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PERSPECTIVE

Bacterial multi-solute transporters

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Bacterial membrane proteins of the SbmA/BacA family are multi-solute transporters that mediate the uptake of structurally diverse hydrophilic molecules, including aminoglycoside antibiotics and antimicrobial peptides. Some family members are full-length ATP-binding cassette (ABC) transporters, whereas other members are truncated homologues that lack the nucleotide-binding domains and thus mediate ATP-independent transport. A recent cryo-EM structure of the ABC transporter Rv1819c from *Mycobacterium tuberculosis* has shed light on the structural basis for multi-solute transport and has provided insight into the mechanism of transport. Here, we discuss how the protein architecture makes SbmA/BacA family transporters prone to inadvertent import of antibiotics and speculate on the question which physiological processes may benefit from multi-solute transport.

Keywords: ABC transporter; antibiotics uptake; *Mycobacterium tuberculosis*; non-specific uptake; transport mechanism

Hydrophilic antibiotics that exert their function inside the cell, such as aminoglycosides and some antimicrobial peptides, enter the cytoplasm *via* membrane transporters that have evolved to support a physiologically relevant function. In some cases, the transported antimicrobial molecule is chemically related to the transporter's physiological substrate, for instance peptide antibiotics that are taken up by peptide transporters [1,2]. In other cases, membrane proteins that facilitate import of antibiotics appear not to be specific for a particular chemical class of compounds and instead transport multiple, structurally unrelated hydrophilic compounds. The best characterized proteins with such a multi-solute transport activity are members of the SbmA/BacA family, which are widespread in Proteobacteria and Actinobacteria. SbmA from *Escherichia coli* is an integral membrane protein with unassigned physiological function [3], while BacA in *Sinorhizobium meliloti* is important for establishing effective symbiosis with leguminous plants [4]. These two proteins do not belong to the ATP-binding cassette (ABC) transporter family, because they lack the characteristic nucleotide-binding domains (NBDs) [5]. However, they may be considered as truncated versions of ABC transporters as their sequences share significant similarity to the transmembrane domains (TMDs) of two poorly characterized subgroups of ABC transporters: ExsE/YddA proteins and BacA-like ABC transporters (Figs 1 and 2). The latter group includes Rv1819c from *Mycobacterium tuberculosis*, which is needed for the maintenance of chronic infection in mice [6]. Similar to Sbm A and BacA, Rv1819c mediates the uptake of antimicrobial peptides and other toxins, such as bleomycin [6]. Yet, Gopinath *et al.* [7] found that the *rv1819c* gene is also essential for the import of vitamin B12 (cobalamin) in *M. tuberculosis*, which may be an indication of the physiological function of the protein. Notably, *M. tuberculosis* does not contain any of the well-characterized bacterial membrane transporters for vitamin B12 (BtuCDF, ECF-CbrT, BtuM) [8–13].

The mechanism by which structurally unrelated hydrophilic solutes are accepted for transport by a

Abbreviations
ABC, ATP-binding cassette; NBDs, nucleotide-binding domains; TMDs, transmembrane domains; MccB17, microcin B17; ECF, energy-coupling factor.

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single SbmA/BacA family protein remained a conundrum until recently, when a structure of Rv1819c was solved [14]. Here, we discuss the discovery, structure, proposed mechanism of transport and potential physiological roles of multi-solute transporters.

Discovery of multi-solute transport activity by SbmA and BacA

The *sbmA* gene was discovered in a study on the sensitivity of *E. coli* to the antimicrobial peptide microcin B17 (MccB17), and the gene name is an acronym for sensitivity to B17 microcin [15]. Disruption of the *sbmA* gene reduced the sensitivity for exogenous MccB17, but did not confer resistance to endogenously produced MccB17, leading to the hypothesis that SmbA has a role in the transport of the peptide [15]. Later studies revealed that SbmA is also required for sensitivity to a variety of other antimicrobial peptides, aminoglycoside antibiotics, peptide nucleic acids and antisense peptide morpholine oligomers [16–21]. *E. coli* mutants with a disrupted *sbmA* gene showed an increase in resistance against all of these molecules. Although structurally unrelated, two common features characterize all SbmA substrates: hydrophilicity and the need to pass the cytoplasmic membrane to exert an antimicrobial activity on intracellular targets [20–25]. Consequently, the role of SbmA in antibiotic sensitivity seems to be that of an importer for structurally diverse hydrophilic antibiotics. Even though SbmA has been extensively investigated for its involvement in antibiotic sensitivity, little is known about its physiological functions.

