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Review

Nutrient acquisition by mycobacteria

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The growth and nutritional requirements of mycobacteria have been intensively studied since the discovery of *Mycobacterium tuberculosis* more than a century ago. However, the identity of many transporters for essential nutrients of *M. tuberculosis* and other mycobacteria is still unknown despite a wealth of genomic data and the availability of sophisticated genetic tools. Recently, considerable progress has been made in recognizing that two lipid permeability barriers have to be overcome in order for a nutrient molecule to reach the cytoplasm of mycobacteria. Uptake processes are discussed by comparing *M. tuberculosis* with *Mycobacterium smegmatis*. For example, *M. tuberculosis* has only five recognizable carbohydrate transporters in the inner membrane, while *M. smegmatis* has 28 such transporters at its disposal. The specificities of inner-membrane transporters for sulfate, phosphate and some amino acids have been determined. Outer-membrane channel proteins in both organisms are thought to contribute to nutrient uptake. In particular, the Msp porins have been shown to be required for uptake of carbohydrates, amino acids and phosphate by *M. smegmatis*. The set of porins also appears to be different for *M. tuberculosis* and *M. smegmatis*. These differences likely reflect the lifestyles of these mycobacteria and the availability of nutrients in their natural habitats: the soil and the human body. The comprehensive identification and the biochemical and structural characterization of the nutrient transporters of *M. tuberculosis* will not only promote our understanding of the physiology of this important human pathogen, but might also be exploited to improve tuberculosis chemotherapy.

Introduction

The growth and nutritional requirements of mycobacteria have been intensively studied since the discovery of *Mycobacterium tuberculosis* (Koch, 1882). These studies have resulted in an overwhelming body of literature on the physiology of mycobacterial metabolism in the years before the dawn of molecular biology (Edson, 1951; Ramakrishnan *et al.*, 1972; Ratledge, 1982). For example, the utilization of many solutes such as carbohydrates, alcohols, carboxy acids, fatty acids and amino acids by mycobacteria was examined by measuring oxygen consumption (Edson, 1951). It is striking that the identity of many transporters for essential nutrients of *M. tuberculosis* and other mycobacteria is still unknown despite considerable progress in the development of genetic methods for mycobacteria (Kana & Mizrahi, 2004; Machowski *et al.*, 2005). Recently, in particular carbon metabolism of mycobacteria has attracted renewed interest since the observation that *M. tuberculosis* relies on the glyoxylate cycle for survival in mice (McKinney *et al.*, 2000; Munoz-Elias & McKinney, 2005). This indicates that *M. tuberculosis* uses lipids as the main carbon source during infection. On the other hand, genes that encode a putative disaccharide transporter were found to be essential for *M. tuberculosis* during the first week of infection (Sassetti & Rubin, 2003), indicating that *M. tuberculosis* may switch its main carbon source from carbohydrates to lipids with the onset of the adaptive immune response. Obviously, this requires that *M. tuberculosis* has the capacity to take up these particular carbohydrates and lipids. However, both the carbon sources and the corresponding uptake proteins are unknown for *M. tuberculosis* inside the human host. Surprisingly little is known about the identity and properties of many other transporters for essential nutrients, even for *in vitro*-grown *M. tuberculosis* or for *Mycobacterium smegmatis*, which is often used as a fast-growing, non-pathogenic model organism. However, recent progress has been made in understanding the uptake of some nutrients in both *M. smegmatis* and *M. tuberculosis*. For example, proteins have been identified and characterized that are involved in uptake of several nutrients such as phosphate (Gebhard *et al.*, 2006; Peirs *et al.*, 2005; Vyas *et al.*, 2003; Webb, 2003; Wolschendorf *et al.*, 2007), sulfate (Wooff *et al.*, 2002) and some amino acids (Seth & Connell, 2000; Talaue *et al.*, 2006). This review focuses primarily on the uptake of solutes that are considered to be major nutrients for mycobacteria. The

Two supplementary figures showing the genetic organization of *M. smegmatis* carbohydrate transporters of the ABC, PTS, MIP, SSS and MFS protein families are available with the online version of this review.

presence of two lipid barriers in mycobacteria imposes considerable constraints for the transport of solutes from the exterior of the cell to the cytoplasm and has important consequences for the physiology and pathogenesis of mycobacteria. Previous reviews have covered inner-membrane transporters of *M. tuberculosis* (Content *et al.*, 2005). Considering the fact that mycobacteria have elaborated a very efficient permeability barrier outside the inner membrane (Brennan & Nikaido, 1995; Niederweis, 2003), the current knowledge about nutrient uptake across both inner and outer membranes in mycobacteria is summarized and evaluated. A putative link between outer-membrane transport and growth rate of mycobacteria has recently been discussed (Cox & Cook, 2007).

The cell envelope of mycobacteria

Permeability barriers in mycobacterial cell envelopes

Nutrient uptake mechanisms obviously depend on the permeability barriers imposed by the cell envelope. Therefore, it is necessary to review the current status of the research about the cell envelope of mycobacteria. The terms are defined as follows (Beveridge, 1995; Beveridge & Kadurugamuwa, 1996; Graham *et al.*, 1991): The *cell wall* consists of the periplasm, the peptidoglycan layer and, for Gram-negative bacteria, the outer membrane. The periplasmic space represents an extracytoplasmic compartment confined between the plasma membrane and an outer structure (outer membrane versus a peptidoglycan–teichoic acid–protein network for Gram-negative and Gram-positive bacteria, respectively). The *periplasm* is composed mostly of soluble components. The *cell envelope* comprises the inner membrane and the cell wall. The components of the cell envelopes of both Gram-positive and Gram-negative bacteria have recently been visualized by cryo-electron microscopy (Matias *et al.*, 2003; Matias & Beveridge, 2005, 2006).

