Outer membrane pore protein prediction in mycobacteria using genomic comparison

Nancy Mah,¹ Carolina Perez-Iratxeta² and Miguel A. Andrade-Navarro¹

Correspondence

Nancy Mah
nancy.mah@mdc-berlin.de

¹Max Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany

²Ottawa Hospital Research Institute, 501 Smyth Road, Ottawa, ON K1H 8L6, Canada

Proteins responsible for outer membrane transport across the unique membrane structure of *Mycobacterium* spp. are attractive drug targets in the treatment of human diseases caused by the mycobacterial pathogens, *Mycobacterium tuberculosis*, *M. bovis*, *M. leprae* and *M. ulcerans*. In contrast with *Escherichia coli*, relatively few outer-membrane proteins (OMPs) have been identified in *Mycobacterium* spp., largely due to the difficulties in isolating mycobacterial membrane proteins and our incomplete understanding of secretion mechanisms and cell wall structure in these organisms. To further expand our knowledge of these elusive proteins in mycobacteria, we have improved upon our previous method of OMP prediction in mycobacteria by taking advantage of genomic data from seven mycobacteria species. Our improved algorithm suggests 4333 sequences as putative OMPs in seven species with varying degrees of confidence. The most virulent pathogenic mycobacterial species are slightly enriched in these selected sequences. We present examples of predicted OMPs involved in horizontal transfer and paralogy expansion. Analysis of local secondary structure content allowed identification of small domains predicted to perform as OMPs; some examples show their involvement in events of tandem duplication and domain rearrangements. We discuss the taxonomic distribution of these discovered families and architectures, often specific to mycobacteria or the wider taxonomic class of *Actinobacteria*. Our results suggest that OMP functionality in mycobacteria is richer than expected and provide a resource to guide future research of these understudied proteins.

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INTRODUCTION

Mycobacteria are responsible for some of the most terrible human diseases including leprosy and tuberculosis (Cosma *et al.*, 2003). However, not all mycobacteria are pathogenic to humans despite their considerable genomic similarity. Part of their variable properties in infective ability and specificity are likely to be related to their variable cell wall (Brennan & Nikaido, 1995). Outer-membrane proteins (OMPs) are an important component of the mycobacterial cell wall, yet only a few of these have been studied in mycobacteria so far (Niederweis *et al.*, 2010). OMPs are

transmembrane proteins that form a beta-barrel structure consisting of amphipathic beta strands and are secreted into the periplasmic space and inserted into the outer membrane to act as channels (Faller *et al.*, 2004). This type of protein is therefore an important target for antibacterial therapy and an object of study for the elucidation of the mechanisms of pathogenicity (Niederweis, 2008). However, currently there is evidence of just some mycobacterial OMPs; there is a large amount of evidence for MspA (Stahl *et al.*, 2001) and less for another two *Mycobacterium tuberculosis* proteins: Rv1973 (Song *et al.*, 2008) and Rv1698 (Siroy *et al.*, 2008; Song *et al.*, 2008). This is due to the difficulties not only of culturing mycobacteria but also of identifying these proteins.

Computational methods of OMP detection have been developed and applied to *M. tuberculosis* with relative success (Pajon *et al.*, 2006; Song *et al.*, 2008), but there is room for improvement, especially when it comes to prioritizing targets for research. The increasing number of related mycobacterial genomes offers a unique opportunity to support the predictions by addressing their coherence across orthologues and to pinpoint OMP families specific to pathogenic mycobacteria.

Abbreviations: firsthel, position in amino acids of the most N-terminal predicted transmembrane helix; FracB5, amphiphilicity of B5 strands; GSP, general secretory pathway; MCE, mammalian cell entry; nnscore, prediction of leaderless score from SecretomeP; numB5, number of B5 strands; numB5res, total number of residues in B5 strands; numcys, number of cysteine residues; numpredhel, number of predicted transmembrane helices; OMP, outer-membrane protein; perbeta, overall percentage of residues in beta strands; PDB, Protein Data Bank; pI, isoelectric point; Smean, general secretion score from SignalP; TM, transmembrane helix.

Six supplementary figures and two supplementary tables are available with the online version of this paper.

In this work we accomplished parameter optimization of a previous method that predicts OMPs based solely on their potential to be secreted and to form an amphiphilic beta-barrel (Song *et al.*, 2008). We explored the predictive power of a set of OMP-related properties by contrasting the robustness of the results on the complete proteomes for seven mycobacteria: three obligate pathogens, *M. tuberculosis*, *M. bovis* and *M. leprae*, two facultative pathogens *M. marinum* and *M. ulcerans*, one opportunistic pathogen *M. avium*, and the non-pathogenic *M. smegmatis*.