SbmA is closely related to the BacA protein from the plant symbiont *S. meliloti* (64% sequence identity), where it is involved in development of the nitrogen-fixing bacteroid form of the bacterium, hence the name BacA [4]. Alike SbmA, BacA has been implicated in import of the same antimicrobial peptides and aminoglycoside antibiotics. Many of the known phenotypical traits of the *E. coli sbmA* mutant, such as antibiotic resistance, can be complemented by *bacA* expression and *vice versa* [16]. However, there are also some effects that are only observed in BacA-deficient *S. meliloti*. For instance, *S. meliloti bacA* mutants have increased sensitivity to membrane destabilizing agents, suggesting that BacA has a role in maintaining membrane integrity [16,26].

SbmA and BacA belong to a diversified family of transport proteins

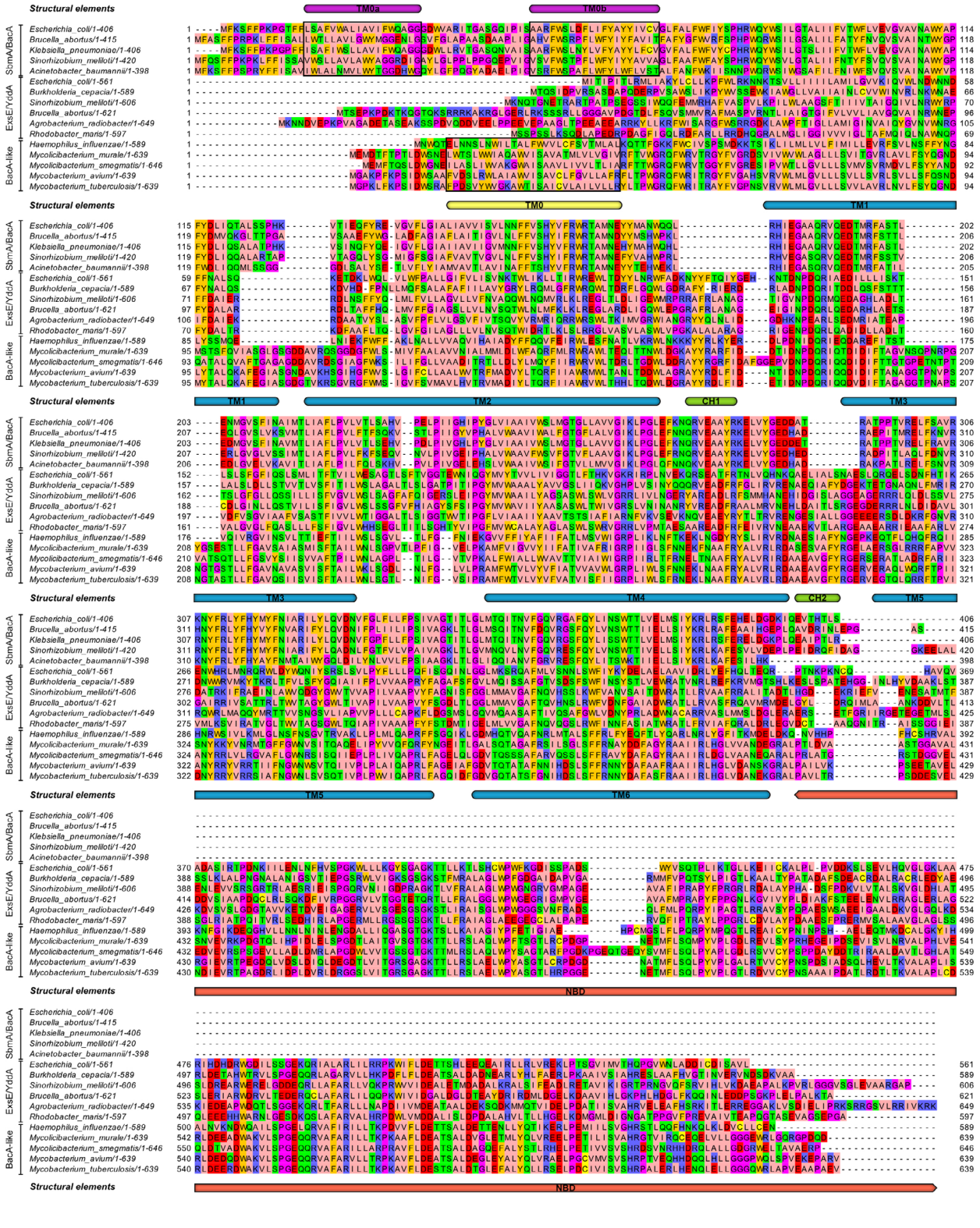
SbmA and BacA are hydrophobic proteins consisting of eight predicted membrane-spanning segments, with no large extramembranous domains (Figs 1 and 2). Size-exclusion chromatography coupled with multi-angle laser light scattering revealed that detergent-solubilized SbmA forms dimers, which was also corroborated by low-resolution structural data [3,27]. Sequence analysis of SbmA and BacA revealed that they belong to a family of membrane proteins that consist of two main groups (Figs 1 and 2): proteins of 320–420 amino acids in length (like BacA and SbmA) that are largely hydrophobic and do not contain extensive hydrophilic or extramembranous domains (found in Proteobacteria), and longer versions that consist of 550–750 amino acids (found in Proteobacteria and Actinobacteria) [6]. The greater length of the proteins in the latter group is caused by the presence of a C-terminal cytosolic extension that is the characteristic hallmark of ABC transporters: the NBD (Fig. 1). The sequence similarity of the transmembrane parts of the long and short proteins is the shared feature between these two groups [28,29].