In microbiology textbooks, mycobacteria are still classified as Gram-positive bacteria. However, it is well documented that mycobacteria, unlike other Gram-positive bacteria, have evolved a very complex cell wall, comprising an arabinogalactan–peptidoglycan polymer with covalently bound mycolic acids of considerable size (up to 90 carbon atoms), a large variety of extractable lipids (Barry *et al.*, 1998; Daffé & Draper, 1998) and pore-forming proteins (Niederweis, 2003). Most of the mycobacterial lipids are constituents of the cell envelope, which provides an extraordinarily efficient permeability barrier to noxious compounds, rendering mycobacteria intrinsically resistant to many drugs (Brennan & Nikaido, 1995). Due to the paramount medical importance of *M. tuberculosis*, the ultrastructure of mycobacterial cell envelopes has been intensively studied for decades by electron microscopy. A thick, electron-transparent zone has been observed in the cell wall in stained thin sections of many mycobacterial species (Mineda *et al.*, 1998; Paul & Beveridge, 1992) and was shown to comprise lipids (Paul & Beveridge, 1994). To account for the remarkable efficiency of the mycobacterial cell wall as a permeability barrier, Minnikin (1982) originally proposed a model (Fig. 1) in which the mycolic acids are covalently bound to the arabinogalactan–peptidoglycan co-polymer and form the inner leaflet of an asymmetrical bilayer. Other lipids extractable by organic solvents were thought to form the outer leaflet of this outer bilayer. X-ray diffraction studies of mycobacterial cell walls showed that the mycolic acids are oriented parallel to each other and perpendicular to the plane of the cell envelope (Nikaido *et al.*, 1993). This provided experimental support for some fundamental aspects of the Minnikin model. Mutants and treatments affecting mycolic acid biosynthesis and the production of extractable lipids showed an increase of cell wall permeability and a drastic decrease of virulence, underlining the importance of the integrity of the cell wall for intracellular survival of *M. tuberculosis* (Barry *et al.*, 1998). These indirect structural, biochemical and genetic

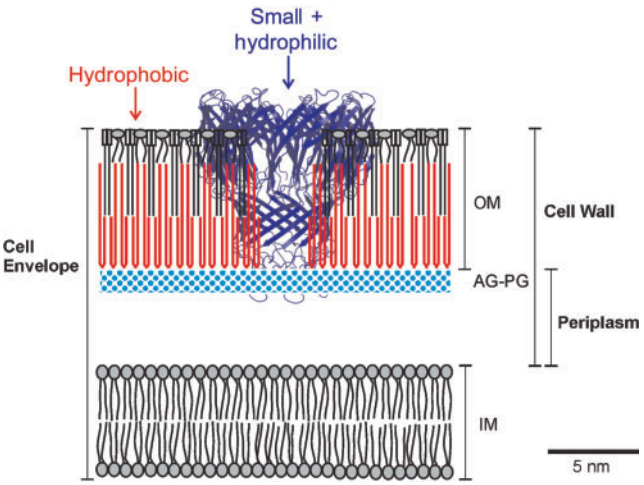

Fig. 1. Transport processes across the mycobacterial cell envelope: schematic representation of the mycobacterial cell envelope consisting of the inner membrane (IM) and the cell wall. This representation is based on the model proposed by Minnikin (1982). Mycolic acids are covalently linked to the arabinogalactan–peptidoglycan (AG-PG) co-polymer and are thought to contribute to the inner leaflet of the asymmetrical outer membrane (OM). A variety of extractable lipids presumably form the outer leaflet of the outer membrane. Note that no experimental data exist for either the spatial distribution of the extractable lipids, or the confirmations and exact locations of the mycolic acids. The porin MspA mediates the uptake of small and hydrophilic nutrients such as sugars (Stephan *et al.*, 2005) and phosphates (Wolschendorf *et al.*, 2007) across the outer membrane of *M. smegmatis*. The representation of MspA is based on its crystal structure (Faller *et al.*, 2004) and was created using the visualization software PyMol (DeLano Scientific). The MspA channel is 9.6 nm long. Approximately 7 nm of the MspA surface is inaccessible to hydrophilic reagents in *M. smegmatis* (Mahfoud *et al.*, 2006). Hydrophobic compounds are assumed to diffuse directly across the outer membrane. The dimensions are approximately to scale.

data are consistent with the existence of an outer lipid bilayer as proposed by Minnikin (1982). However, this model (Fig. 1) faced criticism mainly because electron microscopy of mycobacteria, in particular thin sections thereof, never showed evidence for an additional, outer lipid bilayer (Daffé & Draper, 1998; Draper, 1998). In addition, the exact localization of the extractable lipids and of the mycolic acids is unknown. One of the difficulties in providing additional experimental evidence for this model is the fact that lipid membranes are notoriously poorly preserved during chemical fixation and plastic embedding (Beveridge, 1999). Thus, direct evidence for an outer lipid bilayer in mycobacteria is lacking. Consequently, a periplasmic space as defined above has been regarded as hypothetical for mycobacteria (Daffé & Draper, 1998; Etienne *et al.*, 2005). Recently, cryo-electron tomography of intact *M. smegmatis* and *Mycobacterium bovis* BCG cells revealed two lipid membranes. This is the first visualization of native outer membranes in mycobacteria (Hoffmann *et al.*, 2008). Biologically important consequences are that mycobacteria possess a periplasmic space defined by two membranes, analogous to the situation in Gram-negative bacteria and have periplasmic and outer-membrane proteins that functionalize these cellular compartments.

Principal nutrient pathways across the mycobacterial cell envelope

The unique mycolic acid layer is an extremely efficient permeability barrier protecting the cell from toxic compounds and is generally thought to be the major determinant of the intrinsic resistance of mycobacteria to most common antibiotics, chemotherapeutic agents and chemical disinfectants (Brennan & Nikaido, 1995). The mycolic acid layer is functionally and structurally analogous to the outer membrane of Gram-negative bacteria, a similarity that merits a more detailed comparison. Three general and several specific pathways for transport across the outer membrane of Gram-negative bacteria exist. (i) Hydrophobic compounds penetrate the membrane via the lipid pathway by temporarily dissolving in the lipid bilayer. (ii) Small and hydrophilic compounds diffuse through water-filled protein channels called porins (Nikaido, 1994). Some of these diffusion channels show specificity towards certain classes of compounds such as maltodextrin (Dumas *et al.*, 2000) or nucleosides (Maier *et al.*, 1988). (iii) Polycationic compounds are thought to disorganize the outer membrane, thereby mediating their own uptake in a process termed 'self-promoted uptake' (Hancock *et al.*, 1991). (iv) Certain compounds are specifically taken up by transporter proteins such as FhuA and FepA, which transport iron-loaded siderophores in an energy-dependent process across the outer membrane of *Escherichia coli* (Braun & Killmann, 1999). The nutrient molecules that have crossed the outer membrane need to pass through the periplasm. The mechanisms of these transport processes in *M. tuberculosis* are not known. After passage through the periplasm nutrients are taken up across the inner membrane by specific transporter proteins, often against a concentration gradient, using cellular energy. These inner-membrane transporters of *M. tuberculosis* have been the focus of a recent review (Content *et al.*, 2005).