The relatedness between these species and their pathogenic properties are heterogeneous. The closest genomes by far are those of *M. tuberculosis* and *M. bovis*, but their host ranges are different (*M. bovis* can cause tuberculosis in several mammals, whereas the natural hosts of *M. tuberculosis* are humans). Therefore, both genomes were included in the analysis since we considered that a comparison of OMPs between these two species could lead to insights that help to explain the differences in ability to infect different hosts.

The predictions on these seven genomes directed us to a number of OMP domains present in mycobacteria, most of them exclusive to actinobacteria and without homologues in eukaryotes.

METHODS

Calculation of parameters for outer-membrane protein prediction in mycobacteria. Protein sequences were obtained for seven mycobacterial genomes (Table 1) [ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/], including *M. avium* 104, *M. bovis* AF2122/97, *M. leprae* TN, *M. marinum* M, *M. smegmatis* str. MC2 155, *M. tuberculosis* H37Rv and *M. ulcerans* Agy99. All proteins were predicted to be OMPs based on the two main properties: 1) the ability to be secreted to the outer membrane and 2) the ability to form beta-barrel structures.

Secreted proteins were predicted according to both classical secretion mechanisms (SignalP-v3.0) and non-classical secretion such as twin arginine translocation (TatP-v1.0) or leaderless secretion (SecretomeP-v1.0) (Bendtsen *et al.*, 2004a, b, 2005). Prediction of classically secreted proteins is well-studied and most secreted bacterial proteins are exported in this manner (Malen *et al.*, 2007); therefore predictions determined by SignalP-v3.0 algorithm or the presence of a single predicted transmembrane alpha helix in the first 70 aa of a protein (TMHMM; Krogh *et al.*, 2001) were considered to be superior to other prediction methods. TatP prediction is specific to bacteria and was considered to be next most reliable. SecretomeP prediction was the least specific of all methods, but was still considered to be useful as we were aiming for high recall.

To demonstrate the efficacy of the secretion prediction methods, the algorithms were run on positive and negative validation sets. A set of 53 experimentally verified, classically secreted mycobacterial proteins was obtained from Leversen and co-workers (Leversen *et al.*, 2009). A set of non-classically secreted mycobacterial proteins, including proteins secreted by Tat or SecA2 systems, was assembled by performing a literature search. Negative controls for secretion were represented by 1725 reviewed cytoplasmic proteins from *Mycobacterium* sp. (UniProtKB release 15.3).

In the second step of OMP prediction, the propensity of the proteins to form beta-barrel structures was determined using various beta-strand properties. Secondary structure for all proteins was predicted using Jnet v1.0 (Cuff & Barton, 1999, 2000). Beta-strands of five or more residues (B5 strands) were evaluated for amphiphilicity (FracB5) as previously described (Song *et al.*, 2008). As additional measures of 'betaness', the overall proportion of residues in beta strands (perbeta), the number of B5 strands (numB5) and the total number of residues in B5 strands (numB5res) were recorded. Positive and negative control sets for beta-barrels were used to demonstrate the utility of these parameters to predict beta-barrel structure. Positive validation of beta-barrel structure was represented by 428 bacterial and eukaryotic sequences taken from proteins containing Pfam motifs or bacterial sequences with solved 3D structures annotated as forming a beta-barrel. Negative validation of beta sheet prediction consisted of 90 actinobacterial proteins with solved 3D structure of low beta content. Protein structures were obtained from the Protein Data Bank (PDB; http://www.pdb.org).

Table 1. Predictions for seven genomes

Genome (NCBI Taxon ID)	Habitat	Annotated proteins	Suggested OMP proteins with score ≥ 12 (% of total)	Suggested OMPs unique to a genome*
<i>M. tuberculosis</i> H37Rv (83332)	Obligate pathogen	3991	629 (15.8)	35
<i>M. bovis</i> AF2122/97 (233413)	Obligate pathogen	3920	617 (15.7)	39
<i>M. leprae</i> TN (272631)	Obligate pathogen	1605	242 (15.1)	77
<i>M. marinum</i> M (216594)	Environmental, facultative pathogen	5462	799 (14.6)	184
<i>M. ulcerans</i> Agy99 (362242)	Environmental, facultative pathogen	4160	561 (13.5)	111
<i>M. smegmatis</i> str. MC2 155 (246196)	Environmental, not pathogenic	6716	844 (12.6)	459
<i>M. avium</i> 104 (243243)	Environmental, facultative opportunistic pathogen	5120	641 (12.5)	251
Total		30974	4333 (14)	

*Numbers of predicted OMPs within clusters in other genomic patterns: present in all genomes, 189; present only in obligate pathogens (*M. tuberculosis*, *M. bovis*, *M. leprae*), 48; present only in facultative pathogens (*M. marinum*, *M. ulcerans*, *M. avium*), 66; all other combinations, 2874.

Additional parameters, including the number of cysteine residues (numcys) and the isoelectric point (pI) (BioPerl pI Calculator; <http://www.bioperl.org>) were also evaluated.

