt was observed on day 28 post-infection (Fig. 7A). However, the mutant recovered to wt levels in lungs by day 64 and beyond. Bacterial burden in the spleens of infected mice was also reduced for the mutant by day 28 (Fig. 7B). Conversely to lungs, the mutant remained at significantly reduced levels in the spleen throughout the duration of infection experiments, suggesting impaired dissemination of the *M. tuberculosis ompATb* operon mutant. These experiments indicated that OmpATb, Rv0900 and Rv0901 play no or only a minor role in virulence of *M. tuberculosis*, in contrast to the major growth defect previously observed in mice lungs with the *ompATb* mutant CK69 (Raynaud *et al.*, 2002).

In an effort to resolve this discrepancy, we obtained the *ompATb* mutant strain CK69 described by Raynaud *et al.* (2002) and performed additional infection experiments in mice. Low-dose aerosol infection in BALB/c mice revealed that both at an early time point of infection (day 28) and during the chronic stage of infection (day 65), no attenuation of the mutant (CK69) for growth in lungs or spleens compared to the parent *M. tuberculosis* strain (1424) was observed (Fig. 7C, D). As the original publication had only assessed virulence following intravenous infection (Raynaud *et al.*, 2002), we also determined growth of the *ompATb* mutant strain CK69 and its parent strain 1424 after intravenous inoculation of BALB/c mice. No reduced replication of the *ompATb* mutant CK69 was observed in either lungs or spleens (Fig. S6). Taken together, our *in vivo* experiments with two *ompATb* mutants show that neither OmpATb nor Rv0900 and Rv0901 are required for full virulence of *M. tuberculosis* in a mouse infection model.

DISCUSSION

OmpATb lacks porin activity *in vivo*

In this study we did not detect any porin activity of OmpATb in either *M. smegmatis* or in *M. tuberculosis*. An explanation for the apparent absence of porin activity of OmpATb in mycobacteria despite its *in vitro* channel activity (Alahari *et al.*, 2007, Molle *et al.*, 2006) might be that OmpATb forms channels of very low activity, as has been described for OmpA of *E. coli* and OprF of *Pseudomonas aeruginosa* (Sugawara & Nikaido, 1994,

Sugawara *et al.*, 2006). Detection of low porin activity requires the absence of other general porins in the bacterial strain of interest. Since the porin mutant *M. smegmatis* ML16 still expresses the porin gene *mspB*, albeit at very low amounts (Stephan *et al.*, 2005), it might be difficult to detect very low activity porins in this strain. Another explanation might be that *ompATb* is not functionally expressed in the heterologous host *M. smegmatis*. Indeed, the proteins Rv0900 and Rv0901, which are encoded in the *ompATb* operon, increase expression levels of OmpATb by an unknown mechanism (Fig. 1A). However, these proteins are not required for correct localization of OmpATb (Fig. 2) and they also do not confer any channel activity to OmpATb in mycobacteria (Fig. 3 and 4). Further, ELISA experiments showed that OmpATb is surface-accessible in *M. smegmatis* demonstrating correct localization and indicating proper folding of the protein (Fig. 2). Importantly, the *ompATb* operon mutant *M. tuberculosis* ML163 lacked any detectable uptake defect indicating that OmpATb does not have porin activity in its native environment (Fig. 4). In particular, we did not find significant changes in serine uptake either in the recombinant *M. smegmatis* strain or in the *ompATb* operon mutant of *M. tuberculosis*, in contradiction to earlier results (Raynaud *et al.*, 2002).

The recently solved NMR structure revealed that OmpATb does not form a transmembrane β-barrel or any pore-forming domain (Teriete *et al.*, 2010), supporting our *in vivo* results. This structure does not exclude that OmpATb might be permeable to small ions as observed *in vitro* (Alahari *et al.*, 2007, Molle *et al.*, 2006, Senaratne *et al.*, 1998) based on dynamic conformational changes, as postulated for OmpA of *E. coli* (Hong *et al.*, 2006, Khalid *et al.*, 2008). The recently published model of a heptameric OmpATb pore (Yang *et al.*, 2011) has no continuous hydrophobic outer surface as observed in all outer membrane proteins including MspA. In addition, the height of this OmpATb heptamer was 3 nm, which may be sufficient to span a planar lipid membrane *in vitro*, but is too short to span the ~8 nm thick mycobacterial outer membrane (Hoffmann *et al.*, 2008). Taken together, a critical evaluation of all experiments strongly indicates that OmpATb does not function as a general porin in mycobacteria in contrast to previous findings for another *ompATb* mutant CK69 (Raynaud *et al.*, 2002). However, the apparent permeability defect of the *ompATb* mutant CK69 was not complemented raising the suspicion that it might be due to secondary mutations. The lack of detectable porin activity for OmpATb is also in contrast to MspA, the only well characterized mycobacterial porin, which forms a stable channel (Faller *et al.*, 2004) and enables the diffusion of diverse small molecules including glucose (Stephan *et al.*, 2005), glycerol (Fig. 3), amino acids (Stephan *et al.*, 2005), phosphate (Wolschendorf *et al.*, 2007), antibiotics (Danilchanka *et al.*, 2008) and ferric ions (Jones & Niederweis, 2010). It should be noted that the absence of detectable porin activity in our experiments does not constitute a proof that OmpATb does not have some channel activity which is not detectable under our experimental conditions. Such an activity could be masked by other, yet unidentified pore proteins in *M. tuberculosis*, but would probably not contribute significantly to its outer membrane permeability.

Proteins encoded by the *ompATb* operon are involved in ammonia release by *M. tuberculosis* to adapt to acidic environments

In the absence of any detectable porin activity, what then might be the real function of OmpATb? It was found previously that *ompATb* expression is strongly induced at low pH (Raynaud *et al.*, 2002) indicating that OmpATb might play a role in the adaptation of *M. tuberculosis* to acidic conditions. Here, we show that OmpATb accelerates the secretion of ammonia to neutralize the medium and ultimately enables growth of *M. tuberculosis* under acidic conditions. *M. tuberculosis* does not grow at pH 5.5 (Vandal *et al.*, 2009a), and releases large amounts of ammonia in excess of 5 mM to increase the pH to 6.5 before it enters the exponential growth phase (Fig. 5). This is within the physiologically relevant pH

range as the pH of *M. tuberculosis*-containing phagosomes ranges from pH 4.5 to 6.2, depending on the activation state of the macrophage (Vandal *et al.*, 2009a). Ammonia secretion by *M. tuberculosis* has been known for a long time (Long, 1958, Gordon *et al.*, 1980) and was proposed to play a role in neutralizing acidic pH, in preventing phagosome-lysosome fusion in macrophages (Hart *et al.*, 1987), and diminishing cell-surface expression of MHC class II molecules in macrophages infected with *M. tuberculosis* (Hmama *et al.*, 1998). In this study, we showed that the growth delay of the *ompATb* operon mutant under acidic conditions is shortened by addition of exogenous ammonia (Fig. 5). This partial biochemical complementation provides the first direct evidence that ammonia is indeed required by *M. tuberculosis* to achieve a pH permissive for growth. However, other secreted yet unidentified factors also appear to contribute to growth of *M. tuberculosis* under acidic conditions (Fig. 6).