Transport across mycobacterial outer membranes

Porin-mediated diffusion of hydrophilic solutes

Porins are defined as non-specific protein channels in bacterial outer membranes that enable the influx of hydrophilic solutes (Nikaido, 2003). Channel-forming proteins that are functionally similar to porins of Gram-negative bacteria have been observed in many mycobacteria (Niederweis, 2003) and other closely related genera such as corynebacteria. MspA was discovered as the first porin of *M. smegmatis* (Niederweis *et al.*, 1999). Deletion of *mspA* reduced the outer-membrane permeability of *M. smegmatis* to cephaloridine and glucose nine- and fourfold, respectively (Stahl *et al.*, 2001). These results show that MspA is the major general diffusion pathway for hydrophilic solutes in *M. smegmatis*. Consecutive deletions of the two porin genes *mspA* and *mspC* reduced the number of pores 15-fold compared to wild-type *M. smegmatis*. The loss of porins lowered the permeability for glucose 75-fold and, concomitantly, the growth rate of *M. smegmatis* on plates and in liquid medium dropped drastically (Stephan *et al.*, 2005). This showed for the first time that the porin-mediated influx of hydrophilic nutrients limited the growth rate of porin mutants. However, it is unknown which and how many nutrients are in low supply in the *M. smegmatis* porin mutants. Since MspA could not be expressed in *M. smegmatis* above wild-type levels (Stephan *et al.*, 2005), it is also not clear whether the influx of nutrients really limits the growth rate of wild-type mycobacteria as suggested earlier (Jarlier & Nikaido, 1990). The fact that the lack of the MspA and MspC porins also caused a reduced uptake of phosphates and slower growth on low-phosphate plates (Wolschendorf *et al.*, 2007) indeed suggested that the slow uptake of essential hydrophilic nutrients other than the carbon source may also contribute to the slow growth of *M. smegmatis* porin mutants.

The crystal structure of MspA represents the first such structure of any mycobacterial outer-membrane protein (Faller *et al.*, 2004). The mere existence of these hydrophobic integral pore proteins provides strong, albeit indirect, support for the existence of an outer membrane in mycobacteria because proteins with open channels in the cytoplasmic membrane would be deterimental for any cell for at least two reasons: first, open pores in the inner membrane would lead to an immediate breakdown of the proton gradient and deprive the cell of energy; and second, small hydrophilic solutes such as many metabolic intermediates or nutrients would leak out of the cell. The crystal structure of MspA revealed an octameric goblet-like conformation with a single central channel 10 nm in length (Faller *et al.*, 2004). This structure is different from

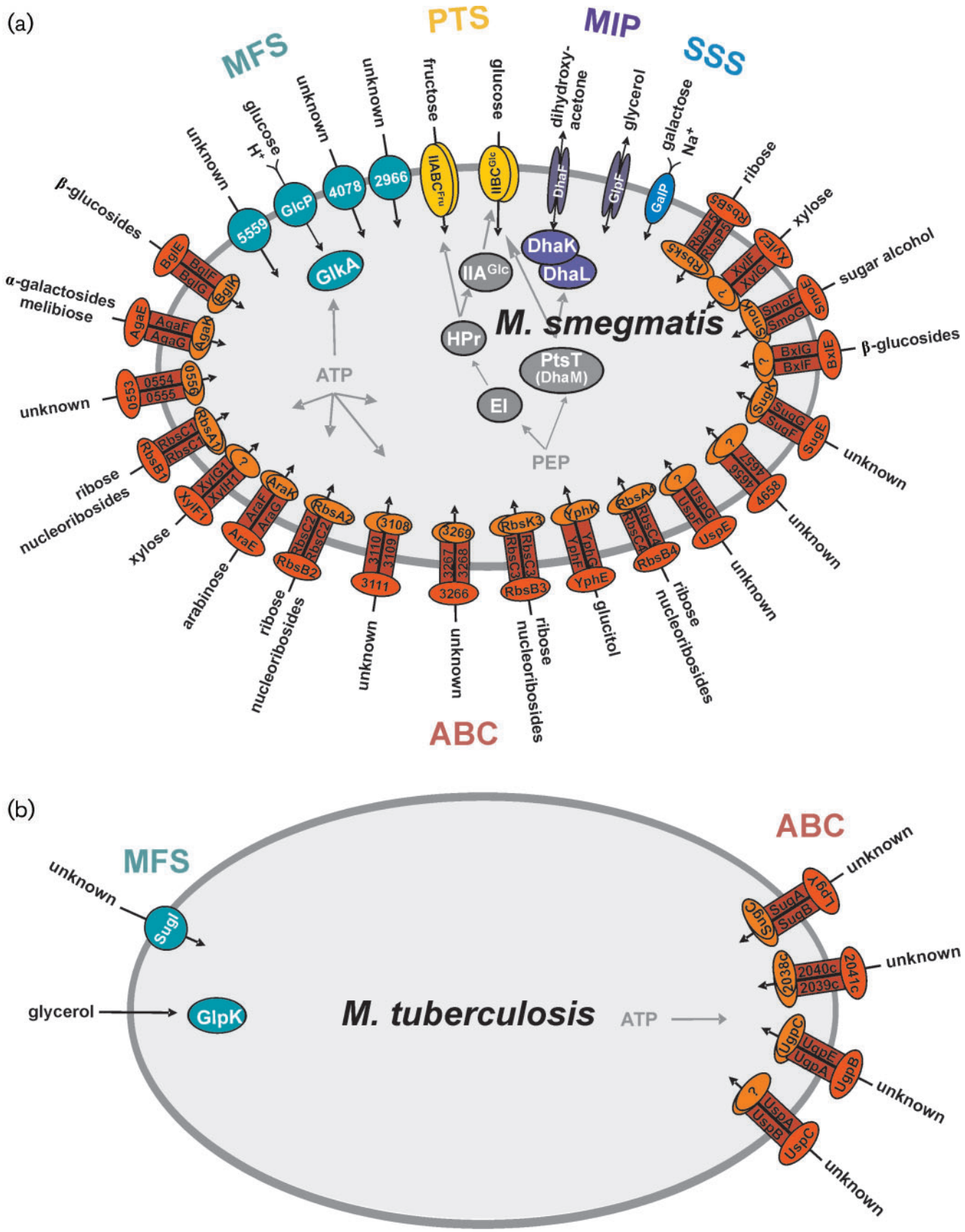
(a)
MFS
PTS
MIP
SSS
ABC
unknown
glucose H+
unknown
unknown
fructose
glucose
dihydroxy-acetone
glycerol
galactose Na+
ribose
xylose
sugar alcohol
β-glucosides
unknown
unknown
ribose nucleoribosides
xylose
arabinose
ribose nucleoribosides
unknown
unknown
ribose nucleoribosides
glucitol
ribose nucleoribosides
unknown
unknown
unknown
β-glucosides
α-galactosides melibiose
5559
GlcP
4078
2966
IIABCFru
IIBCGlc
DhaF
GlpF
GalP
GlKA
IIA Glc
DhaK
DhaL
HPr
PtsT (DhaM)
EI
ATP
PEP
M. smegmatis
(b)
MFS
ABC
unknown
SugI
glycerol
GlpK
M. tuberculosis
ATP
unknown
unknown
unknown
unknown