Initial OMP prediction (termed Method 1) was carried out with similar parameters and thresholds to those previously used by Song *et al.* (2008), namely: FracB5 ≥ 0.19 , perbeta ≥ 0.10 , general secretion score (Smean; from the signal peptide predictor SignalP) ≥ 0.50 or number of predicted transmembrane helices (numpredhel)=1 and position in amino acids of the most N-terminal predicted transmembrane helix (firsthel) ≤ 70 (see Supplementary Table S1, available with the online version of this paper). Here, the OMP prediction method was further refined by using sequence homology information and optimization of the algorithm as described below.

Clustering of mycobacterial sequences. Clustering of the seven mycobacterium genomes listed in Table 1 was carried out using a very strict sequence similarity criterion that enforces all proteins in a cluster to be homologous along their full lengths, ensuring their domain content and architectures are equivalent (Perez-Iratxeta *et al.*, 2007). About 1 % of the sequences were not used because they were too short (<50 aa). The remaining sequences (30 605) were distributed into 11 633 clusters. Pfam motif information (Finn *et al.*, 2008) of representative members of the clusters was retrieved to further characterize them.

Optimization of OMP prediction. To optimize the OMP prediction, two training sets of clusters (with at least five sequences each) were defined: 1) OMP-rich clusters which contained ≥ 80 % OMP-predicted sequences (1151 sequences in 168 clusters) and 2) OMP-poor clusters, which contained ≤ 20 % OMP-predicted sequences (981 sequences in 96 clusters). First, optimization was carried out by applying varying thresholds for OMP prediction on parameters that were not included in Method 1, namely pI, sequence length, numcys, numB5, numB5res, Tat-secretion score (dvalue) and leaderless secretion score (nnscore). Next, the Method 1 thresholds on parameters including FracB5, perbeta, and Smean were optimized. The optimal cut-off values were defined to be those that most reduced the fraction of predicted OMPs in OMP-poor clusters while retaining over 90 % of the predicted OMPs in OMP-rich clusters. The new set of optimized criteria was called Method 2 and was used for the remainder of the analysis.

A scoring system (producing a score ranging from 0 to 16) was used to indicate the confidence of the OMP prediction. Propensity for secretion and beta-barrel formation were given equal weight (maximum of eight points each). Proteins predicted to be secreted by general secretion or Tat mechanisms were awarded eight points. In the absence of these two predictions, proteins were awarded three points if the SecretomeP score > 0.71 . Beta-sheet-related parameters were assessed on: 1) the entire protein length and 2) within a 300 aa sliding window of protein sequence to detect local regions of high beta content. A window size of 300 aa was chosen because this is the size of the beta barrel domain in some known OMP structures (Faller *et al.*, 2004; Song *et al.*, 1996); however, such domains can be formed by association of beta strands from multiple protein monomers, and it can happen that an OMP protein or region is much smaller than 300 aa. One point was awarded for each of the following criteria satisfied: frac ≥ 0.28 , perbeta ≥ 0.11 , numB5 ≥ 3 , numB5res ≥ 17 , to a maximum of eight points.

RESULTS

Optimization of OMP prediction

To optimize our predictions in seven mycobacterial genomes, we clustered their protein sequences and

evaluated the fraction of proteins in each cluster predicted to be an OMP. Contrasting the parameters of OMP-rich and OMP-poor clusters enabled us to further refine the OMP criteria, based on the assumption that all proteins in a cluster should be predicted either as being OMPs or not; and, therefore, that OMP-poor clusters indirectly reflected false positives.

In the first instance, we tested nine parameters that could play a minor role in the initial prediction of OMPs but were not previously used. The fraction of OMP predicted sequences in OMP-rich and OMP-poor groups was monitored when applying increasingly restrictive thresholds in each of these nine parameters (see Supplementary Fig. S1, available with the online version of this paper). Four parameters (pI, numcys, firsthel and dvalue score from TatP) showed little improvement in reducing the number of potential false negatives in the OMP-poor group of clusters. Potential improvements could be made for the remaining five parameters: sequence length, numB5res, numB5, numpredhel, and prediction of leaderless secretion score from SecretomeP (nnscore).

Sequence length was rejected as a constraining factor, as it was undesirable to eliminate short (~100 aa) predicted OMPs potentially composing homologous multimeric structures. Requiring numB5 and numB5res to be a minimum of 3 and 17, respectively, was successful in reducing the number of OMPs in the OMP-poor clusters by > 10 %, while keeping 96 % of the OMPs in the OMP-rich clusters (Supplementary Fig. S1). A rather stringent threshold was used for prediction of leaderless secretion. At nnscore ≥ 0.71 , 56 % of sequences from the OMP-rich clusters were retained, while 74 % of sequences from the OMP-poor clusters were rejected as targets for leaderless secretion. This was not considered to be overly stringent, since proteins with signal sequences, (which would be identified by SignalP-3.0 or TatP-1.0) were likely to have a high nnscore anyway (Bendtsen *et al.*, 2004a).

