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Tripartite ATP-independent periplasmic (TRAP) transporters and tripartite tricarboxylate transporters (TTT): From uptake to pathogenicity

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Author contribution statement

LTR:reviewed the literature, co-wrote the manuscript and co-analysed the bioinformatic data MEB: generated the bioinformatics data and co-analysed it. GT: edited and commented on the drafts. DJK: Conceived the idea and focus of the review, co-wrote and edited the paper.

Keywords

solute transport, Periplasmic Binding Proteins, secondary transporter, high-affinity, Carboxylic Acids

Abstract

Word count: 312

The ability to efficiently scavenge nutrients in the host is essential for the viability of any pathogen. All catabolic pathways must begin with the transport of substrate from the environment through the cytoplasmic membrane, a role executed by membrane transporters. Although several classes of cytoplasmic membrane transporters are described, high-affinity uptake of substrates occurs through Solute Binding-Protein (SBP) dependent systems. Three families of SBP dependant transporters are known; the primary ATP-binding cassette (ABC) transporters, and the secondary Tripartite ATP-independent periplasmic (TRAP) transporters and Tripartite Tricarboxylate Transporters (TTT). The TRAP and TTT systems were last subject to review in 2011 and 2003, respectively. Far less well understood than the ABC family, the TRAP transporters are found to be abundant among bacteria from marine environments, and the TTT transporters are the most abundant family of proteins in many species of B-proteobacteria. In this review, recent knowledge about these families is covered, with emphasis on their physiological and structural mechanisms, relating to several examples of relevant uptake systems in pathogenicity and colonisation, using the SiaPQM sialic acid uptake system from Haemophilus influenzae and the TctCBA citrate uptake system of Salmonella typhimurium as the prototypes for the TRAP and TTT transporters, respectively. High-throughput analysis of SBPs has recently expanded considerably the range of putative substrates known for TRAP transporters, while the repertoire for the TTT family has yet to be fully explored but both types of systems most commonly transport carboxylates. Specialised spectroscopic techniques and site-directed mutagenesis have enriched our knowledge of the way TRAP binding proteins capture their substrate, while structural comparisons show conserved regions for substrate coordination in both families. Genomic and protein sequence analyses show TTT SBP genes are strikingly overrepresented in some bacteria, especially in the B-proteobacteria and some α -proteobacteria. The reasons for this are not clear but might be related to a role for these proteins in signalling rather than transport.

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23 Abstract

24 The ability to efficiently scavenge nutrients in the host is essential for the viability of any 25 pathogen. All catabolic pathways must begin with the transport of substrate from the environment through the cytoplasmic membrane, a role executed by membrane transporters. 26 27 Although several classes of cytoplasmic membrane transporters are described, high-affinity 28 uptake of substrates occurs through Solute Binding-Protein (SBP) dependent systems. Three 29 families of SBP dependant transporters are known; the primary ATP-binding cassette (ABC) transporters, and the secondary Tripartite ATP-independent periplasmic (TRAP) transporters 30 31 and Tripartite Tricarboxylate Transporters (TTT). The TRAP and TTT systems were last subject to review in 2011 and 2003, respectively. Far less well understood than the ABC 32 33 family, the TRAP transporters are found to be abundant among bacteria from marine 34 environments, and the TTT transporters are the most abundant family of proteins in many 35 species of β -proteobacteria. In this review, recent knowledge about these families is covered, 36 with emphasis on their physiological and structural mechanisms, relating to several examples 37 of relevant uptake systems in pathogenicity and colonisation, using the SiaPQM sialic acid 38 uptake system from Haemophilus influenzae and the TctCBA citrate uptake system of 39 Salmonella typhimurium as the prototypes for the TRAP and TTT transporters, respectively. 40 High-throughput analysis of SBPs has recently expanded considerably the range of putative 41 substrates known for TRAP transporters, while the repertoire for the TTT family has yet to be fully explored but both types of systems most commonly transport carboxylates. Specialised 42 43 spectroscopic techniques and site-directed mutagenesis have enriched our knowledge of the 44 way TRAP binding proteins capture their substrate, while structural comparisons show 45 conserved regions for substrate coordination in both families. Genomic and protein sequence 46 analyses show TTT SBP genes are strikingly overrepresented in some bacteria, especially in 47 the β -proteobacteria and some α -proteobacteria. The reasons for this are not clear but might 48 be related to a role for these proteins in signalling rather than transport.