Fig. 2. Transporters for uptake of carbohydrates across the inner membrane of (a) *M. smegmatis* and (b) *M. tuberculosis.* Shown are the transport proteins of the ATP-binding cassette (ABC, red), phosphotransferase system (PTS, yellow), major facilitator superfamily (MFS, green), major intrinsic protein family (MIP, dark blue) and the sodium solute superfamily (SSS, light blue). The derived putative substrates are inferred from *in silico* analyses in combination with experimental data (Titgemeyer *et al.*, 2007). Systems are denoted by the protein name instead of their locus tags (MSMEG_XXXX) if the substrates have been experimentally verified or were predicted with a high likelihood. Note that the outer membranes of *M. smegmatis* and *M. tuberculosis* have been omitted for clarity.

that of the trimeric porins of Gram-negative bacteria, which have one pore per monomer and are approximately 5 nm long (Koebnik *et al.*, 2000). Its structural features define MspA as the founding member of a new class of outer-membrane proteins. The crystal structure also revealed that the constriction zone of MspA consists of 16 aspartates (D90/D91). Thus, the zone of MspA with the smallest diameter is highly negatively charged (Faller *et al.*, 2004). This most likely explains the previously observed preference of MspA for cations (Niederweis *et al.*, 1999). The MspA pore provides an example of how outer-membrane transport proteins can contribute to the selectivity of mycobacteria to particular nutrients.

The existence of channel-forming proteins in *M. tuberculosis* and in *M. bovis* BCG has been demonstrated (Kartmann *et al.*, 1999; Lichtinger *et al.*, 1999; Senaratne *et al.*, 1998). Uptake of serine but not of glycine was reduced in an *ompATb* mutant compared to wild-type *M. tuberculosis.* This was interpreted as proof that OmpATb is a porin, consistent with its apparent channel-forming activity *in vitro* (Raynaud *et al.*, 2002b). However, the overall permeability of the outer membrane of *M. tuberculosis* was reduced at pH 5.5 compared to pH 7.2, although the levels of OmpATb in the outer membrane were strongly increased (Raynaud *et al.*, 2002b). Considering these contradictory results, it is doubtful that OmpATb has significant porin function in *M. tuberculosis* (Niederweis, 2003). The recent observation that a central domain of approximately 150 amino acids is sufficient for the channel activity of OmpATb *in vitro* does not contribute to the understanding of its biological functions (Molle *et al.*, 2006). Pore-forming proteins of *M. tuberculosis* other than OmpATb are not yet known.

Direct diffusion of hydrophobic solutes through the cell membranes

Hydrophobic molecules, in particular nonelectrolytes, can easily diffuse through phospholipid bilayers. However, the lipopolysaccharide-containing outer membrane of Gram-negative bacteria constitutes a considerable permeability barrier that does not allow the penetration of even extremely hydrophobic β-lactam antibiotics (Nikaido *et al.*, 1983). The lipids in mycobacterial cell walls are likely to be organized in a very unusual, asymmetrical bilayer (Nikaido *et al.*, 1993). Differential scanning calorimetry showed that the lipids in mycobacterial cell walls have very high phase transition temperatures in the range of 60–70 °C. This is suggestive of a lipid domain of extremely low fluidity (Liu *et al.*, 1995). Isolated cell walls of corynebacteria, which contain much shorter corynemycolic acids, displayed a much lower temperature transition, suggesting that the fluidity of this lipid bilayer is mainly determined by the mycolic acids. Since direct diffusion of a hydrophobic molecule through a lipid membrane requires that it is dissolved in the lipid phase, the permeability of a particular membrane is directly correlated with its fluidity. This has been demonstrated directly by Nikaido and co-workers (Liu *et al.*, 1996). It is concluded that the mycobacterial outer membrane presents a strong permeability barrier for hydrophobic molecules. On the other hand, there is emerging evidence that fatty acids rather than carbohydrates might be the dominant carbon source of *M. tuberculosis* after the onset of the immune response. This includes the requirement for isocitrate lyase for growth and persistence of *M. tuberculosis* in macrophages and in mice (McKinney *et al.*, 2000), and the induction of expression of genes encoding enzymes involved in the β-oxidation of fatty acids in macrophages (Schnappinger *et al.*, 2003) and mice (Dubnau *et al.*, 2005; Timm *et al.*, 2003). However, the sources and the types of fatty acids that may be utilized by *M. tuberculosis*, as well as the uptake pathways, are unknown.

Transport across the mycobacterial inner membrane

Transporters of carbohydrates

It has been widely documented that the soil bacterium *M. smegmatis* can grow on many carbon sources such as polyols, pentoses and hexoses (Edson, 1951; Franke & Schillinger, 1944; Izumori *et al.*, 1976). A recent comprehensive analysis of carbohydrate uptake systems revealed that *M. smegmatis* has 28 putative carbohydrate transporters (Titgemeyer *et al.*, 2007). The majority of sugar transport systems (19/28) in *M. smegmatis* belong to the ATP-binding cassette (ABC) transporter family (Fig. 2a). *M. smegmatis* further possesses one putative glycerol facilitator of the major intrinsic protein (MIP) family, four sugar permeases of the major facilitator superfamily (MFS), of which one was assigned as a glucose transporter, and one galactose permease of the sodium solute superfamily (SSS). Thus, inner-membrane transport systems for polyols, pentoses and hexoses are predicted to exist in *M. smegmatis* (Fig. 2a; see also Supplementary Figs S1 and S2, available with the online version of this review).