The importance of acid resistance mechanisms for bacterial pathogens is not surprising given that excess internal protons cause intracellular damage to molecules such as proteins and DNA (Foster, 1999). Many enteric bacteria induce expression of genes involved in DNA repair and acid shock response when exposed to acidic conditions (Foster, 1995) and have evolved mechanisms to maintain favorable pH levels to promote growth and survival inside the host. For instance, the F_1F_0-ATPase expels excess protons to maintain intracellular pH and is required for acid-tolerance of *Salmonella typhimurium* (Foster & Hall, 1991). Neutralization of extracellular pH by ammonia is also a common feature in the bacterial response to acid stress. A classic example is *Helicobacter pylori* which produces and transports ammonia to the periplasm to neutralize influxing protons in the very acidic environment of the human stomach (Sachs *et al.*, 2006). In acidic conditions of the oral cavity, *Streptococcus sanguis* secretes an arginine aminopeptidase to release ammonia, while *Streptococcus salivarious* secretes ammonia produced by high levels of urease expression (Quivey *et al.*, 2001). While this study shows that secretion of ammonia plays an important role in adaptation of *M. tuberculosis* to acidic conditions *in vitro*, it is only one of several mechanisms by which *M. tuberculosis* maintains permissive pH levels such as blocking phagosomal fusion with lysosomes and preventing acquisition of the V-ATPase (Vandal *et al.*, 2009a).

How does the *ompATb* operon contribute to ammonia release by *M. tuberculosis*?

Ammonia is a small gas molecule which is thought to be membrane permeable (Antonenko *et al.*, 1997). Thus, protein-mediated transport mechanisms across membranes may not be required. However, highly specific ammonia transporters such as AmtB and some aquaporins (Zheng *et al.*, 2004, Kruse *et al.*, 2006) exist in all three kingdoms of life, indicating that ammonia transport by unmediated membrane diffusion might not be fast enough to support optimal bacterial growth (Soupene *et al.*, 1998). The low fluidity of the mycobacterial outer membrane (Liu *et al.*, 1996) contributes to its extraordinary effectiveness as a permeability barrier (Brennan & Nikaido, 1995). Thus, proteins may be required to accelerate ammonia diffusion across the outer membrane of mycobacteria. However, the apparent lack of porin activity of OmpATb in mycobacteria and identical ammonia release kinetics in wt *M. tuberculosis* and the *ompATb* operon mutant (Fig. 5C) do not support a role of OmpATb in direct ammonia transport. Our experiments rather suggest that *M. tuberculosis* has a mechanism of ammonia release that does not involve OmpATb. Proteins encoded by the *ompATb* operon might be involved in triggering early ammonia secretion by *M. tuberculosis* in acidic environments.

An alternative explanation for the role of OmpATb in acid resistance would be that it is involved in the uptake of a nitrogen source used by *M. tuberculosis* to generate ammonia. In this study, we identified asparagine as the only amino acid efficiently used by *M. tuberculosis* to produce ammonia (Fig. 6). This finding is consistent with the observation

that asparagine is rapidly taken up by *M. tuberculosis* (Lyon *et al.*, 1969) and its particular role as a nitrogen source (Lyon *et al.*, 1974). However, uptake experiments demonstrated that OmpATb does not play any role in uptake of asparagine (Fig. 4D).

The outer membrane permeability of *M. tuberculosis* for glucose and serine is extremely low compared to *M. smegmatis*

The permeability barrier established by the outer membrane is a hallmark of mycobacteria and is crucial for survival of *M. tuberculosis* in the host (Barry, 2001). However, a systematic analysis of the permeability properties of the outer membrane of *M. tuberculosis* is lacking, as is our understanding of which proteins are required for uptake of nutrients and drugs by *M. tuberculosis* (Niederweis, 2008b). Here, we show for the first time a direct comparison of the permeability of *M. tuberculosis* and *M. smegmatis* to several nutrients. Uptake rates of glucose and serine by *M. tuberculosis* were 86- and 11-fold slower compared to wt *M. smegmatis* and were similar to that of the triple porin mutant *M. smegmatis* ML16 (Table 3). These results are consistent with previous observations that the porin activity in detergent extracts of *M. tuberculosis* is much lower than that of *M. smegmatis* (Kartmann *et al.*, 1999). Our experiments confirm that uptake of these solutes is porin-dependent in *M. smegmatis* as shown previously (Stephan *et al.*, 2005). It is unclear whether this is the case for *M. tuberculosis*. Uptake rates for glycerol were similar for *M. tuberculosis* and *M. smegmatis*, although we showed that the highly efficient MspA pore accounts for the vast majority of glycerol uptake in *M. smegmatis* (Fig. 3C). This result combined with the observation that mycobacterial outer membranes have an extremely low fluidity (Liu *et al.*, 1995, Liu *et al.*, 1996) indicate that *M. tuberculosis* probably has a porin that enables rapid influx of glycerol, because the rate of glycerol diffusion through membranes decreases with reduced membrane fluidity (Eze & McElhaney, 1981).

The *ompATb* operon is not required for full virulence in mice

In this study, we show that OmpATb is not required for full virulence of *M. tuberculosis* in mice (Fig. 7 and Fig. S6). Our conclusion is based on assessing replication and persistence of two different *ompATb* mutants, the one described in this report and the one published by Raynaud *et al.* (2002). It is unclear why the previously published *ompATb* mutant did not exhibit, in our hands, the severely attenuated phenotype described earlier (Raynaud *et al.*, 2002). However, after either aerosol or intravenous infection, and both at early and late time points post infection, the *ompATb* mutant *M. tuberculosis* CK69 described by Raynaud *et al.* (2002) did not differ from wild-type *M. tuberculosis* 1424 in terms of initial growth and chronic persistence, thereby corroborating our findings with our *ompATb* operon mutant ML163.

Previous screens did not identify *ompATb* as required for survival in macrophages (Rengarajan *et al.*, 2005), in mice (Sassetti & Rubin, 2003), or under acidic conditions *in vitro* (Vandal *et al.*, 2008). Microarray-based screens of transposon libraries indicated that *rv0900* is required for growth of *M. tuberculosis in vitro* (Sassetti *et al.*, 2003) and in macrophages (Stewart *et al.*, 2005). However, this study shows the the *ompATb* operon mutant does not show a growth defect *in vitro* at neutral pH (not shown) or in mice. These results suggest that *rv0900* does not play a critical role for *M. tuberculosis* growth or viability under those conditions.

Conclusions

The findings of this study indicate that OmpATb is not a general porin, but rather is involved in adaptation of *M. tuberculosis* to acidic environments by mediating ammonia secretion. Clearly, more work is warranted to understand this function of OmpATb and how the other proteins encoded by the *ompATb* operon contribute to this function. However,

ammonia secretion as mediated by the *ompATb* operon does not appear to be required for virulence of *M. tuberculosis* in mice, perhaps because *M. tuberculosis* employs multiple, possibly redundant, mechanisms to resist phagosomal acidification (Stewart *et al.*, 2005, Vandal *et al.*, 2008) or because alternative, albeit delayed mechanisms for ammonia secretion exist in *M. tuberculosis*.

Materials and Methods

Chemicals, enzymes and DNA

Hygromycin B was purchased from Calbiochem. All other chemicals were purchased from Merck, Roche or Sigma at the highest purity available. Enzymes for DNA restriction and modification were purchased from New England Biolabs. Isolation and modification of DNA was performed as described (Ausubel *et al.*, 1990). Oligonucleotides were obtained from Integrated DNA Technologies (Table S1).

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. Mycobacterial strains were grown at 37°C in Middlebrook 7H9 liquid medium (Difco Laboratories) supplemented with 0.2% glycerol, 0.025% Tyloxapol or on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 0.2% glycerol, unless indicated otherwise. *Escherichia coli* DH5α was used for all cloning experiments and was routinely grown in LB medium at 37°C. *M. tuberculosis* strains were grown in Dubos broth or Hartmans de Bont (HdB) minimal medium (Smeulders *et al.*, 1999) or on 7H10 agar plates supplemented with 0.2% glycerol and 10% Middlebrook OADC enrichment (BBL) at 37°C. In HdB medium, $(NH_4)_2SO_4$ was replaced by Na_2SO_4 and 15 mM asparagine was used as a nitrogen source. Antibiotics were used when required at the following concentrations: hygromycin (200 µg ml^{-1} for *E. coli*; 50 µg ml^{-1} for mycobacteria) and kanamycin (50 µg ml^{-1} for *E. coli*; 30 µg ml^{-1} for mycobacteria).

