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The N-Terminal Domain of OmpATb Is Required for Membrane Translocation and Pore-Forming Activity in Mycobacteria▽

Anuradha Alahari,[1] Nathalie Saint,[3,4] Sylvie Campagna,[3,4] Virginie Molle,[5] Gérard Molle,[3,4]* and Laurent Kremer[1,2]

Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Université de Montpellier II et I, CNRS, UMR 5235, Case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France[1]; INSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France[2]; INSERM, U554, 29 Rue de Navacelles, F34090 Montpellier, France[3]; Université de Montpellier I et II, CNRS UMR5048, Centre de Biochimie Structurale, F34090 Montpellier, France[4]; and IBCP, UMR 5086 CNRS, Université de Lyon, 69367 Lyon, France[5]

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OmpATb is the prototype of a new family of porins in *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Although the pore-forming activity of this protein has been clearly established by using recombinant protein produced in *Escherichia coli*, characterization of the native porin has been hampered by the scarce amount of protein present in the *M. tuberculosis* detergent extracts. To this aim, we have developed a protocol to overproduce and obtain high yields of OmpATb in both *Mycobacterium smegmatis* and *M. bovis* BCG. The protein could be extracted and purified from the cell wall fraction and subsequently used for analysis of the pore-forming activity in multichannel and single-channel conductance experiments. Our results indicate that OmpATb produced in mycobacteria presents an average conductance value of 1,600 ± 100 pS, slightly higher than that of OmpATb produced in *E. coli*, suggesting the occurrence of OmpATb in a highly ordered organization within the mycobacterial cell wall. In contrast to OmpATb, a truncated form lacking the first 72 amino acids ($OmpATb_{73-326}$) was essentially found in the cytosol and was not active in planar lipid bilayers. This suggested that the N-terminal domain of OmpATb could participate in targeting of OmpATb to the cell wall. This was further confirmed by analyzing *M. smegmatis* clones expressing a chimeric protein consisting of a fusion between the N-terminal domain of OmpATb and the *E. coli* PhoA reporter. The present study shows for the first time that the N terminus of OmpATb is required for targeting the porin to the cell wall and also appears to be essential for its pore-forming activity.

Mycobacteria are medically important, particularly *Mycobacterium tuberculosis*, which causes about two millions deaths each year in the world (36). The emergence of multidrug-resistant strains poses a serious threat to the treatment and control of tuberculosis. One of the main difficulties to eradicate this infection is due to the weak penetration of drugs in the cell. Mycobacteria are characterized by a highly hydrophobic cell wall in which the lipid and lipid-containing components are present in unusual abundance, constituting 60% of the dry weight of the cell wall (3). This hydrophobicity contributes to the very low permeability of the cell wall, thus preventing the diffusion of small solutes such as hydrophilic drugs (7, 13). The permeability of *M. tuberculosis* is comparable to that of *Pseudomonas aeruginosa*, whose permeability is in turn 100 times lower than that of *E. coli* (7).

The current model of the mycobacterial cell envelope includes the presence of an outer membrane, although mycobacteria are classified as gram-positive bacteria. The outer membrane consists of mycolic acids, which are very long-chain fatty acids attached to a lower layer of arabinogalactan to form a closely packed monolayer (16). Noncovalently bound lipids complement the ordered arrangement of mycolic acids to an asymmetric bilayer (21). Despite the presence of an efficient impermeable barrier, the permeability of small hydrophilic molecules can be ensured by water-filled channel proteins, such as porins. Porin-like proteins that allow diffusion of small and hydrophilic solutes across the unique outer membrane have been identified in the envelope of several mycobacterial species such as *M. chelonae* (35), *M. smegmatis* (34), and *M. phlei* (28), as well as in *M. bovis* BCG (12) and *M. tuberculosis* (9). Unfortunately, the amount of these proteins present in the mycobacterial cell envelope is usually very low, and this has seriously hampered their isolation for subsequent biochemical or structural characterization. BLAST search analysis has allowed identification of a polypeptide resembling the *E. coli* OmpA porin, termed OmpATb, sharing significant sequence similarity in the characteristic carboxy-terminal region (29). A recombinant OmpATb protein was produced and induces ion channels when incorporated in lipid bilayers (29). Moreover, other studies (26) have shown the ability of OmpATb to be the only functional porin at acidic pH, and this feature has been subsequently corroborated with in vitro reconstitution experiments (17). Recent electrophysiological studies with recombinant OmpATb produced in *E. coli* demonstrated that the pore-forming activity of OmpATb was pH dependent and that at low pH values the channels were closed (17). It was therefore proposed that the propensity of channels of native OmpATb to close in acidic pH conditions may participate in the adaptation of the pathogen to survive under acidic conditions such as those encountered within the macrophage phagosomes (26).

* Corresponding author. Mailing address: Centre de Biochimie Structurale, UMR 5048 CNRS, UMR 554 INSERM, 29 Rue de Navacelles 34090 Montpellier, France. Phone: 33 467 417 912. Fax: 33 467 417 913. E-mail: gerard.molle@cbs.cnrs.fr.

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Like OmpA, OmpATb appears to contain a putative signal sequence at its N-terminal end (29) but lacks the precise site at which proteolytic cleavage usually occurs after translocation to the membrane. Senaratne et al. (29) proposed that the hydrophobic α helix between residues 18 and 39 could represent a peptide signal. These authors showed that the production of a truncated OmpATb variant (lacking the 49 first residues) yielded an inactive soluble protein and suggested that expression of this putative signal sequence may play an essential role in the pore-forming activity. We have recently shown that in the absence of any signal peptide, a truncated OmpATb protein lacking its N-terminal 72 residues ($OmpATb_{73-326}$) was found to be soluble in cytosolic compartment and remained inactive in planar lipid bilayers (unpublished data). However, when fused to the *E. coli* OmpA signal peptide, the protein could be targeted to the membrane and exhibited pore-forming activity similar to that of full-length OmpATb in planar lipid bilayers (17). This raises the question about the role of the N-terminal sequence of OmpATb. However, all of these experiments were carried out with recombinant proteins produced in *E. coli*, whose membrane environment is substantially different from that in mycobacteria.