In the second instance, the five parameters initially used to predict OMPs were varied and the fraction of predicted OMPs was recorded. This analysis suggested fine adjustments in the cut-off values for amphiphilicity (FracB5), proportion of residues in beta strands (perbeta), and general secretion score (Smean) (see Supplementary Fig. S2 available with the online version of this paper).

The optimized criteria were applied to the dataset, expanding the number of predicted OMPs, compared with the original method (Table 1 and Supplementary Table S1). Up to this point, the optimization had been carried out by varying parameters on an individual basis. A scoring system was implemented to summarize the effect of all the optimized parameters (with eight points for beta-barrel formation and eight points for secretion, see Methods). Assuming that most of the sequences in OMP-rich clusters should actually be OMPs, and that sequences in OMP-poor clusters should not be OMPs, a threshold of OMP score=12 to accept an OMP prediction was found to be

optimal (Fig. 1; see Supplementary Fig. S3a, available with the online version of this paper). At this threshold, 94 % of sequences from OMP-rich clusters are classified as OMPs, while 89 % of the sequences in the OMP-poor clusters are rejected as OMPs.

Validation of signal sequence and beta-barrel prediction in mycobacteria

None of the secretion prediction programs were specifically designed to predict signal sequences in *Mycobacterium* spp., although the SignalP neural net predictions were based on Gram-positive bacteria, and the TatP server was trained on both Gram-negative and Gram-positive sequences. Mycobacteria are classified as Gram-positive, despite the fact that the mycobacterial outer membrane has distinct properties not found in either functionally classified Gram-negative or Gram-positive bacteria (Hett & Rubin, 2008). Therefore, it was important to test these algorithms for their ability to detect signal sequences in mycobacteria.

Using known cytoplasmic and known mycobacterial proteins secreted by the general secretory pathway (GSP), it could be shown that the optimized cut-off ($S_{\text{mean}} = 0.54$) was sufficient to correctly predict secretion in 93 % of the known GSP proteins and reject 98 % of the cytoplasmic proteins (Supplementary Fig. S3b, available with the online version of this paper). Secretion by the non-classical Tat system could be predicted at a cut-off dvalue of 0.36 in 79 % of the known Tat-secreted mycobacterial sequences,

while rejecting 98 % of the cytoplasmic proteins for secretion (Supplementary Fig. S3c, available with the online version of this paper).

Prediction of leaderless secretion in mycobacteria at the chosen cut-off $\text{nnscore} \geq 0.71$ correctly identified 50 % (6/12) of known leaderless secreted proteins (Supplementary Fig. S3d, available with the online version of this paper), which included proteins secreted by the recently described bacterial export system ESX-1 and the SecA2 (Sec-independent) system. Eighty-one percent of the known cytoplasmic proteins were predicted as being secreted in this instance, making the leaderless secretion prediction the least precise of all three secretion prediction methods. As a result, less emphasis was placed on leaderless secretion scores in the OMP prediction.

Prediction of beta-barrel structures was based on beta-sheet content and the amphiphilicity of predicted beta strands (computed as described by Song *et al.*, 2008). As a measure of the protein's propensity to form beta-barrel structures, beta-barrel scores were calculated globally (over the whole protein) and locally (sliding window) for a maximum score of eight (see Methods). For a beta-barrel score ≥ 6 , a total of 97 % of known bacterial OMP and 90 % of annotated beta-barrel proteins were correctly identified as containing beta-barrels, whereas non-beta-barrel structures were predicted in 74 % of solved sequences lacking certain beta-barrel structure (Fig. 2).

After the optimization stage, we were able to correctly identify 90 % (27/30) of known bacterial OMPs with high scores (score ≥ 14 ; see Supplementary Fig. S4 available with the online version of this paper) corresponding to strong predictions by using Method 2. Among the three OMPs missed by Method 2, there were two from *Rhodobacter* spp. Although one of them (PORI_RHOBL) contained sufficient beta structure for a beta-barrel, it was predicted to be secreted by leaderless secretion resulting in a weak OMP prediction (OMP score=11). OmpG from *E. coli* was also not identified as an OMP by this method, due to a lack of beta-strand prediction from Jnet v1.0.

The selection criteria of both the optimized Method 2 and the previous method show substantial differences between the results (Supplementary Table S1). When applying them to seven mycobacterial genomes (see Methods), 3340 proteins were predicted to be OMPs by both methods. A total of 993 proteins were newly predicted by Method 2 whereas 406 proteins predicted to be OMPs by Method 1 have now been rejected as false positives.