Construction of *ompATb* expression vectors for mycobacteria and *E. coli*

To construct the *ompATb* operon (*ompATb-rv0900-rv0901*) expression plasmid pML763 driven by the native promoter of *ompATb*, the *ompATb-rv0900-rv0901* operon and the adjacent upstream 1.0 kb promoter region was amplified from *M. tuberculosis* genomic DNA using the primer pair 1091/1149 and ligated into pML003 (Song *et al.*, 2008), which was digested by *Xba*I and *Swa*I. The p_{smyc}-*ompATb* operon expression plasmid pML1450 was constructed by removal of the *ompATb-rv0900-rv0901* operon via digestion of pML763 using *Hpa*I and *Hind*III, and ligation into similarly digested pML003. The N-terminally truncated *ompATb*$_{73-326}$ expression plasmid was constructed by PCR amplification of *ompATb*$_{73-326}$ from pML752 with the primer pair 2277/2184. A second PCR amplification of the product using the primer pairs 2276/2184 allowed for addition of a shine-delgarno sequence, which was followed by digestion with *Pac*I and *Hind*III, and ligation into similarly digested pML003.

To construct an integrative expression vector for *ompATb*, the plasmid pCV125 (Steyn *et al.*, 2003) containing the integrase gene and attachment site (*attP*) of the mycobacteriophage L5 and a kanamycin resistant gene was used. The p_{imyc}-*ompATb* fragment was removed from pML588 by *Xba*I and *Cla*I digestion, and ligated into similarly digested pCV125. The resulting plasmid was named pML759 (Table 2) and integrated into the L5 *attB* site of *M. tuberculosis* ML163 (Δ*ompATb::loxP*). The complemented strain was named ML168 (Δ*ompATb::loxP*, *attB*::pML759). All plasmid constructions were verified by restriction enzyme digestion and sequencing and are listed in Table 2, while all strains are listed in Table 1.

To construct a C-terminally his-tagged *ompATb* overexpression vector for *E. coli*, the *ompATb* gene was amplified from pML588 by PCR using the primers 960 and 962 (Song *et al.*, 2008). This fragment was digested with *Nco*I and *Swa*I and ligated into similarly digested pET28b+ (Novagen), resulting in pML591.

Overexpression of *ompATb* in *E. coli*

For protein expression and purification, a 1 L culture was grown to OD_{600} of 0.6-1.0, and induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. Bacteria were harvested by centrifugation, resuspended in 20 ml lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) and sonicated on ice for 15 min. Then, the cell suspension was centrifuged at $12,000 \times g$ for 15 min at 4°C. The supernatant was removed, filtrated through a 0.22 μm filter, and loaded onto a Ni^{2+} charged resin column (HIS-Select™ Spin Columns, Sigma) according to the manufacturer's protocol. Bound proteins were washed (50 mM Tris-HCl, 100 mM NaCl, 5 mM imidazole, pH 8.0) and then eluted from the column using elution buffer (50 mM Tris-HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0).

Construction of an unmarked *ompATb* deletion mutant of *M. tuberculosis*

To construct a mutant of *M. tuberculosis* lacking *ompATb*, fragments of approximately 1,000 bp of DNA up- and downstream of *ompATb* were amplified by PCR from chromosomal DNA with the oligonucleotide pairs 779/780 and 982/983, respectively. The restriction sites for *Asc*I and *Swa*I were introduced into the upstream fragment while *Pac*I and *Bfr*BI sites were introduced into the downstream fragments. The individual sequences were digested and ligated into the similarly digested temperature sensitive replication vector pML563 (Song *et al.*, 2008) flanking the *loxP-hyg-loxP* cassette, and the resulting plasmid was named pML564. The cloned fragments were sequenced to ensure the absence of PCR errors.

The plasmid pML564 was transformed into *M. tuberculosis* H37Rv and selected on 7H10/OADC/Hyg plates at 37°C. After three weeks, a single colony was picked and inoculated into 10 ml of 7H9/OADC/Hyg medium and incubated at 37°C on a shaker to an OD_{600} of 1.0. Dilutions from 1×10^3 to 1×10^6 were plated on 7H10/OADC/Hyg plates and incubated at 41°C. After three weeks, 8 colonies were picked and separately transferred into 30 ml of 7H9/OADC/Hyg medium and cultured at 41°C to prepare chromosomal DNA. All eight clones were correct single cross-over candidates (SCO) and confirmed by southern blot analysis. One SCO candidate was named *M. tuberculosis* ML157. The SCO ML157 was grown in 7H9/OADC/Hyg medium to an OD_{600} of 1.0. A series of 10-fold dilutions were plated on 7H10/OADC/Hyg medium supplement with 2% sucrose and incubated at 41°C. After four weeks, 8 single colonies were picked and cultured in 7H9/OADC/Hyg media at 41°C to prepare chromosomal DNA. All eight clones were correct double cross-over candidates (DCO) and confirmed by southern blot analysis. One DCO was named *M. tuberculosis* ML160 (Δ*ompATb::loxP-hyg-loxP*). The Cre recombinase expression vector pCreSacB1 (a kind gift from Dr. Adrie Steyn, University of Alabama at Birmingham) was used to excise the *loxP*-flanked hygromycin cassette from the chromosome of *M. tuberculosis* ML160. The plasmid pCreSacB1 was transformed into *M. tuberculosis* ML160 and selected on 7H10/OADC/Kan plates. After three weeks, 8 single colonies were transferred into 10 ml of 7H9/OADC/Kan medium and cultured at 37°C to an OD_{600} of 1.0. Whole cell PCR analysis was performed to confirm that the *loxP-hyg-loxP* cassette was removed from the genome using the primer pair 976/961. Three out of these 8 colonies were confirmed as having lost the *loxP-hyg-loxP* cassette. Then, a series of 10-fold dilutions from a single colony having lost the *loxP-hyg-loxP* cassette was plated on 7H10/OADC plates containing 2% sucrose and incubated at 37°C to counter-select against pCreSacB1. After three weeks, 24 single colonies were streaked in parallel on 7H10-OADC, 7H10/OADC/Kan

and 7H10/OADC/Hyg plates to confirm the loss of the *hyg* cassette and pCreSacB1. Twenty out of these 24 candidates failed to grow on plates containing kanamycin, confirming the loss of pCreSacB1 in these candidates. Colony PCR was performed using the primer pair 976/961 and one amplified fragment was submitted for sequencing. Sequencing results confirmed that this colony was an unmarked Δ*ompATb* mutant and was subsequently named *M. tuberculosis* ML163 (Δ*ompATb*::*loxP*). In this mutant, 689 bp within the 981 bp *ompATb* gene were replaced by 41 bp representing the *loxP* site.

A specific probe (1128 bp) for *ompATb* was amplified by PCR from chromosomal DNA of *M. tuberculosis* using the primer pair 781/782. The genomic DNAs of the deletion candidates were digested with *Pst*I, and Southern blot hybridization was performed as previously described (Stephan *et al.*, 2004).