Of the long proteins, Rv1819c from *M. tuberculosis* has been characterized best. Rv1819c has overlapping functional characteristics with SbmA. Heterologous expression of the *rv1819c* gene restores sensitivity of the *E. coli sbmA*-deficient strain to antimicrobial peptides, as well as to the antibiotic bleomycin [6]. Mutant variants in which the ATPases are inactivated fail to complement, showing that the sensitivity to the antibiotics depends on the presence of functional NBDs [14]. In addition to antibiotics uptake, Rv1819c also mediates uptake of vitamin B12, which underlines the multi-solute transport characteristics [7,14].

Members of the SbmA/BacA family are predominantly found in bacteria, yet the human lysosomal transporter ABCD4, which is involved in cobalamin transport from the lysosomal lumen to the cytoplasm, has been identified as a distant homologue [7,30]. Whether this ABC transporter also operates as a multi-solute transporter remains to be determined.

It was initially thought that the activity of SbmA from *E. coli* would depend on complex formation with a separately encoded NBD to form a complete ABC transporter, but such an NBD has never been identified [27]. Instead, *in vivo* uptake of fluorescently labelled substrates was shown to depend on a proton gradient instead of the internal ATP concentration [27] indicating that SbmA is a secondary transporter. Intriguingly, SbmA does not require the presence of NBDs to form the dimeric assembly which is characteristic for ABC transporters.