Therefore, the present study sought to characterize the pore-forming activity of recombinant OmpATb produced in mycobacteria and compare it with the activity obtained from recombinant OmpATb produced in *E. coli*. This was successfully achieved by setting up a new method to express and purify OmpATb from the cell walls of two different mycobacterial species (*M. bovis* BCG and *M. smegmatis*). We also addressed the role of the N-terminal sequence in OmpATb channel activity and its possible participation in targeting the porin to the cell wall.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* TOP-10 (Invitrogen) was used for cloning of the *ompATb* constructs. All strains were grown and maintained in LB medium at 37°C. When required, media were supplemented with 25 μg of kanamycin/ml. *M. bovis* BCG strain Pasteur 1173P2 and *M. smegmatis* mc^2155 were transformed by electroporation as described previously (30). Clones were selected on Middlebrook 7H10 agar plates supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco) and 25 μg of kanamycin/ml and then incubated at 37°C for 2 weeks for *M. bovis* BCG or 3 to 4 days for *M. smegmatis*. Recombinant mycobacteria were grown in Sauton's medium supplemented with 25 μg of kanamycin/ml at 37°C with shaking.

Cloning and overexpression of OmpATb and $OmpATb_{73-326}$ in mycobacteria. A standard PCR strategy was used to amplify the *M. tuberculosis* H37Rv *ompATb* coding sequence, using *Vent* DNA polymerase and the following primers: *ompATb*-5′ (5′-CCA TAT GGC TTC TAA GGC GGG TTT GGG-3′; contains a NdeI site [underlined]) and *ompATb*-3′ (5′-AAA GCT TGT TGA CCA CGA TCT CGA CGC G-3′; contains a HindIII site [underlined]). The PCR product was first ligated into the pCR-Blunt II TOPO vector (Invitrogen). After restriction with NdeI/HindIII, the *ompATb* insert was purified and ligated to the pVV16 expression vector cut with NdeI/HindIII (6). This plasmid is a derivative of pMV261 (32), containing both a kanamycin and a hygromycin resistance cassette, harboring the *hsp60* promoter, as well as a His tag for the expression of C-terminal His-tagged fusions proteins. The resulting expression vector was designated pVV16::*ompATb*. To construct pVV16::$ompATb_{73-326}$, pVV16::*ompATb* was restricted with SmaI (unique site located just upstream of the GASAL motif in the protein sequence) and HindIII. The SmaI/HindIII insert was then gel purified and cloned into the pVV16 digested with MscI/HindIII. All plasmid DNAs were validated by verifying the nucleotide sequence. Both constructs were used to transform *M. bovis* BCG and *M. smegmatis*.

Immunoblotting and antisera. Mycobacterial cultures (10 ml) were harvested, and the cells were resuspended in 0.8 ml of phosphate-buffered saline (PBS) and disrupted by using a bead beater (MM301; Retsch). Protein concentrations in crude cell lysates were determined using the BCA protein assay reagent kit (Pierce). Equal amounts of protein (20 μg) or, alternatively, equal volumes of subcellular fractions were then separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and transferred onto a Protran nitrocellulose transfer membrane (Schleicher & Schuell). The membranes were then saturated with 1% bovine serum albumin in PBS–0.1% Tween 20 and probed by incubation overnight with a 1:25,000 dilution of a rabbit anti-OmpATb antiserum raised against the *M. tuberculosis* protein (29). After extensive washing, the membranes were then incubated with anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (dilution 1:7,000; Promega). Alternatively, membranes were probed by using HisProbe-HRP (Pierce) along with the SuperSignal West Pico Chemiluminescent substrate (Pierce) as recommended by the manufacturer. Monoclonal antibodies to *M. tuberculosis* KatG (IgG1, clone IT-57) were used at a 1:1,000 dilution and served as a marker for the detection of cytoplasmic contamination in the cell wall fraction (27).

Purification of OmpATb and $OmpATb_{73-326}$ from mycobacteria. Mycobacteria were harvested from large-scale cultures (4 to 6 liters), and cells were resuspended in PBS containing an anti-protease cocktail (Roche), as well as DNase and RNase. Cells were then disrupted by using a French pressure cell, and subcellular fractionation was performed essentially as described previously (23). Briefly, cell lysates were centrifuged twice at $1,000 \times g$ to remove unbroken cells and insoluble material. Lysate supernatants were then centrifuged at $20,000 \times g$ at 4°C to separate the soluble cytoplasmic fraction from the cell wall. The cell wall fraction was washed three times with PBS in order to remove cytoplasmic contaminants. Both fractions were resuspended in an identical volume of buffer.

For purification of the truncated recombinant $OmpATb_{73-326}$, the supernatant obtained at $20,000 \times g$ containing the recombinant protein was directly incubated with Ni-NTA agarose suspension (QIAGEN). The protein-resin complex was packed into a column and washed extensively with 20 mM Tris-HCl (pH 8), 300 mM NaCl, and 10 mM imidazole. The protein was then eluted with 5 ml of 20 mM Tris-HCl (pH 8), 300 mM NaCl, and 400 mM imidazole. For purification of OmpATb, the cell wall pellet was resuspended and incubated at room temperature for 1 h in a buffer containing 20 mM Tris-HCl (pH 8), 300 mM NaCl, 2% OPOE (*n*-octyl-polyoxyethylene), and anti-proteases. The final suspension was centrifuged at 4°C for 1 h at $20,000 \times g$ and the supernatant was incubated with Ni-NTA agarose. The protein-resin complex was washed extensively with 20 mM Tris-HCl (pH 8), 300 mM NaCl, 10 mM imidazole, and 0.5% OPOE, and the protein was eluted with the same buffer in the presence of 400 mM imidazole.

The protein solutions were concentrated on Centricon (Amicon) before being loaded onto a gel filtration column (Superdex 75). The elution buffer consisted of 20 mM Tris-HCl (pH 8) and 150 mM NaCl for $OmpATb_{73-326}$ or 20 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.5% OPOE for OmpATb. The eluted fractions were analyzed by SDS-PAGE. Protein concentrations were determined by measuring the absorbance at 280 nm (Nanodrop; Labtech) prior to the addition of an anti-protease cocktail (Roche). The N-terminal sequences of the different proteins were determined by automated Edman degradation (477 Protein Sequencer; Applied Biosystems).