Preparation of detergent extracts from *M. smegmatis* and *M. tuberculosis* and analysis by Western blot

10 ml cultures of *M. smegmatis* or *M. tuberculosis* expressing *ompATb* genes were grown to high density, normalized to an OD_{600} of 3.0, washed in TBS containing 1% SDS (pH 7.2), and concentrated in 1 ml TBS containing 1% SDS lysis buffer. Cells in lysis buffer were transferred to glass bead Lysing Matrix Tubes (MP Biomedicals) and disrupted using a FastPrep FP120 (BIO101/Savant) at $6,000 \times g$ for 90 sec. Whole extracts were incubated at 40°C for 2 h at 800 rpm shaking in a Thermomixer R (Eppendorf). Cell debris was removed by centrifugation at 4°C for 10 min at $13,000 \times g$ and the supernatant was used for subsequent Western blot analysis.

Proteins were separated in a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane using a standard protocol (Ausubel *et al.*, 1987). OmpATb was detected with a rabbit antiserum against OmpATb ((Senaratne *et al.*, 1998); gift from Dr. Philip Draper) while RNA polymerase was detected with the mouse anti-*E. coli* RNAP mAb 8RB13 (Neoclone). A horseradish peroxidase-conjugated goat-anti-rabbit antibody (Sigma) or goat-anti-mouse antibody (Sigma) served as the secondary antibody for OmpATb and RNAP, respectively. The blot was developed using luminol substrates (ECL, Pierce) and detected in an EpiChemi[3] Darkroom (UVP BioImaging system). Purified recombinant $rOmpATb_{His}$ from *E. coli* served as a standard reference.

Growth of *M. smegmatis* strains on agar plates

Cultures of *M. smegmatis* SMR5/pMS2, ML16/pMN016, ML16/pMS2, ML16/pML003 or ML16/pML1450 were grown to an OD_{600} of about 1.0, filtered through a 5 μm filter, and dilutions were plated on minimal HdB medium supplemented with 0.2% glycerol. After 4 days of incubation at 37°C, pictures of colonies were taken under white light at $10 \times$ magnification using a Stemi 2000-C stereomicroscope (Zeiss) and an AxioCam MRc camera (Zeiss).

Uptake experiments

Glucose, glycerol and serine uptake measurements were carried out as described before for glucose (Stephan *et al.*, 2005) with minor modifications. The cells were grown on Middlebrook 7H10 plates containing 0.1% Tween 80 at 37°C. The cells were scraped off the plates, suspended in Middlebrook 7H9 medium and filtered through a 5.0 μm filter (Sartorius). The cell suspension was used to inoculate 100 ml of Middlebrook 7H9 medium for *M. smegmatis* or Dubos medium for *M. tuberculosis* containing 1 mM of either glucose, glycerol, serine, or asparagine. The cells were harvested at an OD_{600} of 0.6 by centrifugation ($3750 \times g$ at 4°C for 10 min), washed twice in uptake buffer (50 mM Tris-HCl pH 6.9, 15 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.05% Tween 80) and resuspended in the same buffer. Radio-

labelled [^{14}C]glucose (GE healthcare) and non-labelled glucose, [^{14}C]glycerol (GE healthcare) and non-labelled glycerol, [^{14}C]serine (GE healthcare) and non-labelled serine, or [^{3}H]asparagine (Moravek Biochemicals and Radiochemicals) and non-labelled asparagine, were mixed and added to cell suspensions to obtain final concentrations of 20 μM accordingly. The mixtures were incubated at 37°C and 1 ml samples were removed at the indicated times. The cells were collected on a 0.45 μm Spin-X centrifuge tube filter (Costar) by mixing with an equal volume of 10% buffered formalin phosphate (Fisher) containing 0.1 M LiCl, and counted in a liquid scintillation counter (Beckman). The uptake rate was expressed as nmol/mg cells (dry weight).

Whole cell enzyme-linked immunosorbent assay (ELISA)

To examine the cellular localization of OmpATb, an enzyme-linked immunosorbent assay (ELISA) using whole cells (Huff *et al.*, 2009) was employed with modifications. 10 ml cultures of *M. smegmatis* expressing *ompATb* were diluted to an OD$_{600}$ of 1.0, harvested by centrifugation, and washed in 10 ml TBST (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM MgCl$_2$, 0.025% tyloxapol). Cells were concentrated in 1 ml TBST and 200 μl were transferred in 4 replicates to a U-bottom 96-well microtiter plate (Becton Dickinson). The plate was centrifuged at $3200 \times g$ for 5 min at 4°C and cells were blocked with 2.5% skim milk (Difco) in TBST for 1 hour at 37°C. Cells were then washed twice with TBST and incubated for 1 hour at 37°C with an anti-OmpATb antiserum (Raynaud *et al.*, 2002). Following three washes with 200 μl TBST ($3200 \times g$, 5 min, 4°C), cells were then incubated with an anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase and incubated for 1 hour at 37°C. Cells were then washed three times in TBST ($3200 \times g$, 5 min, 4°C) and resuspended in 100 μl substrate buffer (0.1 M glycine, 1 mM ZnCl$_2$, 1 mM MgCl$_2$, 1% 4-nitrophenyl phosphate (*p*NPP)). After incubation for 2 hours at 37°C, the reaction was stopped with the addition of 100 μl 2 M NaOH. The plate was centrifuged and 100 μl of the supernatant was transferred to a flat-bottom 96-well microtiter plate (Nunc). Phosphatase activity was quantified by reading the absorption at 405 nm using a microplate reader (Synergy HT, Bio-TEK Instrument Inc, USA).

pH-dependent growth of *M. tuberculosis* at pH 7.2 and pH 5.5

M. tuberculosis ML163 (Δ*ompATb::loxP*) was transformed with either the integrative *ompATb* expression vector pML759 (p$_{imyc}$-*ompATb*), replicative vector pML588 (p$_{imyc}$-*ompATb*) or pML763 (p$_{native}$-*ompATb* operon). The pML759 plasmid was integrated into the genome after plating on 7H10/OADC/Kan plates and confirmed by Western blot. This resulting strain was named *M. tuberculosis* ML168 (Δ*ompATb::loxP*; *attB::ompATb*). *M. tuberculosis* H37Rv wt, ML163, ML168 and the strains complemented with replicative vectors were inoculated into 10 ml 7H9 Middlebrook medium and grown to an OD$_{600}$ of 1.0. Cells were then harvested, washed twice with sterilized Millipore water, and transferred into 200 ml Hartmans de Bont (HdB) minimal medium (Smeulders *et al.*, 1999) (pH 5.5, without OADC) supplemented with 15 mM asparagine as the sole nitrogen source. The initial OD$_{600}$ for all cultures was 0.01. Samples of 3 ml were taken every day from each culture for 20 days and filtrated through 0.22 μm filters before measuring the pH using a pH meter (Symphony, VWR) and the ammonia concentration as described below. Samples of 1 ml were taken from the cultures every day and mixed with equal volume of formalin for 30 min. The OD$_{600}$ of these samples was measured using a spectrophotometer (SmartSpec™ Plus, Biorad).

Chemical analysis of the culture medium of *M. tuberculosis*

To identify chemical differences in the supernatants, wt *M. tuberculosis* and the Δ*ompATb* mutant ML163 were grown in Dubos medium for 14 days. Samples of 5 ml were removed every day from each culture and filtrated through two polyvinylidene fluoride-membrane

(PVDF) membrane filters (0.22 μm, Millipore) to remove live cells and kept at −20°C before analysis. These samples were filtered again through Nylon Luer-Lock membrane filters (0.22 μm, Roth) and then injected into a gas chromatograph with a mass selective detector (HP 5971A MSD). Ammonia was separated from other compounds using a capillary column containing a cross-linked polyethyleneglycol matrix (HP Innowax) and identified by comparison with analytical GC/MS data available from a mass spectral data base (NIST, 2008).