The existence of ATP-independent transporters (lacking NBDs) that are homologous to the TMDs of



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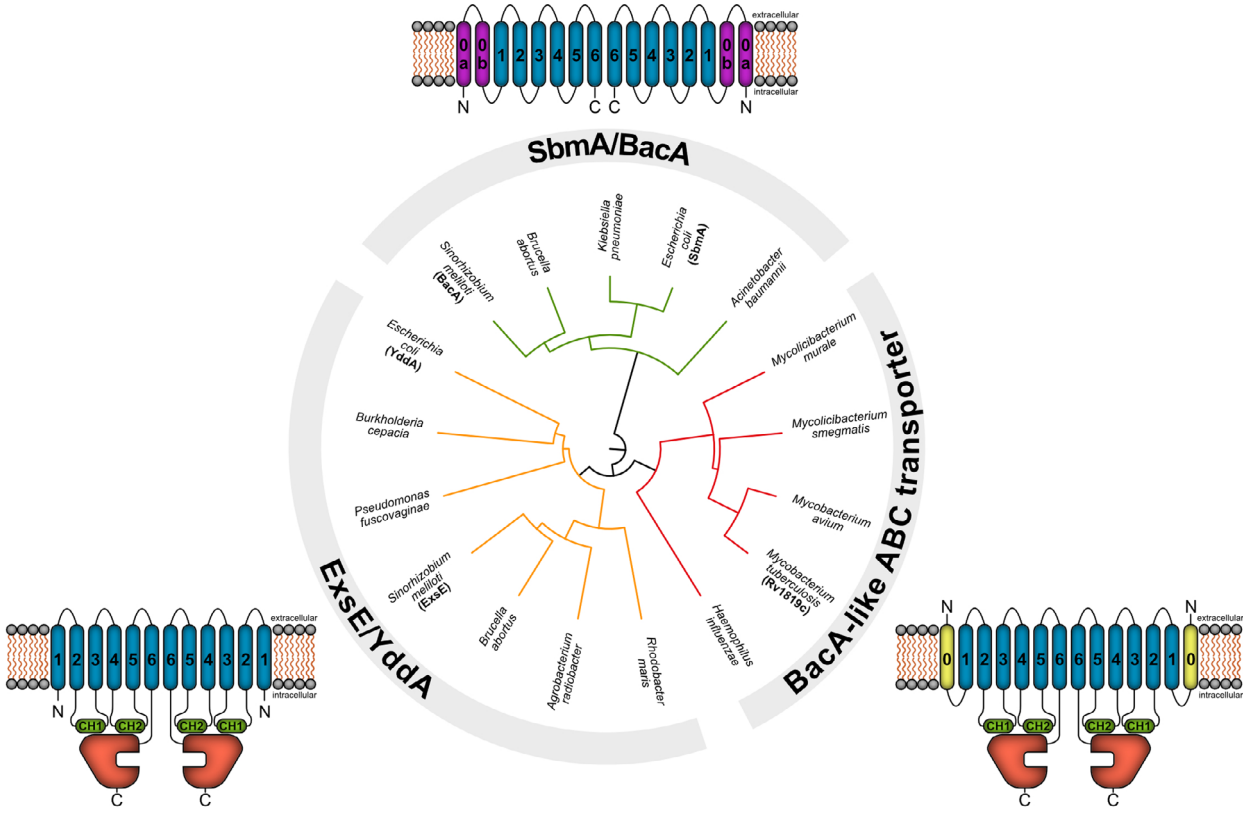

Fig. 2. Phylogenetic tree and membrane topology models of multi-solute transporters belonging to the SbmA/BacA transporter family. The phylogenetic tree was generated from a multisequence alignment in Clustal Omega [55] with the transmembrane segments of representatives of the SbmA/BacA transporter family, and visualized using FIGTREE v.1.4.4 (available from http://github.com/rambaut/figtree/). Topology models were determined using PRODIV-TMHMM [57]. All illustrations were generated with AFFINITY DESIGNER v.1.8.3.

full ABC transporters is rare but not unique. For instance, within the energy-coupling factor (ECF) transporter family (Type III ABC transporters) most members form complexes of two membrane subunits and two NBD subunits for ATP-dependent transport [31]. However, some organisms encode only one of the integral membrane subunits (named the S-component) which has been shown to transport substrates alone [8,32]. Another example is the ABC transporter LmrA, a multidrug efflux pump, that has been engineered into a proton–drug symporter by deleting the NBDs [33].