Purification of recombinant OmpATb and $OmpATb_{73-326}$ from *E. coli*. A His-tagged OmpATb recombinant protein fused to the *E. coli OmpA* signal peptide (known to target fusion proteins to the outer membrane) was produced by constructing pETSIG-*ompATb* (17). This vector was then introduced into *E. coli* BL21(DE3)omp8 strain (24). Because this strain is devoid of numerous porins, it is particularly useful for the production and subsequent characterization of heterologous porins, avoiding eventual contamination with endogenous *E. coli* porins. Recombinant OmpATb and $OmpATb_{73-326}$ produced in *E. coli* (OmpATbEC and $OmpATb_{73-326}$EC, respectively) were purified as reported earlier (17).

Construction of an OmpATb N-terminal/PhoA hybrid protein. The genomic DNA region of *M. tuberculosis* H37Rv corresponding to the N-terminal 74 amino acids of the *ompATb* gene was PCR amplified by using pVV16::*ompATb* plasmid as a template. A vector-derived forward primer, pMVfwd (5′-GCC CGG CCA GCG TAA GTA G-3′) and a reverse primer, omphoA-Xho (5′-AGA CTC GAG CAA CGC AGA AGC GCC-3′), were used. The PCR product carried a XhoI site at the 3′ end (underlined) and the NdeI site of the original pVV16-*ompATb*. It included the region encoding the GASAL amino acid sequence. The PCR product was digested with NdeI and XhoI, used for ligation with the *phoA* gene sequence, and cloned in pVV16 as follows. Genomic DNA of *E. coli* strain TOP-10 was used as a template to amplify the *phoA* gene omitting the first 141 bp that include sequences encoding the PhoA signal peptide. Two pairs of primers were used for this reaction. The first pair consisted of the forward primer, phoANde (5′-GCC CGG ACA CCA CAT ATG CCT GTT CTG G-3′) and reverse primer phoArev 5′-TCG CCA AGC TTA TTT CAG CCC CAG AGC GGC-3′) that have sites (underlined) for NdeI and HindIII, respectively.

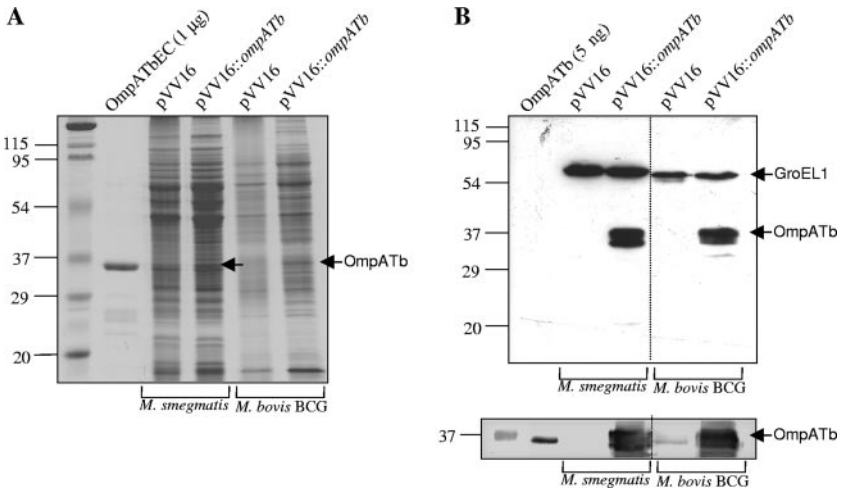

FIG. 1. Overexpression of OmpATb in mycobacteria. *M. smegmatis* and *M. bovis* BCG were transformed with either the empty pVV16 or pVV16::*ompATb* that permits production of OmpATb as a C-terminal His-tagged fusion protein. Recombinant mycobacteria were disrupted, and lysates equivalent to 20 μg of total protein were separated by SDS-PAGE. (A) Coomassie blue-stained gel showing overexpression of OmpATb in crude cell lysates of mycobacterial strains carrying plasmids as indicated. Purified recombinant OmpATb from *E. coli* was used for comparison of molecular size. The numbers on the left indicate sizes of the molecular weight markers. (B) Corresponding immunoblots using either a His-Probe-HRP along with a chemiluminescent substrate that reveals the presence of His-tagged proteins (upper panel) or anti-OmpATb antiserum (lower panel). The GroEL1 is detected in the crude lysates by the virtue of the naturally occurring C-terminal His residues (22).

This amplified product was cloned in pVV16 to obtain a pVV16::*phoA′*. The second pair of primers consisted of the forward primer, phoAXho (5′-GCC CGG ACA CTC GAG ATG CCT GTT CTG G-3′), which has an XhoI site (underlined) and the same reverse primer phoArev. The XhoI site of the forward primer allowed an in-frame ligation with the 5′ region of *ompATb* amplified as described above. The hybrid construct consisting of N-terminal part of OmpATb from *M. tuberculosis* and C-terminal part of PhoA from *E. coli* thus generated was cloned into plasmid pVV16 digested with NdeI and HindIII to obtain pVV16::*omphoA*. *M. smegmatis* mc^2155 was transformed with the plasmids pVV16: *phoA′* or pVV16::*omphoA*. The *M. smegmatis* clones were plated on indicator plates of LB medium containing 40 μg of BCIP (5-bromo-4-chloro-3-indolylphosphate)/ml that would yield blue colonies if the clones overexpress an active phosphatase (10). All plasmid DNAs were validated by verifying the nucleotide sequence. Recombinant clones were validated by diagnostic PCRs carried out on cells with appropriate primers.

Alkaline phosphatase activity assay. Cells from exponentially growing *M. smegmatis* cultures in Sauton's medium were collected by centrifugation at 5,000 rpm for 10 min and concentrated tenfold. For the alkaline phosphatase assay, 10 to 50 μl of the concentrated cell slurry was resuspended in 0.9 ml of buffer AP (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl), and 100 μl of the substrate solution consisting of 0.2 M *p*-nitrophenol phosphate was added. The reaction was allowed for 30 min at 37°C and stopped by the addition of 100 μl of solution containing 0.8 M KH_2PO_4 and 0.1 M EDTA. The cells were separated by centrifugation at 13,000 rpm for 2 min, and the supernatant was read spectrophotometrically at 410 nm. The optical density (OD) from a control reaction that was missing the cells served as a blank and was subtracted from the OD readings for other reactions. Enzyme activity is expressed in arbitrary units of OD_{410}/ml of culture/min.