To quantitatively determine ammonia concentrations, we used a spectroscopic method based on the specific formation of the yellow/brownish adduct $HgO \bullet Hg(NH_2)I$ upon addition of Nessler's reagent to an ammonia-containing solution. Formation of the adduct in the supernatants of *M. tuberculosis* cultures was determined directly by measuring the absorbance at 520 nm (London *et al.*, 1974). The analytical procedure was done as described earlier (Yuen & Polland, 1954). A linear calibration curve was obtained using bis-ammonium salt of citric acid.

As a control experiment to determine whether the compound which reacted with Nessler's reagent was volatile, the supernatants of *M. tuberculosis* were adjusted to pH >12 using NaOH and distilled by high-vacuum or increased temperature using a Schlenk apparatus in combination with a trap cooled with liquid nitrogen. Then, the ammonia content of the distillate was determined photometrically using Nessler's reagent as described above.

Enzymatic determination of ammonia in culture supernatants of *M. tuberculosis*

To determine the ammonia concentration in culture supernatants, wt *M. tuberculosis*, the Δ*ompATb* mutant ML163, and the ML163 complemented strains with pML588 or pML763 were grown in HdB medium for 30 days. Samples of 3 ml were removed every day from each culture and filtrated through two PVDF membrane filters (0.22 μm, Millipore) to remove cells. These samples were frozen at −20°C before analysis. The ammonia concentration in these samples was determined by using an enzymatic method based on the synthesis of glutamate from 2-oxoglutarate and ammonia by glutamate dehydrogenase. The oxidation of nicotinamide-adenine dinucleotide (NADH) during this reaction is stoichiometric to the amount of ammonia and is measured by the loss of absorbance of NADH at 340 nm. The method was used as described in the manufacturer's protocol (Roche).

pH-dependent expression of *ompATb* in *M. tuberculosis*

Two cultures of wt *M. tuberculosis* H37Rv were grown in Dubos medium at pH 7.2 and 5.5 at 37°C. The pH of the medium was adjusted to pH 7.2 or 5.5 using 1 M NaOH or 1 M HCl before autoclaving. Samples of 20 ml were harvested at an OD_{600} of approximately 3.0 by centrifugation and resuspended in 2 ml PEN buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, pH 7.2) containing 1% SDS, and mixed with 1 ml glass beads (Q-Bio Gene). The cells were disrupted using the Fastprep FP120 beater (BIO101) twice at $6,000 \times g$ for a total of 90 seconds, and incubated for 2 h at 40°C while shaking. The cell debris was removed by centrifugation at $16,000 \times g$ for 20 min and the detergent extracts were analyzed by gel electrophoresis. The proteins were then blotted on a polyvinylidene fluoride-membrane, and detected by a rabbit anti-OmpATb antiserum as described previously (Song *et al.*, 2008).

Utilization of amino acids as sole nitrogen sources by *M. tuberculosis*

To test which amino acids are utilized as nitrogen sources by *M. tuberculosis* under acidic conditions, wild-type H37Rv was grown in 200 ml HdB media (pH5.5, with OADC) supplemented with or without asparagine, aspartic acid, glutamine, glutamic acid, serine,

arginine, methionine, proline, alanine, histidine, glycine, phenylalanine, leucine, valine, threonine, tyrosine, tryptophan, cysteine, isoleucine or lysine in parallel. The amino acid was added as the sole nitrogen source in HdB media. The optical density, pH and ammonia concentration were measured every two days as described above. For ammonia measurements, only the cultures supplemented with asparagine, glutamine, aspartic acid and glutamic acid were measured because no visible growth was found in the cultures supplemented with the other 16 amino acids.

Transcription of the *ompATb-rv0900-rv0901 operon*

M. tuberculosis RNA extraction and cDNA preparation were performed as described previously (Hillmann *et al.*, 2007). The primer pairs 976/977, 959/782, and 959/983 (Table S1) were used to amplify the *ompATb-rv0900-rv0901* fragments as indicated in Fig. S3. RNA and chromosomal DNA as controls were used as PCR templates to amplify fragments of the *ompATb-rv0900-rv0901* operon.

Mice infection experiments

Balb/c mice (female, 4-5 weeks old) were infected by aerosols with *M. tuberculosis* H37Rv wt and the isogenic *ompATb* operon mutant ML163. The cells were resuspended in sterile phosphate-buffered saline (PBS) at an OD_{600} of 0.2. Ten ml cell suspensions were loaded into a nebulizer of an aerosol chamber containing 40 mice. This provided a 1000 CFU/ml inoculum in lungs for both strains. After 0, 2, 4, 8 and 12 weeks of infection, the mice were sacrificed. Lungs and spleens were homogenized in 0.8 ml PBS by bead-beating, diluted 100x and 0.1 ml suspensions were plated on 7H10/OADC plates. The colonies on agar plates were counted after 1 month of incubation at 37°C.

Female, 6 to 8 week-old, specific pathogen-free BALB/c mice were purchased from Charles River Laboratories (Sülzfeld, Germany) and maintained in individually ventilated cages (IVC, Ebeco, Castrop-Rauxel, Germany) under biosafety level III conditions. All animal experiments performed were in accordance with the German Animal Protection Law and were approved by the Animal Research Ethics Board of the Ministry of Environment, Nature Protection and Agriculture (Kiel, Germany).

For infection experiments using the previously published wt and *ompATb* mutant strains, cultures of wt *M. tuberculosis* (strain 1424) or the *ompATb* deletion mutant CK69 (Raynaud *et al.*, 2002) were grown to mid-log phase, harvested, aliquoted, and frozen at −80°C. After thawing, viable cell counts were determined by plating serial dilutions on Middlebrook 7H10 agar supplemented with 10% bovine serum (Biowest, Nuaillé, France). Aerogenic infections with approximately 200 CFU of the wild type or the mutant strains were carried out in a Glas-Col aerosol infection device (Glas-Col, Terre-Haute, IN). Actual inoculum size was confirmed 24 h after infection by determining the bacterial burden in undiluted lung homogenates of three infected mice per strain. For intravenous infection, 0.2 ml of a bacterial suspension containing approximately 1×10^5 CFU in PBS were injected into a lateral tail vein. The inocula were confirmed 24 h post infection by determining the bacterial load in undiluted lung homogenates and in serial ten-fold dilutions of spleen and liver homogenates (three infected mice per strain). To follow the course of infection, the bacterial load in lungs and spleens was determined at days 28 and 65 after infection. Organs from five animals per time point and strain were aseptically removed, weighed and homogenized in distilled sterile water containing 0.05% Tween 80. Ten-fold serial dilutions of organ homogenates were plated on Middlebrook 7H10 agar supplemented with 10% bovine serum and incubated at 37°C for 21 days. Colonies on plates were enumerated and results are expressed as $\log_{10}$ CFU per organ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

OPOE	*n*-octylpolyethylene oxide
wt	wild-type
ELISA	enzyme-linked immunosorbent assay