Structural insight into multi-solute transport

A recent structure of Rv1819c from *M. tuberculosis,* determined by single particle electron cryo-microscopy, has provided a first glimpse into the mechanism of multi-solute transport [14]. It is noteworthy that it was necessary to inactivate the protein by a glutamate-to-glycine substitution at the C terminus of the conserved Walker B motif in the NBD to obtain a biochemically well-behaving preparation of the protein. The mutation caused Rv1819c to be trapped in an ATP-bound

Fig. 1. Multiple sequence alignment of representative members of the multi-solute transporter family. The multiple sequence alignment was performed with Clustal Omega [55] and was visualized in JALVIEW v.2.11.1.0 [56]. The Zappo colour scheme is used to show the physiochemical properties. Structural elements are highlighted as follows: the region corresponding to conserved transmembrane helices (TM) 1–6 among the members of the multi-solute transporter family is indicated by blue bars; the region corresponding NBD is indicated by a red bar; the region corresponding to the CH 1 and 2 is indicated by a green bar; the region corresponding to the additional N-terminal transmembrane helices (named TM0a and TM0b) among the SbmA/BacA transporters is indicated by purple bars and boxes; and the region corresponding to the additional transmembrane helix TM0 from Rv1819c is indicated by a yellow bar and a box. All illustrations were generated with AFFINITY DESIGNER v.1.8.3 [Serif (Europe) Ltd, Nottingham, UK].