Table 1. Inner-membrane sugar transporters of *M. smegmatis*

	Family and predicted substrate(s)	Gene designations	Locus tag*	Representative homologues†/ comments	References‡
	ABC				
1	β-Glucosides, chitobiose, disaccharides	*nagB1 bglA bglR sugK bglEFGK*	0501–0508	*nagB* SCO5236, *bgl* SCO0670, *deoR*, *rbsK E. coli*, *abcEFG* genes are distantly related to many ABC permease genes, *msiK* SCO4240	Bertram *et al.* (2004), S, C
2	α-Galactosides, melibiose	*agaRZSXPAEFGK mspB*	0509–0517	SCO5848–5851, SCO0538–0541, *msiK* SCO4240	Bertram *et al.* (2004); Brinkkötter *et al.* (2000), S
3	Unknown	*abcEFGK*	0553–0556	Distant similarity to many ABC permease genes	S, C
4	Ribose, xylose	*rbsA1C1B1 gatY sugK rbsR1 pfkB*	1372–1378	SCO6009–6011, *gatY* SCO5852, *pfkB* SCO3197, distantly related to ribose and xylose ABC transporters	Bell *et al.* (1986); Bertram *et al.* (2004), S, C, T, B
5	Xylose	*xylF2G2H2*	1704–1706	*E. coli xylFGH*	Daley *et al.* (2005); Sumiya *et al.* (1995), C
6	Arabinose	*pho araR araGFKEBDA*	1707–1715	*E. coli ytf* operon, *B. subtilis araABD*	Sa-Nogueira *et al.* (1997), B, C
7	Ribose, ribonucleosides	*gap pgk tpiA secG urf rscA deoC rbsC2A2rbsR2 sugK sugD rbsB2 ppc pgl opcA zwf tal tkt*	3084–3103	*rbsH* SCO2747, *rbsA* SCO2746, *rbsB E. coli*	Bell *et al.* (1986); Bertram *et al.* (2004), S, C
8	Unknown	*abcKGFE fabG sugK*	3108–3113	Distant similarity to many ABC permease genes	Bertram *et al.* (2004), S, C
9	Unknown	*abcR sugK abcEFGK1K2 sugK rpiB*	3264–3272	Distant similarity to many ABC permease genes, SCO0580, *E. coli rpiB*	Bertram *et al.* (2004); Sørensen & Hove-Jensen (1996), S, C
10	Ribose	*rbsB3R3C3A3*	3598–3602	SCO2747, *B. subtilis* ribose operon	Woodson & Devine (1994)
11	Sorbitol	*yphREKFB*	3998–4002	*E. coli yph* operon	Bertram *et al.* (2004), C
12	Ribose	*rbsA4C4B4R4*	4170–4174	*E. coli* ribose operon *rbsCBR*	Bell *et al.* (1986), C
13	Unknown	*uspGFE*	4466–4468	*M. tuberculosis usp* operon	Braibant *et al.* (2000), T
14	Unknown	*abcRFGE*	4655–4658	Distant similar to many ABC permease genes	S, C
15	Unknown	*sugKGFE*	5058–5061	*M. tuberculosis sug* operon	Braibant *et al.* (2000), T
16	β-Xyloside	*bglG bxlRAEFG*	5142–5147	*S. coelicolor bxlEFG2*	Bertram *et al.* (2004)
17	Sugar alcohol	*smoKGFER*	5571–5574	*S. coelicolor smo* operon	Bertram *et al.* (2004)
18	Xylose	*xylG1F1E1A1R1*	6018–6022	*E. coli xyl* operon	Sumiya *et al.* (1995)
19	Ribose	*rbsR5P5K5G5F5E5*	6798–6805	SCO0723, SAV5702, SCO2747	Bertram *et al.* (2004), S, C
	PTS				
20	Fructose	*ptsH fruAKR ptsI*	0084–0088	*S. coelicolor ptsH fruAKR ptsI*	Bertram *et al.* (2004); Nothaft *et al.* (2003), S
21	*N*-Acetylglucosamine glucose, trehalose	*ptsG crr nagB2A ptsR*	2116–2120	*S. coelicolor nagE2 crr nagAB*	Bentley *et al.* (2002); Nothaft *et al.* (2003)
22	Dihydroxyacetone	*ptsT dhaLKFR*	2121–2125	*S. coelicolor gyl* operon, *E. coli dhaKLM*	Bertram *et al.* (2004); Gutknecht *et al.* (2001), S, C
	MIP				
23	Glycerol	*glpK2RFK1D*	6756–6760	*S. coelicolor gyl* operon	Bertram *et al.* (2004), S
	SSS				
24	Galactose	*galPRTK*	3689–3692	*S. coelicolor gal* operon	Bertram *et al.* (2004)

Table 1. cont.

	Family and predicted substrate(s)	Gene designations	Locus tag*	Representative homologues†/ comments	References‡
	MFS				
25	Unknown	*sugP1*	2966	Distant similarity to sugar permeases of the MFS	S, C, B, T
26	Unknown	*sugP2*	4078		
27	Glucose	*glcP*	4182	*S. coelicolor glcP*	van Wezel *et al.* (2005)
28	Unknown	*sugP3*	5559		

*Only the numbers of the locus tags msmeg_XXXX of the *M. smegmatis* mc^{2}155 genome are shown. Locus tags are taken from the revised genome annotation by TIGR (http://www.tigr.org).

†This column contains information on representative homologues for which experimental information is available.

‡The following genome servers were used for BLASTP analysis: B, *B. subtilis* (http://genolist.pasteur.fr/SubtiList); C, *E. coli* (http://genolist.pasteur.fr/Colibri); S, *S. coelicolor* (www.avermitilis.ls.kitasato-u.ac.jp/blast_local/index.html); T, *M. tuberculosis* (http://genolist.pasteur.fr/TubercuList).

M. smegmatis did not grow on lactose, maltose and sucrose as a sole carbon source (Titgemeyer *et al.*, 2007). Franke & Schillinger (1944) obtained the same result for lactose and maltose, but observed respiration of *M. smegmatis* in the presence of sucrose. *M. smegmatis* has at least three inner-membrane transport systems with significant similarities to other bacterial disaccharide transporters (Table 1, Fig. S1). However, the substrate specificities of the transporters encoded by *msmeg0501–0508* and *msmeg0509–0517* are not known. Growth of bacteria on disaccharides as sole carbon sources requires enzymes that cleave the disaccharide and release the monosaccharides for further metabolism. The absence of proteins similar to known bacterial β-D-galactosidases (LacZ of *E. coli*, BgaB of *Bacillus circulans*, MbgA of *Bacillus megaterium*, LacA of *Streptomyces coelicolor*) provides a molecular explanation for the inability of *M. smegmatis* to utilize lactose as a sole carbon source. By contrast, *M. smegmatis* has six homologues (MSMEG3191, 3577, 4901, 4902, 4685, 6477) of MalL of *Bacillus subtilis*, which hydrolyses maltose, longer maltodextrins up to maltohexose, isomaltose and sucrose (Schönert *et al.*, 1999), and of the cytoplasmic trehalase TreC of *E. coli*, which cleaves trehalose 6-phosphate (Rimmele & Boos, 1994). It is conceivable that these enzymes are used in trehalose metabolism, considering the unusual importance of trehalose in mycobacteria (Murphy *et al.*, 2005; Woodruff *et al.*, 2004) and the observation that trehalose was the only disaccharide that was used by *M. smegmatis* as a sole carbon source. However, it cannot be excluded that some of the enzymes with similarities to TreC and MalF have roles in pathways distinct from trehalose metabolism.