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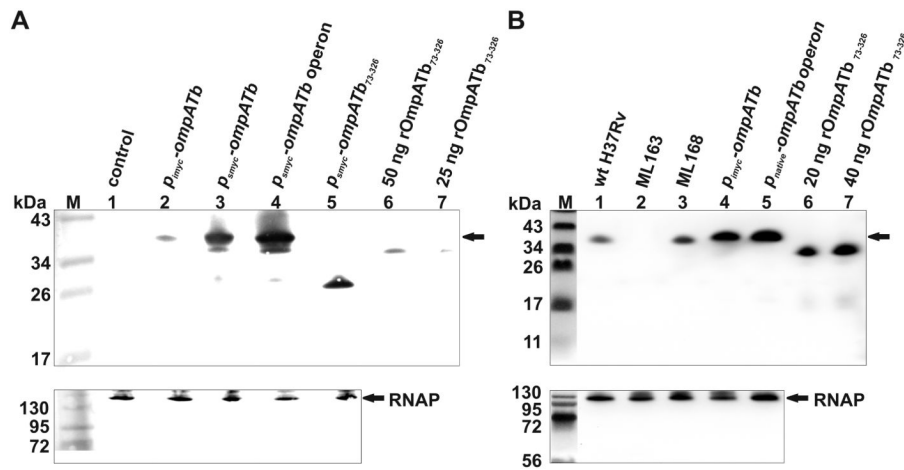
Fig. 1. Expression of *ompATb* genes in mycobacteria

A: Western blot analysis of *ompATb* expression in the porin mutant *M. smegmatis* ML16 (Δ*mspA* Δ*mspC* Δ*mspD*). Proteins of *M. smegmatis* ML16 were extracted with 1% SDS, separated on a 10% SDS-polyacrylamide gel, and transferred to a PVDF membrane. Upper panel: OmpATb was detected with an OmpATb-specific antiserum (Senaratne *et al.*, 1998). Lanes: M, Pre-Stained Protein Ladder; 1, ML16/pMS2 (empty vector); 2, ML16/pML588; 3, ML16/pML003; 4, ML16/pML1450; 5, ML16/pML2334; 6, 50 ng rOmpATb$_{73-326}$ purified from *E. coli*; 7, 25 ng rOmpATb$_{73-326}$ purified from *E. coli*. Arrow denotes full-length OmpATb. Lower panel: *M. smegmatis* RNA polymerase (RNAP) was detected on the same blot with a monoclonal antibody.

B: Western blot analysis of *ompATb* expression in *M. tuberculosis* strains. Proteins were extracted, separated, blotted, and detected as described above. Lanes: M, Pre-Stained Protein Ladder; 1, wt *M. tuberculosis* H37Rv; 2, ?*ompATb* strain ML163 (?*ompATb::lox*P); 3, ML168 (ML163::*ompATb*); 4, ML163/pML588 (p$_{imyc}$-*ompATb*); 5, ML163/pML763 (*ompATb* operon); 6, 20 ng rOmpATb$_{73-326}$ purified from *E. coli*; 7, 40 ng rOmpATb$_{73-326}$ purified from *E. coli*. Arrow denotes full-length OmpATb. Lower panel: *M. tuberculosis* RNAP was detected on the same blot with a monoclonal antibody.
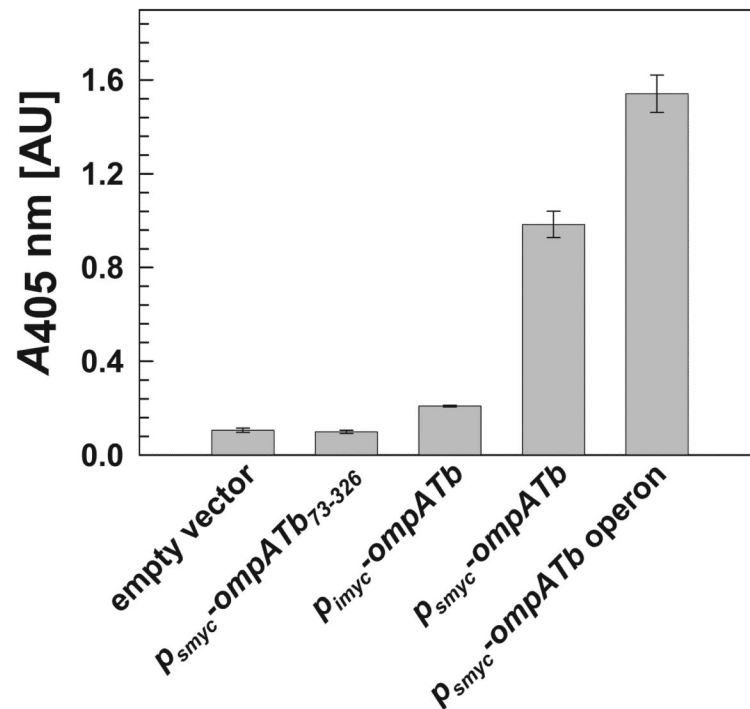
Fig. 2. Surface accessibility of OmpATb in *M. smegmatis* by whole cell ELISA
Surface-accessible OmpATb was probed for on whole cells of *M. smegmatis* ML16 using a
rabbit OmpATb-specific antiserum. Binding of a secondary alkaline phosphatase conjugate
antibody was followed by addition of *p*NPP and subsequent absorbance readings at 405 nm.
From left to right, 1st bar: *M. smegmatis* ML16 (Δ*mspA* Δ*mspC* Δ*mspD*) with the empty
vector control pMS2; 2nd bar: ML16/pML2334; 3rd bar: ML16/pML588; 4th bar: ML16/
pML003; 5th bar: ML16/pML1450. Data were obtained from four replicates and error bars
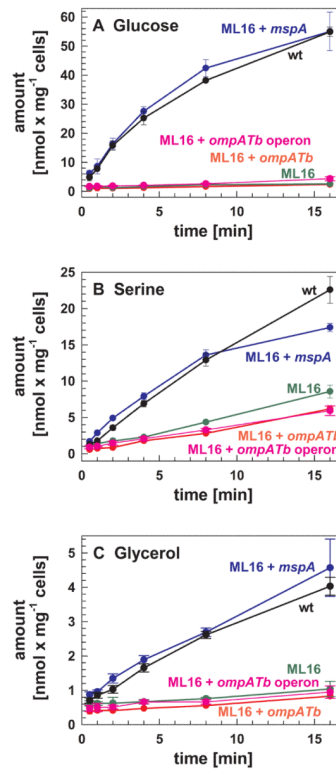represent standard errors.

Fig. 3. OmpATb does not contribute to uptake of hydrophilic solutes across the outer membrane of *M. smegmatis*

Accumulation of [^{14}C]glucose (**A**), [^{14}C]serine (**B**) and [^{14}C]glycerol (**C**) by *M. smegmatis* SMR5/pMS2 (wt, black), ML16 with the empty vector pMS2 (ML16, green), ML16/pMN016 (ML16+*mspA*, blue), ML16/pML003 (ML16+*ompATb*, red) and ML16/pML1450 (ML16+*ompATb* operon, pink) was measured. All assays were performed at 37 °C at final glucose, serine or glycerol concentrations of 20 μM. Uptake experiments were performed in triplicate and are shown with standard deviations.
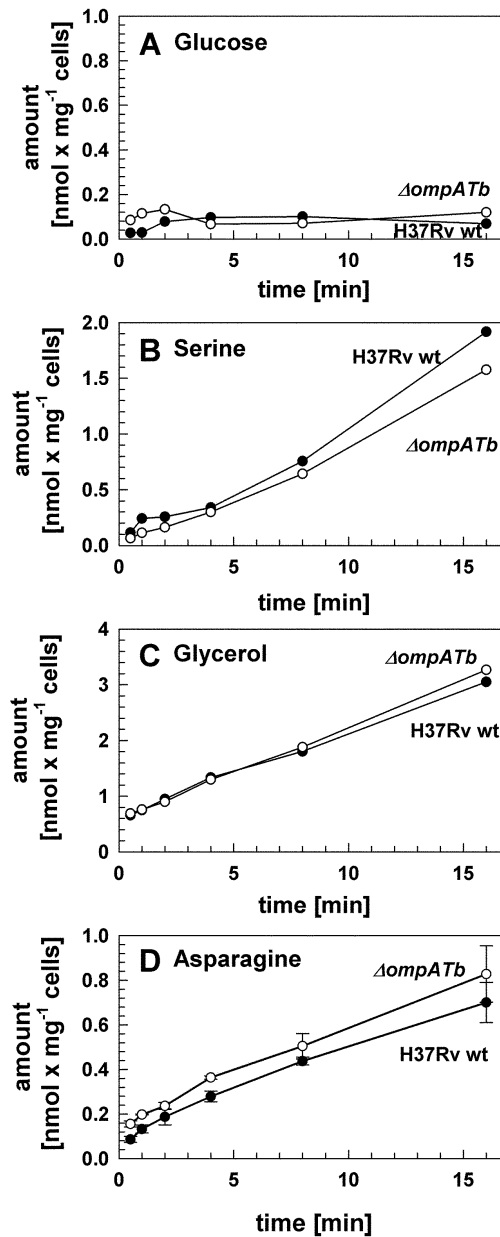
Fig. 4. OmpATb does not have porin activity in *M. tuberculosis* and is not involved in uptake of asparagine