Conductance experiments. The technique described by Montal and Mueller (18) was applied in order to form virtually solvent-free planar lipid bilayers for macroscopic and single-channel conductance experiments. The membranes were formed over a 100- to 150-μm hole in a Teflon film (10 μm thick), pretreated with a 1:40 mixture (vol/vol) of hexadecane-hexane, separating two half cells. Lipid monolayers from a 5-mg/ml azolectin solution (Soybean azolectin IV-S from Sigma) were spread on the top of an electrolyte solution (1 M KCl, 10 mM HEPES, and citric acid depending on the pH) in both compartments. Bilayers were formed by lowering and raising the level of electrolyte in one or both sides. A voltage was applied by an Ag/AgCl electrode on the *cis* side.

For the macroscopic conductance experiments, doped membranes were subjected to slow voltage ramps (10 mV/s), and the transmembrane currents were amplified (BBA-01; Eastern Scientific, Rockville, MD). The current-voltage curves were stored on a computer and analyzed with Scope software (Bio-Logic, Claix, France).

For single-channel recording, potentials were applied, and the currents were amplified simultaneously by using a BLM 120 amplifier (Bio-Logic). Single-channel currents were stored on a CD (DRA 200; Bio-Logic) for off-line analysis. All experiments were carried out at room temperature. The data were filtered at 1 kHz before being digitized at 11.2 kHz for analysis.

RESULTS

Overexpression of OmpATb in *M. bovis* BCG and *M. smegmatis*. At low external pH, OmpATb is thought to provide the primary general diffusion pathway for hydrophilic compounds through the hydrophobic outer membrane of *M. tuberculosis*. In order to overproduce and purify OmpATb in a homologous host, a His tag was fused to the C terminus of the OmpATb polypeptide, and the recombinant protein was expressed from the constitutive *hsp60* promoter, both in *M. bovis* BCG and in *M. smegmatis*. The advantages of using *M. smegmatis* are that it lacks an OmpATb-related protein, thus providing a clean genetic background, and that it is a fast-growing species. Recombinant mycobacteria were pelleted and lysed, and protein expression was analyzed by SDS-PAGE. As shown in Fig. 1A, lysates from *M. bovis* BCG or *M. smegmatis* carrying pVV16::*ompATb* produced an intense band comigrating with recombinant OmpATb purified from *E. coli* (17), which was not observed in the strains harboring the empty pVV16. The identity of these proteins was further confirmed by Western blot analysis using the HisProbe-HRP, along with a chemiluminescent substrate, to reveal the presence of His-tagged proteins (Fig. 1B). A band of higher molecular weight was also present in all lysates, presumably corresponding to the GroEL1 protein, which has been shown to possess a natural His tag at its C terminus (22). Both *M. bovis* BCG and *M. smegmatis* carrying pVV16::*ompATb*, but not the control strains, showed the presence of two similar-sized forms of His-tagged OmpATb. The identity of these double bands was established by their positive immunoreactivity to rabbit anti-

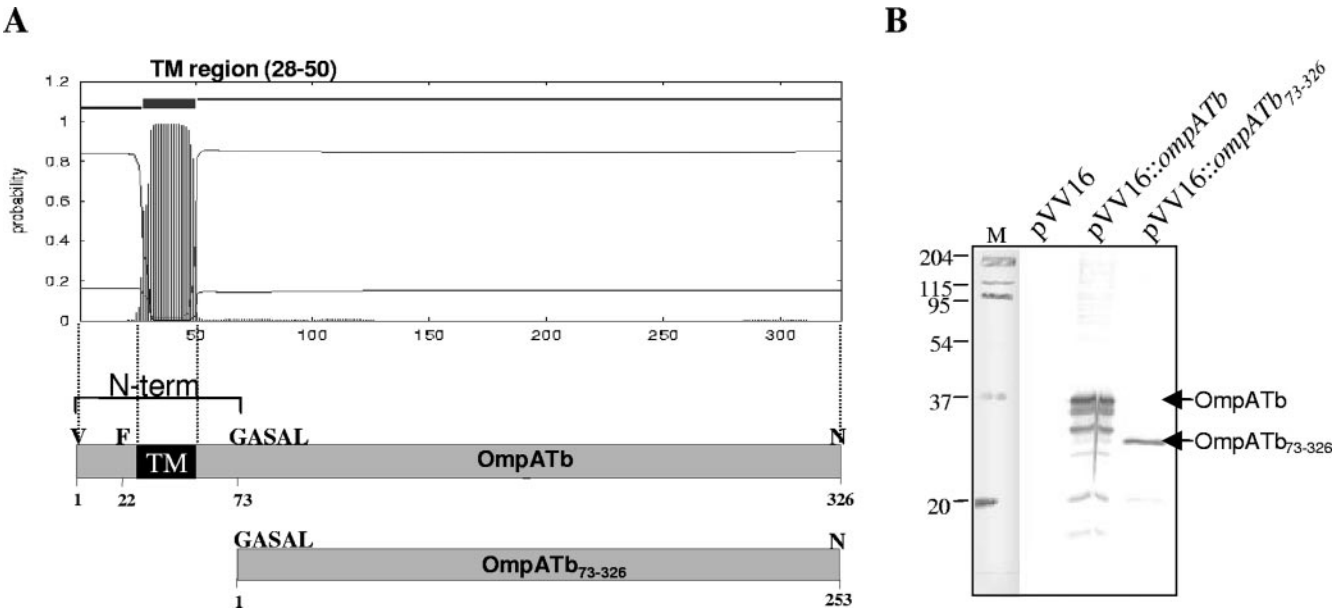

FIG. 2. Overexpression of $OmpATb_{73-326}$ in *M. smegmatis*. (A) Graphical representation of OmpATb as predicted by TMHMM 2.0 software (http://www.cbs.dtu.dk/services/TMHMM-2.0/). One transmembrane helix (TM) was predicted to be present in the N terminus of the protein (residues 28 to 50) (upper panel). The N terminus of OmpATb, lacking in $OmpATb_{73-326}$, comprises the first 72 amino acids and includes the TM region (lower panel). (B) Western blot analysis of crude lysates of recombinant *M. smegmatis* strains overexpressing either OmpATb or $OmpATb_{73-326}$. Equal amounts (20 μg) of total lysates were loaded in each lane, resolved on SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-OmpATb antiserum.