Bioinformatic analysis of the genome of *M. tuberculosis* H37Rv revealed four ABC-type transporters and one permease of the MFS class for carbohydrates (Titgemeyer *et al.*, 2007) (Fig. 2b). These ABC transporters have been described earlier in a global analysis of the *M. tuberculosis* genome (Braibant *et al.*, 2000; Content *et al.*, 2005). It is obvious that *M. tuberculosis* is poorly equipped with carbohydrate transport systems in comparison to *M. smegmatis*. Two of the operons, the *lpgY–sugABC* and the *uspABC* operons, are highly conserved between the two species. The proteins of the ABC^{Sug} and of the ABC^{Usp} systems share between 62 % and 80 % similar amino acids, compared to only 25–30 % similar amino acids for the ABC^{Ugp} and the Rv2038c/Rv2039c/Rv2040c/Rv2041c systems. The similarities of all four ABC systems to known transporters outside the genus *Mycobacterium* is so low (<25 %) that substrates of these transporters cannot be predicted (Titgemeyer *et al.*, 2007).

The ABC^{Sug} sugar transport system was predicted to be essential for virulence of *M. tuberculosis* in mice based on transposon site hybridization (TraSH) experiments (Sassetti & Rubin, 2003). Previously, it was suggested that this permease may transport maltose or maltodextrins (Borich *et al.*, 2000; Braibant *et al.*, 2000). However, the similarities both of ABC^{Sug} and of the corresponding substrate-binding protein LpgY to the maltose transporters and periplasmic maltose-binding proteins MalE of *E. coli* and *S. coelicolor* are very low (<25 %). Thus, it is questionable whether maltose is the substrate of ABC^{Sug}. This conclusion is supported by the fact that neither *M. smegmatis*, which has a highly similar ABC^{Sug} system, nor *M. tuberculosis* (Edson, 1951) grows on maltose as a sole carbon source. It has to be noted that similar uncertainties exist about the substrate specificities of the four other carbohydrate uptake systems of *M. tuberculosis*, including the ABC^{Usp} transporter that was proposed to transport sn-glycerol 3-phosphate based on low protein similarities (Braibant *et al.*, 2000; Content *et al.*, 2005). The SugI transporter of the MFS class shows distant sequence similarity to the glucose permease GlcP (28 %) of *S. coelicolor* and to the galactose (GalP, 24 %) and arabinose (AraE, 24 %) transporters of *E. coli*. Thus, the SugI system may transport a monosaccharide.

Glycerol is used as the standard carbon source to grow *M. tuberculosis*; however, no uptake system is known or

apparent by sequence similarity (Titgemeyer *et al.*, 2007). Since *M. tuberculosis* grows with a generation time of 24 h and it has been shown that glycerol can directly diffuse through lipid membranes both *in vitro* (Paula *et al.*, 1996) and *in vivo* (Eze & McElhaney, 1981), it is conceivable that the rate of glycerol intake by passive diffusion may be sufficient for growth. Incoming glycerol would then be converted by glycerol kinase (GlpK) to glycerol 3-phosphate to enter the route of central carbon metabolism (Fig. 2b). *M. tuberculosis* has one putative glycerol kinase that shows a high similarity to the two glycerol kinases of *M. smegmatis* (MSMEG6759, 77 %, and MSMEG6756, 57 %) and to the two glycerol kinases from *S. coelicolor* SCO0509 (75 %) and SCO1660 (59 %).

Comparison of inner-membrane sugar transporters of *M. smegmatis* and *M. tuberculosis*. The analysis of the carbohydrate uptake proteins in the genomes of *M. smegmatis* and *M. tuberculosis* confirms the very early phenotypic observations that the saprophytic mycobacteria have a much broader spectrum of substrates that they can use as sole carbon and energy sources (Edson, 1951). It is striking that the genome of *M. tuberculosis* has only five permeases for carbohydrate uptake compared to the 28 of *M. smegmatis*. This suggests that the phagosome does not provide an environment rich in diverse sugars. The tantalizing conclusion is that an experimental analysis of the substrate specificity of the inner-membrane carbohydrate transporters of *M. tuberculosis* may reveal the carbon sources available in the phagosome of human macrophages and/or in other cellular hideouts of *M. tuberculosis* inside the human body.

Transporters of lipids

Several lines of evidence strongly suggest that *M. tuberculosis* switches from a carbohydrate to a fat diet after the onset of the adaptive immune response. (i) Biochemical studies suggest that in chronically infected lung tissues, fatty acids may be a major source of carbon and energy for *M. tuberculosis* (Wheeler *et al.*, 1990). (ii) During the first 10 days of infection of mice, *M. tuberculosis* requires the sugar transporter SugAB for survival (Sassetti & Rubin, 2003). Thereafter, enzymes such as isocitrate lyase and malate synthases are essential for virulence (McKinney *et al.*, 2000). This indicates that lipids are the major carbon and energy source of *M. tuberculosis* because the glyoxylate shunt is required for running the citric acid cycle on acetate, which is produced by degradation of lipids through β-oxidation. (iii) *M. tuberculosis* possesses four genes encoding putative phospholipases C, *plcA*, *plcB*, *plcC* and *plcD*. These genes are required for virulence of *M. tuberculosis* in mice (Raynaud *et al.*, 2002a). The fact that the phospholipases C are attached to the cell wall by lipid anchors argues for a role of these enzymes in the controlled release of fatty acids, probably from phospholipids of the phagosomal membrane. However, while several proteins have been identified in *M. tuberculosis* as being involved in the transport of lipids from the cytoplasm to the periplasm (Jackson *et al.*, 2007), the proteins involved in the transport of lipids across the outer membrane are as yet unknown. By contrast, the mechanisms that govern the uptake of exogenous fatty acids are well established in *E. coli* (Dirusso & Black, 2004). When the cell encounters long-chain fatty acids in the environment, these bind to outer-membrane protein FadL and via a ligand-induced conformational shift within the protein, are transported into the periplasmic space. The more acidified environment of the periplasmic space promotes the formation of uncharged fatty acid molecules, which partition into and flip across the inner membrane. Within the cytosol, the acyl-CoA synthase FadD partitions into the inner membrane, where it functions in the vectorial esterification of the long-chain fatty acids (Dirusso & Black, 2004). Consistent with the importance of lipid uptake, *M. tuberculosis* possesses numerous homologues of FadD proteins (Trivedi *et al.*, 2004).