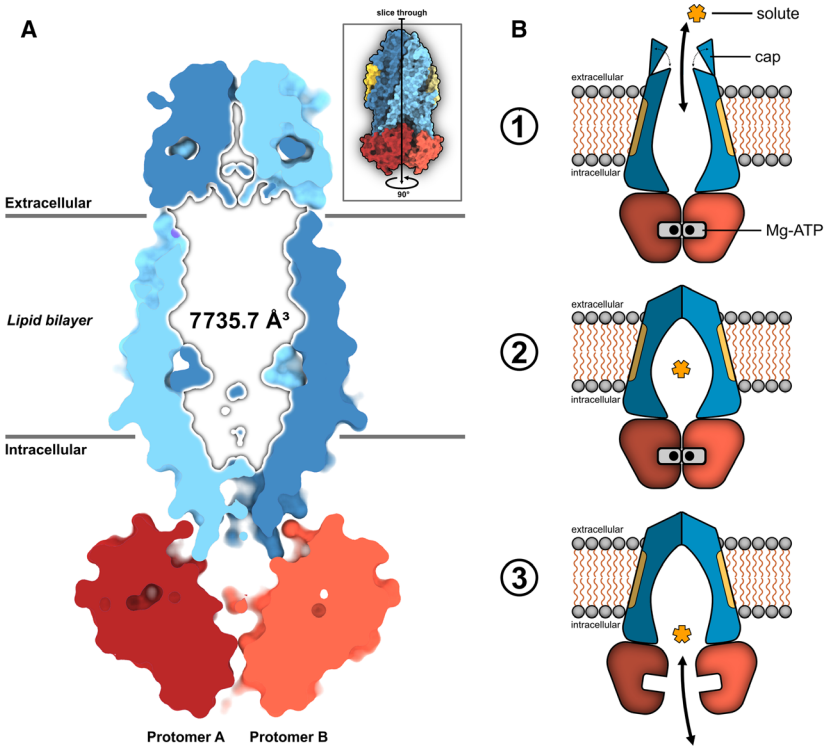

Fig. 3. Structural and mechanistic insights in Rv1819c from *M. tuberculosis*. (A) Large cavity of Rv1819c. A cross section of Rv1819c is shown revealing the cavity (PDB: 6TQF). The inset shows the position of the slice through, and the position of the lipid bilayer is indicated as lines. (B) Proposed transport mechanism. Cross sections of the transporter are illustrated. In the ATP-bound state, the external gate may be flickering between a hypothetical ATP-bound outward-facing open state (1) in which the extracellular gate is open, thus allowing a solute (illustrated as an asterisk) to enter into the large cavity, and the ATP-bound occluded state (PDB: 6TQF), with the alleged solute trapped in the large cavity. (3) Upon ATP hydrolysis, the release of inorganic phosphate and/or ADP presumably opens the intracellular gate (2), which releases the solute into the cell. The transporter then returns to the starting conformation (1) upon binding to ATP. ChimeraX 1.0 was used to generate the cross section and the full structure of Rv1819c in surface representation [58]. All illustrations were generated with AFFINITY DESIGNER v.1.8.3.

conformation, which is of importance for the mechanistic interpretation of the structure.

Rv1819c has the typical architecture of a type IV ABC transporter (Thomas, C. *et al.*, *FEBS Lett*. 2020, submitted for this special issue). This architecture was previously described as the 'ABC exporter fold', but the new name 'type IV ABC transporter fold' is now preferred because many proteins with this architecture do not have exporter functions [7,14] (Thomas, C. *et al.*, *FEBS Lett*. 2020, submitted for this special issue). Two identical Rv1819c protomers form a symmetrical dimeric assembly, with both NBDs and TMDs interacting (Fig. 3A). Like all type IV ABC transporters, the Rv1819c protomer has a core of six membrane-spanning segments per TMD, but it also contains an additional membrane-spanning helix at the N terminus. Compared to the short proteins in the family (SbmA, BacA), the core of six membrane-spanning segments is conserved (Fig. 1). The short transporters SbmA and BacA also have an N-terminal extension, in these cases containing two predicted transmembrane helices (Fig. 1), but the sequences of the extensions of Rv1819c and SbmA/BacA are not conserved (Fig. 1). The functional role of the extra N-terminal helices is yet to be elucidated. Similar to other type IV ABC transporters, Rv1819c contains two short helical segments per protomer in the cytoplasmic loops connecting TM3 and TM4, and TM5 and TM6, respectively (Fig. 1). These so-called coupling helices (CH) mediate noncovalent interaction between the NBDs and the TMDs and ensure conformational coupling between the two domains upon ATP binding and hydrolysis [34]. Remarkably, the CH appear to be absent from the short proteins in the family (Fig. 1), consistent with the absence of NBDs in these transporters.

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The most remarkable structural feature of Rv1819c is that the protein possesses a massive occluded cavity, with a volume of over 7700 Å^3, which spans the entire thickness of the lipid bilayer (Fig. 3A). The surface of this water-filled cavity is lined with polar and negatively charged residues. Although the cavity is occluded in the ATP-bound conformation of the inactive mutant protein, it is likely to open alternately to the cytoplasmic and extracellular side of the membrane during ATP-driven transport activity, *via* gates on either side (Fig. 3B). In the ADP-bound or nucleotide-free state, the internal gate is expected to be open, similar to what has been observed in other ABC transporters [34]. Opening of the external gate may occur in the ATP-bound state, by 'flickering' between the occluded and outward-open conformations, similar to what was found for the peptide exporter McjD, where single-molecule FRET experiments revealed that the external gate can open with short dwell times in the ATP-bound state [35]. It is possible that solutes can simply diffuse into the water-filled cavity when the external gate is open, get trapped when the gate closes and diffuse away on the other side of the membrane when the internal gate opens, which may explain the multi-solute transport activity of the transporter. It cannot be excluded that the reverse flow of compounds from the cytoplasm to the surrounding environment is also facilitated by the protein.