OmpATb antibodies (Fig. 1B, lower panel). In addition, these antibodies also recognized OmpATb in the crude extract of control *M. bovis* BCG but not in control *M. smegmatis*, a finding that is in agreement with our previous observations (17) and the fact that the *ompATb* homologue is present in *M. bovis* BCG but absent from the *M. smegmatis* genome. Recombinant OmpATb from *E. coli* (OmpATbEC) reacted strongly with the anti-OmpATb polyclonals but not with the HisProbe-HRP since the His_6 tag has been removed by cleavage with thrombin (17).

Overexpression of $OmpATb_{73-326}$ in *M. bovis* BCG and *M. smegmatis*. It was previously shown that trypsin digestion of purified OmpATb from *E. coli* revealed a 26-kDa peptide with pore-forming activity which, following Edman degradation, was found to start with the GASAL sequence, indicating that trypsin cut OmpATb between Arg72 and Gly73 (17). It is expected that upon translocation of the protein across the inner membrane of mycobacteria, signal peptides of the proteins are cleaved off by signal peptidases (2). Although OmpATb is a cell wall-associated protein, our previous results suggest that the N terminus of OmpATb is not removed (17). It is therefore not clear to what extent the channel properties of recombinant OmpATb are influenced by the presence of the N-terminal peptide, although it was previously demonstrated that the purified $OmpATb_{73-326}$ from recombinant *E. coli* lacking the putative N-terminal peptide did not show channel activity (17, 29). We therefore investigated the role of the N-terminal sequence of the protein with respect to cell wall targeting and its eventual participation in the pore-forming activity of the protein. Analysis of the OmpATb amino acid sequence clearly revealed the presence of a transmembrane region (residues 28 to 50, Fig. 2A), although it does not contain a consensus signal peptide (25). To examine the role of this N-terminal sequence, the region corresponding to the first 72 amino acids comprising the putative transmembrane domain was removed, and the truncated protein, designated $OmpATb_{73-326}$, starting with the GASAL sequence was expressed in *M. smegmatis* (Fig. 2A). *M. smegmatis* was transformed with pVV16::$ompATb_{73-326}$, and the expression of $OmpATb_{73-326}$ in the crude lysates was analyzed by immunoblotting using the anti-OmpATb antibodies. Figure 2B clearly shows the presence of the truncated protein. No band comigrating with $OmpATb_{73-326}$ was found in the lysate of *M. smegmatis* overproducing the full-length porin, indicating that the first 72 amino acids are not removed in mycobacteria. Similar results were obtained when we analyzed the crude lysate of *M. bovis* BCG harboring pVV16::$ompATb_{73-326}$ (data not shown).

Subcellular localization and purification of OmpATb and $OmpATb_{73-326}$ in recombinant *M. bovis* BCG and *M. smegmatis* strains. Subcellular fractions of *M. smegmatis* overproducing either OmpATb or $OmpATb_{73-326}$ were probed for the presence of these proteins. As expected for a porin, OmpATb was found to be present in the cell wall fraction (Fig. 3, upper panel). The cytosolic preparation also showed the presence of this overexpressed protein and contained additional bands, presumably corresponding to degradation products. Further, the lack of contamination from cytoplasmic components in the cell wall fraction was ascertained by probing the fractions with monoclonal antibodies raised against the cytosolic catalase-peroxidase KatG of *M. tuberculosis* (27). KatG was only detected in the cytosolic fractions and not in the cell wall fractions, thus validating the purity of the cell wall preparations (Fig. 3, lower panel). In contrast to OmpATb, $OmpATb_{73-326}$ was exclusively found in the cytosol but not in the cell wall.

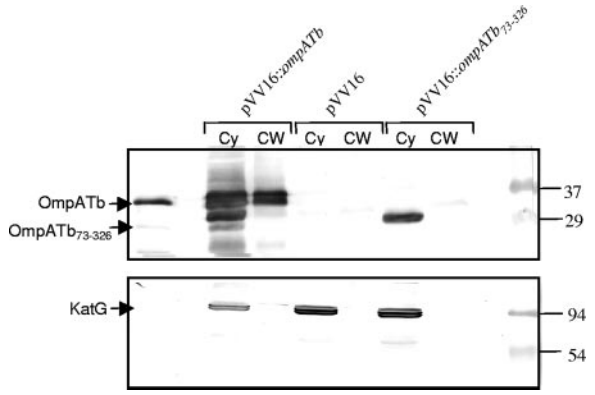

FIG. 3. Subcellular localization of OmpATb and OmpATb$_{73-326}$ in *M. smegmatis*. Recombinant *M. smegmatis* strains overproducing either OmpATb or OmpATb$_{73-326}$ were lysed and fractionated to separate the cytoplasm (Cy) from the cell wall (CW). The fractions were resuspended into the same volume of buffer and 20 μl of each fraction were subjected to SDS-PAGE, electroblotted onto a nitrocellulose membrane, and probed with anti-OmpATb antiserum (upper panel) or monoclonal anti-KatG (lower panel) antibodies.

Similar results were obtained with the corresponding *M. bovis* BCG recombinant strains (data not shown), except that additional signals from the endogenous OmpATb were also detected in the cell wall fraction. These results suggest that the N-terminal domain of OmpATb is required for cell wall targeting.

OmpATb was next purified to homogeneity from cell wall extract in the presence of OPOE and isolated by affinity chromatography on Ni-containing beads. Figure 4A shows the purity of the *M. smegmatis* and *M. bovis* BCG cell wall-purified OmpATb proteins. Because OmpATb$_{73-326}$ was exclusively found in the supernatant, it was therefore purified from the soluble fraction.