Identification of OMPs

Table 1 specifies the number of sequences and predicted OMPs. The seven genomes analysed have a genomic size in the 4000–5000 range except for the small *M. leprae* genome (1605 genes, an extreme case of genome downsizing; Cole *et al.*, 2001) and the larger *M. smegmatis* genome (6716 genes). When considering the proportion of predicted

Fig. 1. OMP scores for sequences in OMP-rich and OMP-poor clusters. The minimum score for OMP prediction was set to ≥ 12 (vertical dotted line). At this threshold, 94 % of sequences from OMP-rich clusters (black line) were classified as OMPs, while 85 % of the sequences in the OMP-poor clusters (red line) were rejected as OMPs.

Fig. 2. Beta-barrel prediction scores for control sequences. Positive controls for beta-barrels include 428 bacterial or eukaryotic proteins from Pfam or PDB with annotated beta-barrel structures and solved structure information. Negative controls include 90 actinobacterial sequence fragments with low beta content, as determined from solved structures in PDB. At beta-barrel score ≥ 6 , 97 and 90% of known bacterial OMPs and annotated beta-barrels, respectively, are predicted to be beta-barrels, and 74% of low-beta-sheet-content sequences are predicted to be without beta-barrel structure.

OMPs, it is interesting to note that the three obligate pathogenic organisms (*M. tuberculosis*, *M. bovis*, and *M. leprae*) have the largest (15.1–15.8%) while the opportunistic pathogen *M. avium* and the non-pathogenic species *M. smegmatis* have the lowest (12.5–12.6%) values.

We showcase the results of our method with some examples of newly predicted mycobacterial OMPs. Because the taxonomic distribution of a protein can give an indication of its functional relevance, we have categorized the examples by this property. Our selection of examples was facilitated by the clustering used for the optimization of the method, e.g. when searching for OMPs present in all seven mycobacteria. The complete results are included in Supplementary Table S2, available with the online version of this paper.

OMPs present in *M. tuberculosis* but not in *M. bovis*

Though *M. tuberculosis* and *M. bovis* are very closely related (they both belong to the *M. tuberculosis* complex) and share many proteins with 100% identity, they have obvious differences regarding pathogenicity. It is therefore interesting to find OMPs in *M. tuberculosis* that have no equivalent in *M. bovis*. Two outstanding examples are *M. tuberculosis* proteins mce3c (Rv1968; 410 aa) and mce3e

(Rv1970; 377 aa) encoded by two of six genes from the putative mce3 operon Mce3A–F. Both Mce3C and Mce3E proteins have been found to react with antibodies from serum of TB patients (Ahmad *et al.*, 2004) and have one predicted mammalian cell entry (MCE) domain each (at positions 38–114 and 36–112, respectively).

The presence of the MCE domain in these sequences is relevant because many of the genes encoding proteins with this domain have been shown to be expressed during natural infection of *M. tuberculosis* and it is thought that they are related to mycobacterial pathogenicity (Ahmad *et al.*, 1999). *M. tuberculosis* has a total of 24 genes encoding proteins with this domain arranged in four mce operons whose first two genes encode integral membrane proteins and are followed by six genes encoding proteins containing the MCE domain (Cole *et al.*, 1998). In our previous work (Song *et al.*, 2008) we predicted that 23 out of 24 of *M. tuberculosis* MCE genes were OMPs. The present method predicts all 24 MCE genes in *M. tuberculosis* H37Rv as OMPs with a score of 16 (the maximum possible). We speculate that the MCE domain is actually a beta-barrel characteristic of OMPs, which is coherent with their proposed role at the mycobacterial cell surface (Flesselles *et al.*, 1999).

OMPs present in the *M. tuberculosis* complex but not in all mycobacteria

We observed many OMP clusters with members in *M. tuberculosis* and *M. bovis* but these were missing in all or some of the five species outside the *M. tuberculosis* complex. This can be due to either genes being invented (or horizontally transferred) within the mycobacteria lineage or to selective gene loss (as in the massive pseudogenization that occurred in the *M. leprae* genome; Cole *et al.*, 2001). Here we show examples of each of these.

An OMP unique to *M. tuberculosis* and *M. bovis*. Rv1351 is a 109 aa protein of *M. tuberculosis* that we predict to be an OMP. The *M. bovis* orthologue is 100% identical and there are no homologous sequences in the other five mycobacteria analysed in this work or outside mycobacteria (no PSI-BLAST hits with an E-value below 8.3). We also predict that the gene next to it, Rv1352 (encoding a 123 aa protein), is also a small OMP. According to the STRING database (Jensen *et al.*, 2009), these two predicted OMPs are in an operon conserved between *M. tuberculosis* and *M. bovis*, which includes upstream genes Rv1348 and Rv1349 (two uncharacterized ABC transporter ATP-binding proteins) and fabG2/Rv1350 [predicted as 3-ketoacyl-(acyl-carrier-protein) reductase]. Therefore, these two predicted OMPs seem to form part of an *M. tuberculosis*–*M. bovis*-specific operon and although they are rather small, they could form a barrel by multimerization, which would explain the need to co-express them in an operon. Such an operon could carry out a function inherent to the *M. tuberculosis* complex. The three genes, Rv1348, Rv1349 and Rv1350, are essential

genes for growth of *M. tuberculosis* as determined by Sasseti *et al.* (2003).