The cavity in Rv1819c has very similar hydrophilic surface properties as that of the peptide exporter McjD [36], but there are also notable differences between the two proteins. First, McjD is selective for the peptide Mccj25, while Rv1819c displays multi-solute transport activity. Second, the ATPase activity of McjD is stimulated by the binding of substrate, whereas the (basal) ATPase activity of Rv1819c, albeit essential for transport, is not stimulated by the transported substrates vitamin B12 and bleomycin [14]. This observation suggests that there is no specific interaction between the transporter and the substrates and that uncoupled, facilitated diffusion takes place. Such a mechanism contrasts sharply with what has been found for most ABC transporters, where substrate translocation is coupled to ATP hydrolysis allowing the movement of transported molecules uphill against their electrochemical gradients. A notable exception is the cystic fibrosis transmembrane regulator, which belongs to the ABC transporters superfamily, but is an ATP-gated chloride channel rather than a transporter [37]. Finally, the volume of the cavity in McjD matches the size of the transported substrate, while the cavity in Rv1819c is much larger than any of the known substrates (for instance, it measures ~ 7 times the volume of a vitamin B12 molecule). It is therefore possible that Rv1819c has a basal activity of cycling through inward-open, occluded and outward-open states to allow packages of solutes to be exchanged between the cytosol and external environment.

A structure of the human lysosomal cobalamin transporter ABCD4 that is distantly related to Rv1819c has also been solved recently [38]. The ABCD4 structure revealed a wide-open gate on the luminal side of the lysosomal membrane (an outward-open conformation). In this state, the protein captures vitamin B12, which is then transported to the cytoplasm. Even though the gate is open, no specific binding site for vitamin B12 was found. This observation may suggest that ABCD4 is not specific for vitamin B12 and might also have multi-solute transport activity, which remains to be investigated. Although there are no structural data on other members of the family, it is tempting to speculate that multi-solute transport activity must be linked to large water-filled cavities and the absence of specific binding sites.

Members of the SbmA/BacA family can be considered as the counterpart of multidrug efflux pumps, many of which also have the type IV ABC transporter fold, for example P-glycoprotein [39]. Similar to multi-solute transporters, these pumps recognize and transport a large variety of structurally diverse compounds (for reviews, see Ref. [39,40]). Unlike multi-solute transporters, these compounds are hydrophobic and transported in the outward direction. Instead of a hydrophilic cavity, the efflux pumps contain hydrophobic pockets [41], usually of much smaller volumes than the hydrophilic cavity observed in Rv1819c.

Physiological roles of SbmA/BacA family proteins

The role of SbmA from *E. coli* in conferring sensitivity to antibiotics has been investigated extensively, but little is known about the physiological function of this transporter. There is a slightly better understanding of the role of BacA in *S. meliloti,* a plant nodule-inducing bacterium that develops into nitrogen-fixing bacteroides inside nodule cells. BacA is essential for the symbiosis between *S. meliloti* and leguminous plants [4]. *S. meliloti* differentiation into bacteroides is tightly controlled by the host *via* the release of nodule-specific cysteine-rich (NCR) peptides. NCR peptides comprise a large number of different peptides that are expressed in infected nodule cells, in distinct subsets during different stages of colonization [42,43]. Due to the large variety of NCRs, it is conceivable that they could regulate multiple intracellular processes and that BacA

mediates import of all these peptides. Some NCR peptides also possess antimicrobial activity by compromising the integrity of the cytoplasmic membrane. *S. meliloti* mutants with disrupted *bacA* genes are hypersensitive to these NCRs and quickly perish due to a loss of membrane integrity, accompanied by a change in the lipid A composition of the outer membrane [26,44]. Exactly how BacA influences membrane integrity remains unclear [44–46].

With regard to Rv1819c from *M. tuberculosis*, it is likely that vitamin B12 be a physiological substrate [7]. While specific vitamin B12 transporters have evolved in other bacteria (BtuCDF, BtuM, ECF-CbrT), these transporters are absent in *M. tuberculosis* [8,9,47]. Taking into account that dedicated transporters are more efficient in vitamin B12 scavenging and transport than unspecific ones, vitamin B12 uptake is unlikely the sole physiological role of Rv1819c. Other members of the SbmA/BacA family have not been studied much, and it remains to be determined if they are multi-solute transporters as well [48].