The two bands also observed in SDS-PAGE analyses of OmpATbs (Fig. 4A, lanes 5 and 6) were separately analyzed by Edman degradation to determine the N-terminal residues. Both the upper bands corresponded to the expected peptide sequence, ASKAGLGQ, starting with the second amino acid, while the N-terminal peptide of the lower bands was FYRGSP, starting at position 22 (Fig. 2A). This short peptide does not resemble a typical signal peptide and, moreover, it precedes the transmembrane domain. It is possible that the loss of the 21 first residues of OmpATb is due to the action of proteolytic enzymes on the overexpressed proteins. It is very unlikely that removal of the first 21 residues occurs during the process of purification since this doublet is also found in the native, nonrecombinant OmpATb from crude lysates of wild-type *M. bovis* BCG (17). As expected, purified OmpATb$_{73-326}$ displayed a unique band in agreement with Edman sequencing (Fig. 4A, lanes 1 and 2).

In the purified OmpATb preparations, faint bands of ~70 kDa were observed on the SDS-PAGE gel (Fig. 4B, lane 1). Significantly, these bands reacted positively with the anti-OmpATb antiserum (Fig. 4B, lane 2). These results suggest that purified OmpATb appears to exist in an oligomeric state.

The N-terminal domain of OmpATb allows translocation of PhoA across the cell membrane. *E. coli* PhoA has been extensively studied and used as a reporter for the identification of secreted proteins (1, 15) because it is an exported enzyme that is enzymatically active only after its translocation into the bacterial periplasmic space. If an appropriate signal sequence is fused to the truncated *E. coli phoA′*, lacking its signal peptide sequence, the resulting fusion protein will be exported and can be assayed enzymatically. Such *phoA′* gene fusions have been used to study exported proteins in *M. smegmatis* (33). We have used this strategy to investigate whether the N-terminal region of OmpATb, which appeared to be necessary for localizing the OmpATb to the extracytoplasmic compartment, would be sufficient to permit PhoA activity. A hybrid protein, termed OmphoA, containing the N-terminal 74 amino acids of OmpATb fused to PhoA′, which is missing its own signal peptide, was generated (Fig. 5A, upper panel). When pVV16::*omphoA* was introduced in *M. smegmatis*, expression of the fusion protein yielded blue colonies, while the colonies of cells carrying pVV16: *phoA′* were colorless (Fig. 5A, lower panel), suggesting that the PhoA′ was only active when fused to the N-terminal domain of OmpATb. Further, the alkaline phosphatase activity assayed in whole cells of *M. smegmatis* clones carrying the OmphoA fusion was found to be about 50 times higher than that of the clones expressing the truncated PhoA′ or those carrying the empty vector (Fig. 5B). Taken together, these data indicate that the N-terminal part of OmpATb is necessary and sufficient for membrane translocation.

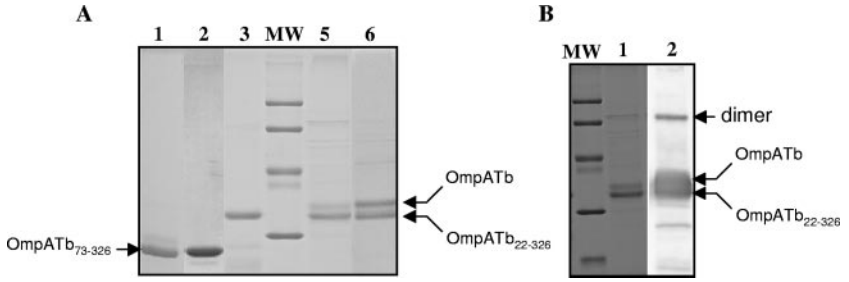

FIG. 4. Purification of OmpATb and OmpATb$_{73-326}$ produced in mycobacteria and *E. coli*. (A) Coomassie blue-stained gel showing OmpATb$_{73-326}$BCG (lane 1), OmpATb$_{73-326}$SMEG (lane 2), OmpATbEC (lane 3), OmpATbBCG (lane 5), and OmpATbSMEG (lane 6). Proteins (15 μg) were separated by SDS–12% PAGE. The molecular mass standards used were, from top to bottom, 97, 66, 45, and 30 kDa. (B) Immunoreactivity of the purified proteins. Lane 1, Coomassie blue-stained gel of OmpATbBCG; lane 2, immunoblot of OmpATbBCG probed with anti-OmpATb antibodies. The molecular mass standards used were from top to bottom: 97, 66, 45, 30, 20.1 kDa.