A mycobacterial OMP with horizontal transfer. *M. tuberculosis* Rv1914c (135 aa) is predicted as an OMP with orthologues in *M. bovis*, *M. ulcerans* and *M. marinum* but without apparent equivalents in *M. leprae*, *M. avium* or *M. smegmatis*. Curiously, the only match in the database outside mycobacteria is a very clear hit (>50 % identity) on a distant bacteria, the proteobacterium *Geobacter uraniireducens* (sequence GI:148265072, 135 aa). This suggests an event of horizontal transfer of this gene between mycobacteria and geobacteria. One could speculate that the function of this OMP would not be associated with pathogenicity given its presence in both pathogenic and non-pathogenic mycobacteria (and in *G. uraniireducens*). Incidentally, Rv1914c was one of 224 genes found to be deleted in one or more clinical isolates of a H37Rv strain from San Francisco (Tsolaki *et al.*, 2004).

C4: a novel putative OMP domain that occurs as a tandem repeat. *M. tuberculosis* Rv2270 (175 aa) defines a family with orthologues in five of seven mycobacteria tested (missing in *M. smegmatis* and *M. leprae*) and corynebacteria. This implies that the gene originated in *Corynebacterineae* and that there was a selective loss of this gene within some members of the mycobacteria lineage, indicating that it is not essential for them.

Sequence analysis indicated that the family contains a C-terminal 120 aa domain (that we termed C4 for its conserved four cysteines, see Supplementary Fig. S5, available with the online version of this paper), which is present in two other protein families, one where the domain is tandemly repeated (with orthologues in all seven mycobacteria, e.g. *M. tuberculosis* Rv3835), and another where it is combined with an N-terminal Ser/Thr kinase C domain (present exclusively in a series of *Actinomycetales* species, e.g. *Stackebrandtia nassauensis* GI:229864975, 577 aa; see Supplementary Fig. S5).

The prediction of *M. tuberculosis* Rv2270 as containing a lipoprotein anchor signal may invalidate the OMP function, but the predicted C4 domain has high beta content and high amphiphilicity; its involvement in variable domain architectures suggests that it can be used as a biological module.

Present in all seven mycobacterial genomes

We found a total of 588 clusters with sequences from all seven mycobacteria, and 61 of these were predicted to be OMP families. These families are likely to represent proteins important for all mycobacteria but possibly not for pathogenicity, since they are present both in pathogenic and in non-pathogenic organisms. We present two interesting cases below.

ACT: an actinobacteria OMP domain greatly expanded in *Corynebacterineae*. Rv0431 is an *M. tuberculosis*

predicted OMP with orthologues in all seven mycobacterial species tested. Sequence analysis indicated that the family contains a C-terminal domain of about 100 aa (we name this ACT for the names given to the proteins where it is present: Alanine rich, CpsA, Tuberculin related) present in five *M. tuberculosis* sequences that define five families (see Supplementary Fig. S6, available with the online version of this paper). In three of the five families the ACT domain is preceded by a predicted domain of around 170 aa of unknown function (PFAM LytR_cpsA_psr).

The ACT domain is present in some genera outside but close to mycobacteria, chiefly *Nocardioides* and *Corynebacterium*, but not all species have the five sequences and *M. avium* has an extra copy of one of the five. These results suggest that the ACT domain was invented before the divergence of mycobacteria, corynebacteria and nocardioles. Its high level of duplication and a number of gene losses and duplications in mycobacteria suggest that it confers some kind of low-specificity functional advantage.

An OMP essential for mycobacteria growth. Rv0227c is another predicted OMP in a cluster with orthologues in all seven mycobacterial genomes investigated. The proteins in this cluster have no known function, and closer analysis by PSI-BLAST revealed that there are distant homologues in nocardia and corynebacteria. The protein itself is characterized by a signal peptide with a predicted cleavage point before the first TM helix and a 300 aa beta-domain surrounded by two TMs. Mutagenesis and comparative genomic analyses have identified Rv0227c as being a 'core' mycobacterial gene, required for optimal growth (Marmiesse *et al.*, 2004; Sasseti *et al.*, 2003).

Example OMPs identified by new criteria

Method 2 included two predictive features that have not been used before: export signals other than those reported by SignalP and a window analysis of secondary structure. These allowed the identification of many extra OMPs compared with our previous work. Here, we present two examples of OMPs detected by each of these new criteria.