Accumulation of [^{14}C]glucose (**A**), [^{14}C]serine (**B**), [^{14}C]glycerol (**C**) and [^{3}H]asparagine (**D**) by *M. tuberculosis* wt (closed circles) and ML163 (Δ*ompATb*, open circles) was measured. All assays were performed at 37 °C at final glucose, serine, glycerol and asparagine concentrations of 20 μM.
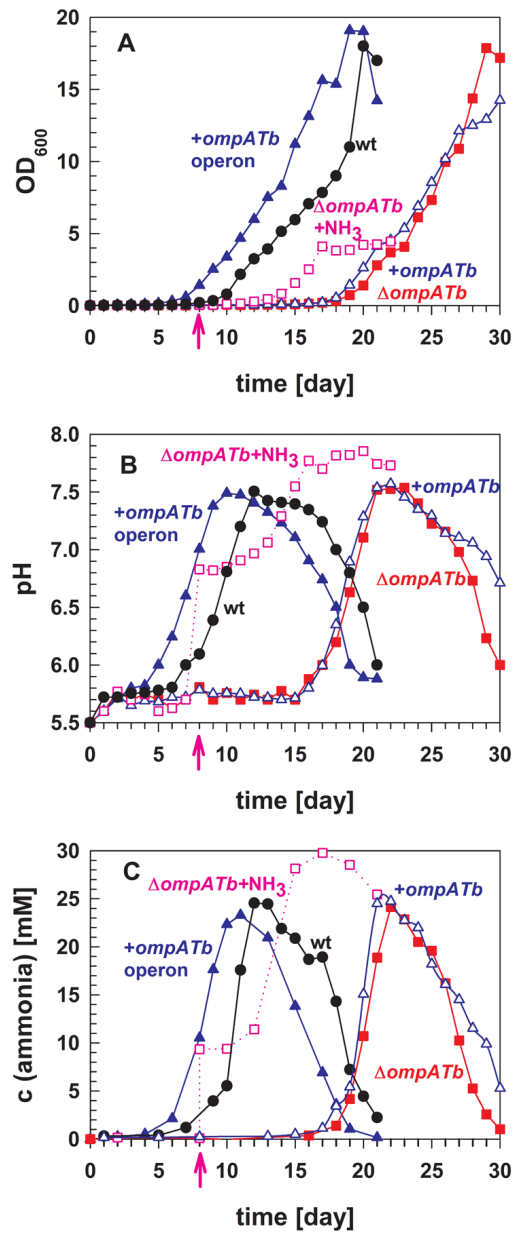
Fig. 5. OmpATb-dependent growth, pH neutralization and ammonia secretion by *M. tuberculosis*
M. tuberculosis H37Rv (wt, black circles), the mutant ML163 (Δ*ompATb,* closed red squares), the pML588 complemented mutant (+*ompATb,* open blue triangles) and the pML763 complemented mutant (+*ompATb* operon, closed blue triangles) were grown in HdB medium (pH 5.5). In parallel, 10 mM exogenous ammonia was added (pink arrows) to the mutant ML163 culture on day 8 (Δ*ompATb*+NH₃, open pink squares). The cell density (OD₆₀₀) of the culture (**A**), pH (**B**) and ammonia concentration (**C**) were measured each day for 30 days.
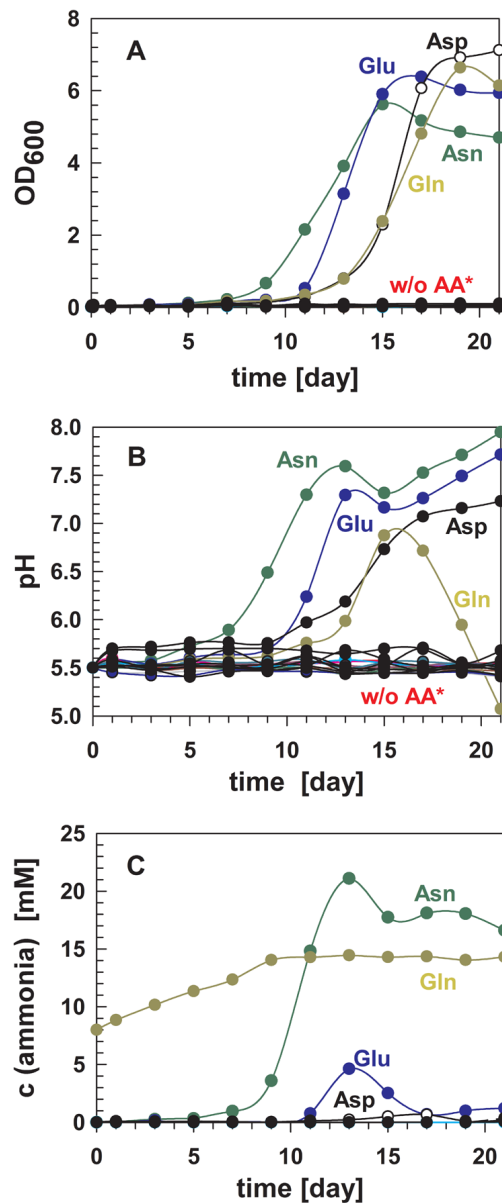
Fig. 6. Asparagine is the primary ammonia source for *M. tuberculosis* H37Rv at acidic pH
M. tuberculosis H37Rv wt was grown in HdB medium (pH 5.5) supplemented with one of the 20 natural amino acids as the sole nitrogen source. The cell density (OD_{600}) of the culture (**A**), the pH (**B**) and the ammonia concentration (**C**) were measured every two days. w/o AA* indicates that *M. tuberculosis* in all cultures except those containing asparagine (Asn), glutamine (Gln), glutamic acid (Glu) and aspartic acid (Asp) gave similar growth curves and pH change to the culture without any amino acids supplemented. Cysteine was removed from graph **B** as it interfered with the assay. For clarity, cultures with amino acid supplements that did not give rise to detectable changes in optical density, pH, or ammonia levels were omitted from graph **C** except for the culture with no amino acid supplements (filled black circles).
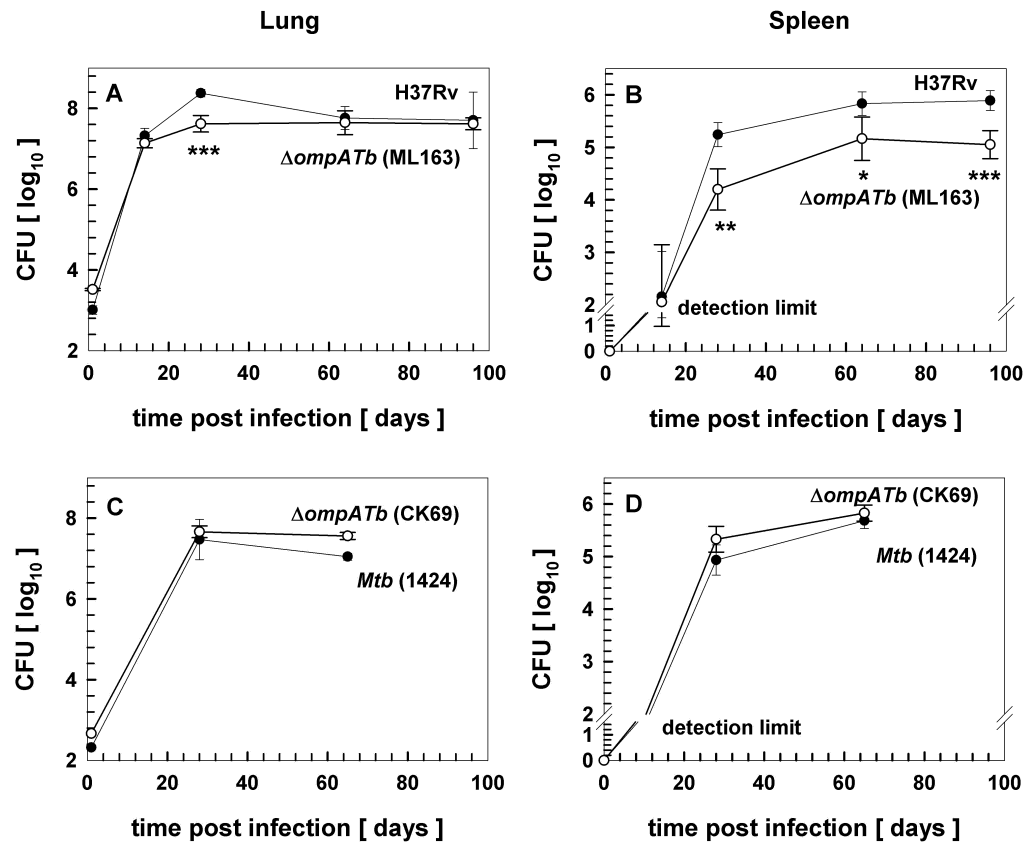
Lung