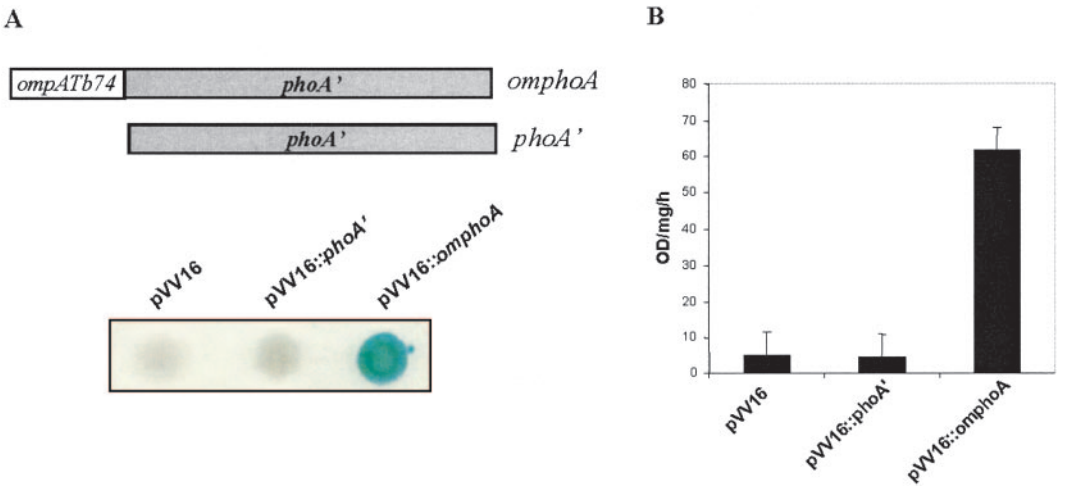

FIG. 5. Expression and translocation of PhoA across the cell membrane in *M. smegmatis*. (A, top) Representation of the *omphoA* fusion construct. The *phoA* gene encoding the *E. coli* alkaline phosphatase has been fused to the DNA sequence corresponding to the first 74 amino acids of OmpATb and designated *omphoA*. *phoA'* corresponds to the *phoA* gene missing its signal sequence. (A, bottom) PhoA activity in *M. smegmatis* harboring pVV16::*omphoA*. Liquid cultures of *M. smegmatis* harboring either pVV16, pVV16::*phoA'*, or pVV16::*omphoA* were spotted onto indicator LB plates containing 40 μg of BCIP/ml, generating a blue-colored bacteria when expressing an active PhoA phosphatase. (B) Alkaline phosphatase activity assayed on whole *M. smegmatis* cells. PhoA activities were measured on intact cells and are expressed as OD_{410}/ml of culture/min. Error bars represent the standard deviation from triplicate analyses.

Pore-forming properties of OmpATb and OmpATb$_{73-326}$. First, the pore-forming activity of OmpATb overproduced in *M. bovis* BCG was assessed and compared to OmpATb produced in *E. coli* (termed OmpATbBCG, and OmpATbEC, respectively), using macroscopic and single-channel conductance experiments. In order to exclude the possibility that the activity could be due to contamination from endogenous OmpATb, all experiments were also carried out with OmpATb produced in *M. smegmatis* (termed OmpATbSMEG), since this strain does not contain the *ompATb* gene.

Multichannel conductances. Azolectin membranes were continuously subjected to a slow voltage ramp between −200 and +200 mV. After the addition of OmpATbSMEG to the *cis* side of the measurement cell, the current remained ohmic in the negative quadrant, whereas a typical voltage closure (Vc) curve was observed in the positive quadrant (Fig. 6). This result unambiguously shows the asymmetric insertion of OmpATb into the membrane. A Vc value of 102 ± 5 mV was determined at pH 5. Similar asymmetric behavior was also observed at pH 7 (data not shown), and the Vc value was about 146 ± 5 mV. These values are close to those found previously for OmpATbEC at these same pH values (17). Interestingly, the Vc curves of OmpATbSMEG (and OmpATbBCG) are in the positive quadrant, whereas with OmpATbEC the Vc curves are in the negative quadrant. This result indicates that the insertion of OmpATbSMEG or OmpATbBCG in the lipid bilayer occurred via the opposite terminus. Moreover, OmpATbEC displayed also an asymmetrical insertion when the His tag in N-terminal position was not removed. In this case, the Vc occurred in the negative quadrant. More importantly, the truncated OmpATb$_{73-326}$ did not show any pore-forming activity as previously observed with the cytosolic OmpATb$_{73-326}$ from *E. coli* (17).

Single-channel conductances. Single-channel experiments were carried out with purified OmpATbSMEG or OmpATb BCG inserted into planar azolectin bilayers. When 5 ng of OmpATbSMEG was added in the *cis* side of the compartment, channels progressively closed when a positive voltage was applied above the Vc value. Figure 7A shows that at pH 7 and with an applied voltage of +166 mV (higher than the Vc value of 146 mV at this pH), the channels close progressively. If the applied voltage was around the Vc value, openings and closings of channels were observed as displayed by OmpATbBCG at pH 6 and at +100 mV (Vc value of 102 mV) (Fig. 7B).

In numerous experiments performed with independent OmpATb preparations, an average conductance value of 1,600 ± 100 pS was measured for both OmpATbBCG and OmpATb SMEG. Only in a few rare cases was a conductance value of 800 pS (which was the main value obtained with OmpATbEC) (17)

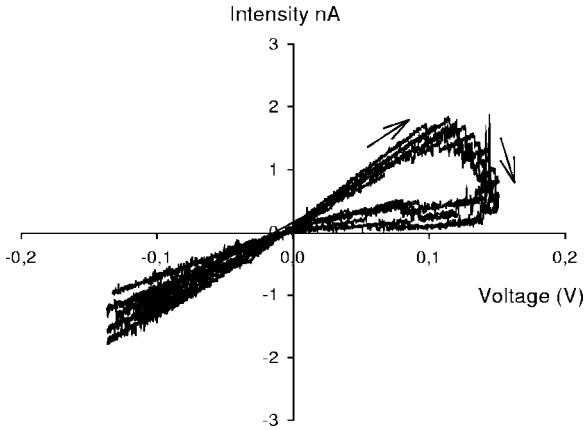

FIG. 6. Macroscopic current-voltage (I/V) curves of OmpATb SMEG in azolectin membranes. I/V curves between −200 and +200 mV at a ramp sweep of 10 mV/s in 1 M KCl and 10 mM HEPES (pH 5) at room temperature. A total of 30 ng of protein was added in the *cis*-side compartment.

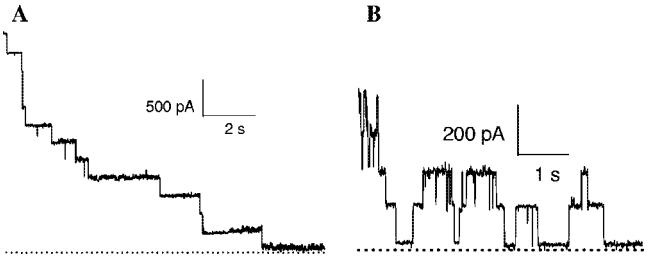

FIG. 7. Single-channel traces for OmpATbs in azolectin bilayers. (A) OmpATbSMEG at +166 mV and pH 7. (B) OmpATbBCG at +100 mV and pH 6. The electrolyte solution was composed of 1 M KCl and 10 mM HEPES. A total of 5 ng of protein was added in the *cis*-side compartment. The dotted lines represent zero current.

observed. It is also noteworthy that, because similar activities were obtained with OmpATbBCG and OmpATbSMEG, the pore-forming activity described in the present study cannot be due to the presence of contaminations, such as endogenous OmpATb. Moreover, the activity of OmpATbSMEG cannot be attributed to contaminations with MspA, the major porin from *M. smegmatis* (5, 20, 31) since MspA exhibits different channel properties with a main conductance value of 4,600 pS in 1 M KCl (20).