An actinobacteria-specific protein with low global beta content. One of our clusters represents a family with members in five of the mycobacteria tested, four of which have OMP scores of 15 (Rv2345, MAV_2041, Mb2374, MMAR_3652; ~660 aa) and one with an OMP score of 13 (MSMEG_4484). Notably absent are sequence homologues in *M. leprae* and *M. ulcerans* (confirmed using PSI-BLAST under default parameters), but we found orthologues of this protein in a wide range of actinobacteria. These proteins contain a predicted Pfam domain of unknown function (DUF477), followed by a predicted TM domain, and a very variable glycine-rich region at the end. The percentage of beta structure of the whole sequence is well

below the threshold of 0.11 that we use for selection. However, the window analysis shows that the DUF447 domain has a high percentage of beta content and high amphiphilicity, potentially characterizing an OMP function (Fig. 3).

Similarity searches uncovered a much shorter second homologue in *M. avium* (MAV_2102), also present in *Mycobacterium intracellulare*, which keeps the N-terminal domain, the predicted TM following it, and a C-terminal domain, but lacks the middle region and the glycine-rich region (Fig. 3). We predict that both the long and the short families are OMPs.

Mycobacteria-specific OMPs secreted by the Tat system. An example found using the predicted Tat-system secretion that would not have been detected using SignalP was Rv2577 from *M. tuberculosis*. This is a predicted OMP with orthologues in *M. marinum* and *M. avium* (all of them with a maximum OMP score of 16), apparently absent from *M. ulcerans* and *M. leprae*. The C-terminal end contains a predicted metallophosphoesterase domain (similarity to COG1409 predicted phosphohydrolases according to database annotations) with clear homologues to other species outside actinobacteria.

In *M. bovis* AF2122/97, the syntenic gene of Rv2577 (529 aa) is separated into two ORFs (Mb2607 and Mb2608) due to a base transversion (G–A), which introduces a stop codon. Mb2607 (83 aa) contains the signal sequence and sufficient beta strand structure for an OMP prediction of perfect score. Mb2608 (434 aa) matches

Rv2577 from position 96 on, so that just 12 amino acids of the *M. tuberculosis* protein are not represented in any of the two *M. bovis* proteins. The complete predicted phosphoesterase domain is intact. The N-terminal region has a high content of potential amphiphilic beta-strand but this extends further to the region of homology to Mb2608. In the absence of sequences with homology to Mb2607 but not to Mb2608, we cannot validate the hypothesis that Mb2607 can form an independent domain, although the gene split suggests this possibility.

Both Mb2607/Mb2608 transcripts have been shown to be upregulated in a virulent strain of *M. bovis* during bacterial replication in macrophages (Blanco *et al.*, 2009). The G–A transversion is absent in avirulent *M. bovis* strains used for human vaccine development (*M. bovis* BCG str. Tokyo 172, *M. bovis* BCG Pasteur 1173P2). The splitting of this gene may extend host-specific modular functions of this protein in *M. bovis* AF2122/97, which is pathogenic to cattle (Garnier *et al.*, 2003).

DISCUSSION

OMPs act as gatekeepers to the external environment. They are exposed as quorum sensors or they act in response to the environment, and are likely to be essential for general survival of the cell. In pathogenic species, the function of the OMPs may play important roles in host–cell interactions that enable the persistence of mycobacterial infection. As such, OMPs are logical drug targets, not only in tuberculosis and leprosy but also in opportunistic

Fig. 3. FracB5 and perbeta in a sliding window for Rv2345. Rv2345 defines a family conserved in actinobacteria and present in the mycobacteria tested, with the exception of *M. leprae* and *M. ulcerans*. (a) Average on a 300 aa window of perbeta and FracB5 for Rv2345. The horizontal lines indicate the thresholds used for these two parameters. The plot suggests that the N-terminal region of Rv2345 contains a highly amphiphilic beta structure. (b) The N-terminal end of Rv2345 and orthologues contains a predicted Pfam domain of unknown function (DUF477). *M. avium* protein MAV_102 represents a different architecture but is potentially a shorter OMP as it keeps the N-terminal domain. Other predicted sequence features for these proteins are: transmembrane helices (boxes), a 300 aa domain (hexagon), a C-terminal domain (oval) and a G-rich amino-acid-biased region (shown by arrow).

infections in immunocompromised patients, which in total kill millions of people worldwide every year and are complicated by new problems such as co-infection with HIV and resistance to drugs (Meya & McAdam, 2007).