Spleen

Fig. 7. Survival of *ompATb* mutants of *M. tuberculosis* in mice

Wt *M. tuberculosis* H37Rv and the isogenic *ompATb* operon mutant ML163 (**A, B**), or wt *M. tuberculosis* 1424 and the isogenic *ompATb* mutant CK69 (Raynaud *et al.*, 2002) (**C, D**), were administered to BALB/c mice by aerosol infection. At indicated time points, lungs (**A, C**) and spleens (**B, D**) were removed from infected mice, homogenized, and the suspensions were plated on solid media. Colony-forming units (CFU) of *M. tuberculosis* wt (closed circles) and the *ompATb* mutants (open circles) were counted and plotted. Two-tailed and paired Student's *t*-tests were performed between wt and the *ompATb* mutant. * indicates *p* value <0.1; ** indicates *p* value <0.05; *** indicates *p* value <0.01. The detection limits were 30 and 100 CFU's in the lung and in the spleen, respectively.

<div align="center">

Table 1

Strains used in this work

</div>

The annotations Cm^R, Sm^R, and Hyg^R indicate that the strain is resistant to the antibiotics chloramphenicol, streptomycin, and hygromycin, respectively.

Strain	Parent strain and relevant genotype	Source or reference
E. coli DH5α	*rec*A1, *end*A1, *gyr*A96, *thi*; *re*/A1,*hsd*R17(r_K^-,m_K^+), *sup*E44, ϕ80Δ*lac*ZΔM15, Δ*lac*Z(YA-argF)UE169	(Hanahan, 1983)
E. coli BL21 Rosetta	F⁻ ompT hsdS$_B$(R_B^- m$_B^-$) gal dcm λ(DE3) pLysSRARE; Cm^R	Novagen
M. smegmatis SMR5	*M. smegmatis* mc²155; Sm^R	(Sander *et al*., 1995)
ML16	*M. smegmatis* SMR5, Δ*mspA*::*FRT*, Δ*mspC*::*FRT*, Δ*mspD*::*FRT*, *attB::loxP*, *FRT;* Sm^R	(Stephan *et al*., 2005)
M. tuberculosis H37Rv	Laboratory strain	ATCC 25618
ML157	*M. tuberculosis, ompATb::pML564*	this study
ML160	*M. tuberculosis, ΔompATb::loxP-hyg-loxP*; Hyg^R	this study
ML163	*M. tuberculosis, ΔompATb::loxP*	this study
ML168	ML163, *attB*::*pML759*	this study

Table 2

Plasmids used in this work

Up- and downstream homologous sequences of genes are subscripted as up and down. "Origin" denotes origin of replication. The annotations Hyg^R and Km^R indicate that the plasmid confers resistance to hygromycin and kanamycin, respectively.

Plasmid	Components and properties	Source or reference
pMS2	ColE1 origin; PAL5000 origin; Hyg^R; 5229 bp	(Kaps *et al.*, 2001)
pMN016	p_{smyc}-*mspA;* ColE1 origin; PAL5000 origin; Hyg^R; 6164 bp	(Stephan *et al.,* 2005)
pMN013	p_{imyc}-*mspA;* ColE1 origin; PAL5000 origin; Hyg^R; 6000 bp	(Mailaender *et al.*, 2004)
pCV125	ColE1 origin; Km^R; L5 *attP;* L5 integrase; 8261 bp	(Steyn *et al.*, 2003)
pML003	p_{smyc}-*ompATb;* ColE1 origin; PAL5000 origin; Hyg^R; 6534 bp	(Song *et al.*, 2008)
pML588	p_{imyc}-*ompATb;* ColE1 origin; PAL5000 origin; Hyg^R; 6357 bp	(Song *et al.*, 2008)
pML763	p_{native}-*ompATb-rv0900-rv0901;* ColE1 origin; PAL5000 origin; Hyg^R; 8023 bp	this study
pML1450	p_{smyc}-*ompATb-rv0900-rv0901;* ColE1 origin; PAL5000 origin; Hyg^R; 7308 bp	this study
pML2334	p_{smyc}-*ompATb*$_{73-326}$; ColE1 origin; PAL5000 origin; Hyg^R; 6292 bp	this study
pML759	ColE1 origin; p_{imyc}-*ompATb;* Km^R; L5 *attP*; L5 integrase; 9457 bp	this study
pML564	ColE1 origin; PAL5000 origin; Hyg^R; *sacB*; *ompATb*$_{up}$, *ompATb*$_{down}$; 10630 bp	this study
pML591	f1 origin; p_{T7}-*ompATb*$_{His}$; Km^R; 6129 bp	this study

Table 3

Uptake rates for *M. smegmatis* and *M. tuberculosis* towards hydrophilic solutes

Quantification of uptake rates for glucose, serine and glycerol by the wt strains *M. smegmatis* SMR5 and *M. tuberculosis* H37Rv as well as the porin mutant *M. smegmatis* ML16. All assays were performed at 37°C at a final solute concentration of 20 μM. The *M. smegmatis* strains contained the empty plasmid pMS2. Uptake rates were determined from the kinetics shown in **Figs.** 3 and 4 for *M. smegmatis* and *M. tuberculosis,* respectively, and are expressed in nmol/min/mg cells.

	SMR5	ML16	H37Rv
glucose	3.43	0.14	0.04
serine	1.41	0.53	0.13
glycerol	0.25	0.06	0.19