DISCUSSION

Mycobacteria are characterized by the presence of a very thick waxy coat that severely restricts the permeability of small hydrophilic molecules (8). The passage of such molecules is therefore strongly dependent on water-filled channels in the outer membrane. The occurrence of outer membrane proteins that are able to form pores, thereby permitting diffusion of these molecules through the mycobacterial cell wall, has been demonstrated (19). Among these, OmpATb, a porin from *M. tuberculosis*, has been shown to be essential for survival in macrophages and virulence in mice. OmpATb appears to be the major porin at low pH, allowing pathogenic mycobacteria to adapt and survive under mildly acidic conditions, such as those found within the phagosome (17, 26). Our previous studies on the structure-function relationship of OmpATb demonstrated that its pore-forming activity is dependent on translocation of the protein across the membrane. Further, while the N-terminal domain is required for this translocation, it could be substituted by the signal peptide of *E. coli* OmpA. However, all of these studies were performed on recombinant porins that were produced in *E. coli*. It remained to be addressed whether the channel properties of these proteins are comparable to porins produced in mycobacteria, especially due to the considerable structural differences between the outer membrane compositions of *E. coli* and the mycobacteria. However, the intracellular concentration of the native OmpATb in *M. tuberculosis* and/or *M. bovis* BCG is prohibitively low to enable its characterization (9, 11, 29). Efforts at purifying OmpATb from electro-elution experiments of different areas from SDS-PAGE have met with limited success.

In the present study we characterize for the first time a mycobacterial porin isolated after overexpression in mycobacterial species. In their native environment, the proteins would be expected to express and translocate correctly. Our method allowed us to obtain about 0.8 mg of OmpATb per liter of culture of *M. bovis* BCG or *M. smegmatis*. The isolated porins were reconstituted in planar azolectin bilayers and tested for their pore-forming properties. In both cases, when a voltage ramp was applied to the artificial membrane, asymmetrical I/V curves were observed with a typical Vc value. The values of the Vc were pH dependent, in a manner similar to that observed previously with OmpATbEC (17). Interestingly, the closings of channels occurred in the positive quadrant. The presence of six positively charged His residues at the C terminus could impede the insertion of the C terminus in the membrane and thus favor the insertion of OmpATb by the N terminus. In contrast, in the case of OmpATbEC, when the His_6 tag was either attached to the N terminus or removed, the Vc occurred in the negative quadrant, suggesting an insertion via the C terminus. Together, these results suggest that the insertion of OmpATbBCG or OmpATbSMEG occurred via their N terminus in planar lipid bilayers. Another explanation could pertain to the presence of lipids associated with OmpATbs as has been shown for MspA (14). The difference in behavior between the recombinant OmpATbs could be due to the differences in the lipidic composition of the mycobacterial cell wall and the *E. coli* outer membrane.

The closings of channel under an applied positive voltage were confirmed by single-channel experiments. Similar conductance values of about 1,600 pS in 1 M KCl were obtained for both OmpATbBCG and OmpATbSMEG. In some rare occasions, a conductance value of 800 pS, close to the one observed with OmpATbEC was found (17). The twofold-increased conductance value of OmpATbs produced in mycobacteria with respect to OmpATbEC suggests the presence of dimers. This is supported by the presence of higher-molecular-weight bands on SDS-PAGE in preparations of purified OmpATbBCG and OmpATbSMEG that were immunopositive with the anti-OmpATb antiserum. The formation of these dimers could be due to the presence of strongly associated lipids with the protein. Indeed, this type of lipid association observed after detergent extraction had been described for MspA (5). An oligomerization process was already observed with MspA from *M. smegmatis*, which displayed different oligomeric bands on SDS-PAGE and presented two states of conductances of 2,300 and 4,600 pS in 1 M KCl (20). With a molecular size of 38 kDa, OmpATb appears to be too small to be able to form a pore as a monomer in the extensive outer membrane of *M. tuberculosis*. X-ray analysis of *M. smegmatis* MspA has revealed that the pore is nearly three times longer than the ones typically found in gram-negative porins, which is in agreement with the considerably greater thickness of the mycobacterial cell wall. In addition, these studies have demonstrated that MspA is present as a homo-octameric goblet-like conformation with a single central channel (4). Taken together, our results suggest that OmpATb is present in an active oligomeric form in mycobacteria. Determination of the three-dimensional structure of OmpATb, which is currently under investigation, is likely to reveal the degree of multimerization of this protein.

In parallel, $OmpATb_{73-326}$ lacking the N-terminal 72 amino acid residues was also produced in *M. bovis* BCG and *M. smegmatis.* This truncated protein was exclusively localized in the cytosol. After extraction, purification, and reconstitution in lipid bilayers, it failed to show any pore-forming activity. The

essential role of the N-terminal domain in the translocation process of OmpATb was further highlighted by the detection of alkaline phosphatase activity in translational fusions between the first 74 amino acids of OmpATb and the signal-peptide-less *E. coli* PhoA. It is noteworthy that this N-terminal sequence appears to be different from a typical signal peptide, which would be characterized by positively charged residues, followed by a long hydrophobic region and a domain containing the cleavage site for the action of a signal peptidase (25). Moreover, unlike most other signal peptides, the OmpATb N-terminal region is not cleaved off after or during membrane translocation. This is in contrast to MspA, whose 27 N-terminal amino acids exhibit the characteristics of a standard signal peptide that is cleaved off to generate a mature porin (20). It is very likely that, in the case of OmpATb, proper folding and/or the multimerization necessary for full activity occurs only when the intact protein is present in the mycobacterial cell envelope.

In conclusion, we have supported and extended our earlier studies on the OmpATb by observing the protein when expressed in its native environment and show that the N-terminal domain is necessary and sufficient for membrane translocation. An interesting application would consist of fusing the OmpATb N-terminal sequence to proteins of interest in order to express them in the mycobacterial cell wall and trigger specific immune responses. Recombinant *M. bovis* BCG strains expressing cell wall-associated antigens would be of particular interest for future vaccine development.

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