Conclusions and Perspectives

Uptake of antibiotics cannot be the function that has determined the rise of the SbmA/BacA family during evolution; thus, a prime question to be answered in the future is why bacterial cells have maintained multi-solute transporters that make them vulnerable to antibiotics. The bacteria containing SbmA/BacA proteins are engaged in parasitic or symbiotic relations with their eukaryotic host [29]. It has been demonstrated that SbmA/BacA proteins in these species are essential for proper host colonization or persistence of infections [6,49,50]. Therefore, it is possible that they evolved for functions involving interactions with eukaryotes. The uptake of NCR peptides by *S. meliloti* may be an indication of such a function. The structure of Rv1819c further supports the possibility that peptides are physiological substrates of SbmA/BacA transporters: nonprotein density is visible in the water-filled cavity of the protein and could originate from bound peptides, picked up during protein production in the heterologous expression host *E. coli* [14]. However, it is unlikely that peptides are the sole substrates of multi-solute transporters, because other uptake systems, such as oligopeptide transporters, might be more suited for specific uptake of peptides.

Another clue for a potential physiological role of multi-solute transport comes from the observation that expression of the *sbmA* gene in *E. coli* and in *Salmonella enterica* is regulated by the extracytoplasmic function sigma factor σ^E [51,52]. This sigma factor governs a signal transduction pathway involved in the expression of genes involved in the envelope stress response in *E. coli* [53,54], including genes coding for periplasmic proteases [51]. SbmA might retrieve from the periplasmic space peptides that are the product of degradation of misfolded proteins. Other (damaged) cell envelope components such as cell wall fragments might be salvaged in the same way by this multi-solute transporter.

A second question to be addressed in the future relates to the transport mechanism. Although alternating access of the cavity to either side of the membrane is plausible for Rv1819c (Fig. 3B), further structural insight in the gating mechanism is needed. This is particularly important for the extracellular gate, since Rv1819c has a unique external cap that makes this gate thicker than in other type IV ABC transporters. The mechanism of energy coupling is also intriguing due to the presence of short and long orthologues in the SbmA/BacA family. The short SbmA variant from *E. coli* requires a proton gradient for transport [27], but it is unclear how coupling of the transport cycle to the proton flux occurs. Similarly, it is not entirely clear why the longer variants in the family have become dependent on ATP hydrolysis. Given that ATP hydrolytic activity of Rv1819c is not stimulated by the transported substrates, coupling between substrate translocation and ATPase activity seems to be weak [14]. Therefore, it is possible that the protein allows passive flux of compounds down their electrochemical gradients and that ATP hydrolysis (in the long proteins) or proton binding and release (in the short proteins) is only necessary for uncoupled gate opening and closure.

A final question that needs to be addressed is to what extent there exists some substrate specificity in the family. It is unlikely that these transporters are completely unspecific, since the chemical properties of the gates and cavity surface may select against the entry of some compounds [14]. For instance, the cavity of Rv1819c has a negative surface potential that could impede entry of negatively charged molecules. Such bias may prevent the escape of precious commodities such as nucleotides from the cytoplasm *via* the transporter. A complete lack of specificity may also lead to exceedingly low transport rates. The presence of one substrate molecule in the cavity (volume of $\sim 0.8 \times 10^{-23}$ L) would correspond to a substrate concentration of ~ 0.2 M. However, compounds like antibiotics and vitamin B12 are present at much lower, often subnanomolar concentrations in the surroundings, leading to mostly empty cavities in case of complete nonspecificity. Even with multiple copies of the

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protein being inserted in the membrane and turnover numbers in the second range, the flux of molecules across the membrane would still be minute. Therefore, some level of binding specificity is necessary to guide the desired solutes into the cavity. Alternatively, accumulation of the compounds to be transported in the periplasm may also increase the rates to functionally relevant levels. In any case, multi-solute transporters appear to be most suitable for transport of compounds needed only in small quantities (such as vitamin B12), because a sufficient flux of bulk nutrients is unlikely to be reached under homeostatic conditions.

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