Existing computational methods to predict beta-barrel OMPs primarily focus on known OMPs in Gram-negative bacteria (Berven *et al.*, 2004; Bigelow *et al.*, 2004; Casadio *et al.*, 2003; Remmert *et al.*, 2009; Zhai & Saier, 2002). Unfortunately, OMPs of the beta-barrel type are almost totally uncharacterized in mycobacteria. Models for Gram-positive and Gram-negative OMPs can only partially extend to mycobacteria due to its unique cell wall construction that eludes clear functional classification as Gram-positive or Gram-negative. For example, the beta-barrels of the OMPs of mycobacteria have to be longer than those typically known from Gram-negative bacteria, in agreement with the greater thickness of the mycobacterial wall (Alahari *et al.*, 2007; Hoffmann *et al.*, 2008; Zuber *et al.*, 2008); this is the case for MspA (Faller *et al.*, 2004). As a result, the study of mycobacterial OMPs has to rely on tools specific to it.

Computational methods can be used to predict OMPs based on two properties: OMPs must form an amphipathic beta-barrel and be secreted. However, in view of the current small number of mycobacterial OMPs that can be used to benchmark such methods, and given the fact that mycobacterial OMPs are expected to be very different to those known outside actinobacteria, we benchmarked our method according to its ability to produce coherent predictions across proteins with high similarity to *M. tuberculosis* proteins.

Accordingly, the method we presented here uses clusters of homologous sequences from seven mycobacterial genomes to optimize OMP prediction, based on the assumption that cross-genomic sequences within the same cluster should share similar properties, and therefore if the majority of sequences within a cluster were predicted to be OMPs, then those sequences that escaped prediction were also likely to be OMP sequences. In this manner, we were able to examine the effect of changing the thresholds of different parameters on the number of predicted OMPs to set final thresholds that reduced the number of spurious OMP predictions in clusters with low OMP content while maintaining OMP predictions for clusters with initially high OMP content. Moreover, we performed a sliding window analysis on all proteins to identify local regions of beta-content within larger proteins with low overall propensity to form beta-barrels. This method predicts practically all known mycobacterial OMPs with close to maximum scores.

We computed a set of 4300 potential OMPs in seven genomes (>600 alone in *M. tuberculosis*). It is unlikely that all of them will be OMPs as current estimations of OMPs in *M. tuberculosis* are in the order of 100s (Niederweis *et al.*, 2010). We do not think that with the current information on mycobacterial OMPs we can devise a more sensitive

scoring system. In any case, we note that this dataset includes a higher proportion of sequences from obligate pathogenic mycobacteria compared with opportunistic or non-pathogenic mycobacteria (15 versus 13 %), suggesting that the set is enriched in genes with a function related to pathogenicity. Many of these proteins, as we have shown, define families specific to *Mycobacteria* or *Actinobacteria* that remain to be functionally characterized.

Our work proposes a number of putative OMP domains. Some of them are reused in multiple domain architectures and duplicated in paralogues (e.g. the ACT domain) or inside genes (e.g. the tandemly repeated C4 domain in Rv3835). Some of these domains or even some of the entire OMP predicted proteins are probably too small to form a beta-barrel by themselves (<150 aa). However, the many cases where such proteins appear together in putative operons (e.g. the mce operons) suggest that they may associate to form a barrel. OMP formation by oligomerization is already suspected in the predicted OMP Rv1698. Rv1698 has been observed to dimerize, and the observation that channel complexes containing Rv1698 have variable conductance states suggests that Rv1698 might form oligomers (Siroy *et al.*, 2008). The formation of self-associations is also a possibility that has been reported. For example, both *M. tuberculosis* MspA and the alpha-haemolysin porin of *Staphylococcus aureus* (from a different phylum: *Firmicutes*) form a beta-barrel with each monomer contributing just a 50 aa loop to the beta-barrel associating as a homo-octamer (Faller *et al.*, 2004) or a homo-heptamer (Song *et al.*, 1996), respectively.

Conclusions

In summary, our results suggest that potential OMPs are a large contributor to the protein baggage of mycobacteria, possibly of actinobacteria in general. Should a large fraction of our predictions be demonstrated experimentally to be OMPs, it would point to this function as an important factor for shaping the evolution, variability and adaptability of these organisms. Using genomic information, we have been able to tune an OMP prediction algorithm and produced a set of OMP predictions for more than 4300 mycobacterial proteins. Their profiles of taxonomic conservation can be used to hypothesize the functional importance and pathogenicity relevance.

We note that while this manuscript was under review, one of our predicted OMPs, Rv0899, which was widely believed to be an OMP, was demonstrated not to form a trans-membrane beta-barrel structure (Teriete *et al.*, 2010). Although this result contradicts our prediction, this indicates that the method produces targets that align well with the expectations of researchers in the field. As new experimental evidence accumulates, we will be able to refine our algorithm. In addition, the expected sequencing of novel mycobacterial genomes will allow us to further complete the picture of the evolutionary history of OMPs and to pinpoint their association with pathogenicity,

hopefully leading to new strategies to combat a number of terrible diseases.

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