Transporters of phosphorus-containing solutes

Phosphorus is indispensable for energy supply, the biosynthesis of nucleic acids and phospholipids, and many other cellular processes. While inorganic phosphate is the preferred source of phosphorus, many bacteria can also take up organic phosphates and release phosphate by the action of periplasmic phosphatases such as PhoA. Gram-negative bacteria employ sophisticated transport mechanisms to acquire phosphorus-containing nutrients from the environment. *E. coli* uses four phosphate transport systems, Pst, Pit, GlpT and UhpT, to translocate inorganic phosphate across the inner membrane (van Veen, 1997). Part of the Pst system is the periplasmic protein PstS, which binds to and transfers phosphate to the transmembrane components PstA and PstC. PstB hydrolyses ATP and delivers energy for phosphate translocation across the inner membrane by PstA/PstC. Pst systems bind and transport phosphate with binding constants and apparent transport K_m values in the submicromolar range. *M. tuberculosis* contains several copies of the genes encoding the Pst system (Braibant *et al.*, 1996). Two Pst components, PstS1 and PstS2, have been shown to be virulence factors in *M. tuberculosis* (Peirs *et al.*, 2005; Sassetti & Rubin, 2003). Further, *M. tuberculosis* contains two genes, *pitA* and *pitB*, which encode putative constitutive inorganic phosphate transporters (Content *et al.*, 2005). The physiological role of the Pit transporters is unclear.

The single *pstSCAB* operon of the fast-growing *M. smegmatis* encodes a high-affinity Pst system with an apparent K_m value of 40 μM phosphate. A second high-affinity phosphate uptake system of *M. smegmatis* is encoded by the *phnDCE* operon (Gebhard *et al.*, 2006). However, even a *phnD pstS* double mutant did not show a reduced phosphate uptake, suggesting the presence of a third high-affinity phosphate uptake system of *M. smegmatis* (Gebhard *et al.*, 2006). Considering the presence

of three high-affinity phosphate uptake systems that are inducible at low phosphate concentration in *M. smegmatis*, it is unclear why a *pstB* mutant showed reduced phosphate transport (Bhatt *et al.*, 2000). Taken together, these results underline the importance of phosphate uptake for mycobacteria. The transcriptional profiles of *M. tuberculosis* and *Salmonella enterica* in infected macrophages revealed that the proteins involved in inorganic phosphate transport are upregulated (Eriksson *et al.*, 2003; Schnappinger *et al.*, 2003), indicating that phosphate levels inside phagosomes of macrophages are indeed limited. Consistent with this conclusion, genes encoding efficient phosphate transport systems were found to be essential for the survival of *M. tuberculosis* in macrophages and mice (Rengarajan *et al.*, 2005; Sassetti & Rubin, 2003). However, it is unknown how inorganic or organic phosphates cross the outer membrane of *M. tuberculosis*. Since direct diffusion of phosphates through model lipid membranes is extremely slow [permeability coefficient of the mono-anion 5×10^{-12} cm s^{-1} (Chakrabarti & Deamer, 1992)] it appears likely that slowly growing mycobacteria also use outer-membrane pore proteins for uptake of phosphate. Indeed, the existence of a porin with anion specificity has been demonstrated (Lichtinger *et al.*, 1999). This porin still awaits discovery.

Transporters of sulfur-containing solutes

Sulfur is essential in cells for biological activities such as translation initiation and maintenance of the redox potential. Transposon insertions in the *cysA* and *subI* genes of *M. bovis* BCG yielded methionine auxotrophs. These mutants were resistant to chromate and did not take up sulfate. These results identified the products of the genes *cysTWA* and *subI* as components of a sulfate permease and indicated that this transporter is the sole sulfate transporter of *M. bovis* BCG (McAdam *et al.*, 1995; Wooff *et al.*, 2002). The sensitivity of sulfate uptake to azide and 1,3-dicyclohexylcarbodiimide are characteristic of ABC transporters. Survival of the *cysA* and *subI* mutants in mice was not different from wild-type *M. bovis* BCG. This indicated that, in the host, methionine may be a more important sulfur source than sulfate for growth of the *M. tuberculosis* complex. This may also explain how *Mycobacterium leprae* remains a pathogen, despite being a natural methionine auxotroph because of its loss of *cysTWA* for sulfate transport (Wood, 1995). An alternative explanation may be that other putative sulfate transporters such as the predicted sulfate permease (SulP) of *M. tuberculosis* are induced *in vivo* and compensate for the loss of the CysTWA transporter (Content *et al.*, 2005).

Transporters of nitrogen-containing solutes

Nitrogen is an essential component of nearly all complex macromolecules in a bacterial cell, such as proteins, nucleic acids and cell wall components. Ammonium is the preferred nitrogen source of many bacteria. In enteric bacteria, diffusion of uncharged ammonia (NH_3) through the cytoplasmic membrane into the cell is sufficient to support growth in the presence of high amounts of ammonium (NH_4^+) in the growth medium. Only when diffusion across the cell envelope becomes limiting for growth is the ammonium transporter AmtB synthesized. Homologues of AmtB exist in *M. tuberculosis* and *M. smegmatis* (Nolden *et al.*, 2001). However, no biochemical data are available for ammonium uptake by mycobacteria and the role of AmtB in this process.

Nitric oxide (NO) is generated in large amounts within macrophages and restricts the growth of *M. tuberculosis*. Nitrate can be produced by oxidation of nitric oxide and is an alternative source of nitrogen for bacteria within the human host. Early work in *E. coli* had suggested that *narK* was involved only in nitrite export (Rowe *et al.*, 1994), and so the homologous *narK2* in *M. tuberculosis* was annotated as a 'nitrite extrusion protein'. More recent work with an *E. coli narK narU* double mutant indicated that the two proteins could transport nitrate into and nitrite out of the cell (Clegg *et al.*, 2002; Jia & Cole, 2005). In *M. tuberculosis*, four genes, *narK1* through *narK3* and *narU*, are homologous to *E. coli narK* and *narU*. Since *M. tuberculosis* is unable to reduce nitrite, which could accumulate to toxic levels, it must then be exported out of the cell. The *M. tuberculosis narK2* was shown to complement this *E. coli* double mutant, supporting a role for *narK2* in nitrate reduction by coding for a transporter of nitrate into and nitrite out of the cell (Sohaskey & Wayne, 2003). Nitrate reduction by *M. tuberculosis* is regulated by control of nitrate transport into the cell by NarK2. It is proposed that NarK2 senses the redox state of the cell, possibly by monitoring the flow of electrons to cytochrome oxidase, and adjusts its activity so that nitrate is transported under reducing, but not under oxidizing, conditions (Sohaskey, 2005). Inhibition of nitrate transport by oxygen has been documented in other bacteria (Moir & Wood, 2001). It is intriguing that *M. tuberculosis*, classified as an obligate aerobe, should have such intricate control of an anaerobic enzyme system. Transcription of *narK2* is controlled by DosR/DevR, which responds to hypoxia and NO (Ohno *et al.*, 2003; Voskuil *et al.*, 2003). Both the transcription of the *narK2* gene and the activity of NarK2 are controlled by similar signals (Sohaskey, 2005).

Transporters of inorganic cations

Metal ions such as Fe^{2+}/Fe^{3+}, Cu^{+}/Cu^{2+} and Zn^{2+} play structural and catalytic roles in metalloenzymes. Genome analysis of *M. tuberculosis* revealed 28 genes encoding a broad repertoire of putative metal ion transporters. They comprise eight families of secondary active transporters and three families of primary active transporters, including twelve 'P'-type ATPases, and represent approximately a quarter of all transporters in this organism. Potential metal ion specificities include K^+, Na^+, Cu^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, $Fe^{2+/3+}$, Hg^{2+}, AsO_2^- and AsO_4^{3-}. Seventeen of these transporters are also

encoded as complete open reading frames in *M. leprae*, suggesting a role in intracellular survival. The properties of these transporters, including the NRAMP orthologue MntH, which transports manganese ions in other bacteria, have been reviewed recently (Agranoff & Krishna, 2004; Content *et al.*, 2005). Here we summarize only the new findings for the uptake of iron by *M. tuberculosis*.

Throughout the living world, iron is contained in the active centres of most redox enzymes. Because iron occurs in the insoluble Fe^{3+} form under oxic conditions (10^{-9} M Fe^{3+} in soil and water) (Ratledge & Dover, 2000), proteins and siderophores with high binding affinity are required to make Fe^{3+} biologically available. *M. tuberculosis* produces salicylate-containing siderophores named mycobactins. The more polar form (carboxymycobactin) is released into the medium, whereas the less polar form (mycobactin) remains cell-associated (Ratledge & Dover, 2000). Upon binding by siderophores, Fe^{3+} is transported into the bacterium and released from the siderophore, possibly by reduction. In most bacteria, Fe^{3+}–siderophore complexes bind to specific receptor proteins on the cell surface and are actively transported into the cytoplasm by specialized proteins that belong to the family of ABC transporters (Braun & Killmann, 1999). The ABC transporter IrtAB is required by *M. tuberculosis* to replicate in iron-deficient medium and to use Fe^{3+}–carboxymycobactin as an iron source, indicating that IrtAB is involved in the transport of Fe^{3+}–carboxymycobactin (Rodriguez & Smith, 2006). Deletion of the *irtAB* genes also reduced the ability of *M. tuberculosis* to survive in macrophages and in the lungs of mice. However, the lack of *irtAB* does not completely eliminate replication of *M. tuberculosis* in iron-deficient conditions, which indicates that other transporters can partially compensate for the lack of IrtAB (Rodriguez & Smith, 2006). Given the importance of iron, this is not surprising because pathogenic bacteria often have multiple pathways for iron acquisition. The genome of *M. tuberculosis* does not reveal other obvious siderophore transporters (Rodriguez, 2006). However, there are numerous ABC transporters for which the substrates are unknown; one or more of these could contribute to iron uptake.

Transporters of amino acids

Many micro-organisms use amino acids as a source of energy and/or nitrogen, and also for biosynthetic purposes. It was shown early in seminal papers by Yabu that D-amino acids are taken up rapidly by mycobacteria while the L-forms are transported at a much lower rate (Yabu, 1967, 1970, 1971). These results can be attributed to the specificity of the inner-membrane transporters for the natural form of amino acids. It was also found early on that *M. tuberculosis* cannot utilize amino acids to support metabolism, in contrast to saprophytic mycobacteria (Edson, 1951). Nevertheless, some amino acids are taken up by *M. tuberculosis* and *M. bovis* BCG (Seth & Connell, 2000). In particular, uptake of arginine was examined because arginine also plays an important role in the cellular immune response as the substrate of the inducible nitric oxide synthase (iNOS), which generates nitric oxide to kill bacterial and parasitic pathogens in macrophages (Chan *et al.*, 2001; Nathan & Shiloh, 2000). Thus, competition between the pathogen and macrophages for arginine has been suggested to contribute to the outcome of infection (Mills, 2001). Not surprisingly, *M. tuberculosis* has several genes encoding putative L-arginine uptake transporters: Rv0522, Rv1979c, Rv1999c, Rv2320c and Rv3253c (Cole *et al.*, 1998). Transport of L-arginine, but not of L-lysine and L-ornithine, was reduced by 70 % in a mutant of *M. bovis* BCG lacking the gene homologous to *rv0522*. This identified Rv0522 (GabP) as an arginine transporter of *M. tuberculosis* (Seth & Connell, 2000). The remaining 30 % of L-arginine transport activity and the uptake of other cationic amino acids by the mutant are probably mediated by other amino acid permeases.

Conclusions

It is apparent that the uptake of nutrients by mycobacteria is not well understood, despite its importance for understanding the physiology and pathogenicity of *M. tuberculosis* and the availability of a wealth of genomic information. The knowledge gap begins with the uncertainty about which are the main essential nutrients of *M. tuberculosis* inside the host. This obviously depends on the location of *M. tuberculosis* inside the human body, which may vary from phagosomes in macrophages and dendritic cells (Russell, 2003) to granulomas (Ulrichs & Kaufmann, 2006) and even fat cells (Neyrolles *et al.*, 2006). There is clearly a need to determine the nutrients available in those different environments and to identify the proteins that are employed by *M. tuberculosis* to take up these nutrients. A solid functional, biochemical and structural analysis of these membrane proteins is a prerequisite to understanding the transport processes across both the inner and outer membrane of *M. tuberculosis*. Comparisons with the saprophyte *M. smegmatis* are not expected to contribute much to understanding the physiology and virulence of *M. tuberculosis*. However, studying nutrient uptake pathways in *M. smegmatis* should reveal the principal mechanisms by which the permeability barriers in mycobacteria can be overcome. Examples are presented in this review. It is striking that *M. tuberculosis* uses far fewer carbohydrate transporters than *M. smegmatis*. Further, the set of outer-membrane porins is apparently also different. These differences probably represent adaptations of *M. tuberculosis* to its slower growth (Cox & Cook, 2007) and to its very different natural habitat.

The comprehensive identification and the biochemical and structural characterization of the nutrient transporters of *M. tuberculosis* will not only promote our understanding of the physiology of this important human pathogen, but might also be exploited to improve tuberculosis chemotherapy. In particular, outer-membrane proteins

offer the tremendous advantage as drug targets that inhibitors may not need to cross the outer membrane, which is an extremely efficient permeability barrier in mycobacteria. In addition, they are likely to represent novel drug targets because they do not appear to show any similarity to other proteins (Faller *et al.*, 2004). It is my hope that this review will stimulate experimental work to examine nutrient transport pathways in mycobacteria.

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