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511. Solute Binding-Protein (SBP) dependant secondary transporters: The TRAP and52TTT systems

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54 Solute Binding-Protein (SBP) dependent transport systems contain, in addition to the membrane proteins, a soluble extra-cytoplasmic protein, located either free in the periplasm 55 56 or anchored to the membrane in the case of Gram-positive bacteria, which binds the substrate 57 with high affinity and specificity, allowing uptake even in very low concentrations of ligands. 58 Three families of SBP dependant transporters are currently known, the composition of which 59 are summarized in Fig. 1. ATP-binding cassette (ABC) transporters use the free energy of 60 ATP binding and hydrolysis to move substrates across the membrane against a concentration gradient. First described in the early 1970's (Kalckar 1971, Willis and Furlong 1974), this 61 62 family is by far the best investigated SBP-dependant transporter family, with the maltose and 63 vitamin B₁₂ uptake systems as the most thoroughly studied models, and was subject of several reviews over recent years (Jones and George 2004, Davidson et al. 2008, Rice et al. 64 65 2014, Maqbool et al. 2015, Wilkens 2015, Locher 2016).

66 The Tripartite ATP-independent periplasmic (TRAP) transporters (TC: 2.A.56) and Tripartite 67 Tricarboxylate Transporters (TTT) (TC: 2.A.80), on the other hand, use ion-electrochemical

68 gradients to move substrates in a symporter mechanism, thus being defined as secondary 69 transporters. These two families are significantly less well understood than ABC systems but 70 share a similar overall protein composition and topology, as well as genomic organization. In additon to the SBP's ("P" subunit in TRAP systems, "C" subunit in TTT), each system is 71 comprised of two transmembrane proteins, one well-conserved 12 transmembrane (TM) 72 domain protein ("M" subunit in the TRAP systems, "A" subunit in TTT) and one poorly 73 conserved 4 TM domain protein ("Q" subunit in TRAP systems, "B" subunit in the TTT) 74 (Forward et al. 1997, Winnen et al. 2003, Thomas et al. 2006, Hosaka et al. 2013) (Fig 1). 75 However, no sequence similarity is found between the corresponding proteins in these 76 77 families, thus representing either a case of convergent evolution (Fischer et al. 2010) or very 78 ancient orthology and divergence (Winnen et al. 2003).

79 Regardless of their lack of sequence similarity, the SBPs from these two families show very 80 similar tertiary structures. They are folded in a 'Venus fly-trap' shape, with two wings 81 composed of one β -sheet containing four to six strands, surrounded by α -helices and 82 connected by a hinge. Opened in the *apo* form, the wings close around the substrate in a very 83 specific manner, binding the substrate tightly in a cleft formed between the two domains. The 84 enclosure of the substrate then allows the protein to interact with the transmembrane domains 85 (Herrou et al. 2007). It is suggested that these two wings were generated by a duplication 86 event in early TTT (and other SBP dependent) transporters (Winnen et al. 2003). 87 Classification of SBPs into related clusters has been proposed, based on their secondary and 88 tertiary structural patterns and their substrate specificities, with the first classification into 89 three distinct types proposed by Fukami-Kobayashi et al. (1999). With the exponential 90 increase in new entries for SBPs in genomic databases due to new sequencing capabilities, it 91 became clear that the separation into three types was too simplistic to comprise SBP 92 diversity, and thus a new model was presented by Berntsson et al. (2010) and recently revised 93 by (Scheepers et al. 2016). Both TRAP and TTT SBP's are contained within the Type II 94 group in the first classification, and inside Cluster E in the latter. This review summarises the 95 evidence of a relationship between these two classes of secondary high-affinity uptake systems and pathogenicity. Additionally, it adds an evolutionary perspective regarding the 96 97 expansion of the TTT family in some pathogens.

98

2. The TRAP Transporter family

99 The first characterization and naming of TRAP transporters was described in *Rhodobacter* 100 capsulatus by Forward et al. (1997), when a solute binding-protein encoding gene was found 101 adjacent to two genes encoding transmembrane proteins of 12 (DctM) and 4 (DctQ) predicted 102 helices. Functional studies showed symport of C4-dicarboxylic acids apparently energized by the proton motive-force. Subsequent studies showed that these systems can transport a variety 103 104 of substrates under different contexts. Detailed reviews about this family were provided by 105 Kelly and Thomas (2001) and Mulligan et al. (2011), and the following sections will focus on 106 more recent insights.

107 **2.1. Substrate diversity of the TRAP family and roles in pathogenicity**

108 The best studied TRAP system is undoubtedly SiaPQM from Haemophilus influenzae (Fig 1 109 and Fig 2a), discovered by Severi et al. (2005) to be involved in the uptake of sialic acid. 110 Sialic acid is a generic name for a class of 9-carbon sugar acids used by most eukaryotic cells 111 in the form of cell surface glycoproteins. For this reason, many pathogens evolved to mimic these surface structures in their own cell envelope, constituting an important virulence factor 112 113 which improves evasion of the human immune system (Bouchet et al. 2003). In H. 114 influenzae, absence of SiaPQM causes loss of sialic acid uptake and lack of incorporation in 115 the lipo-oligossacharide (Allen et al. 2005), and a subsequent study showed an increased 116 susceptibility of this pathogen to human serum and decreased virulence in the chinchilla otitis 117 model (Jenkins et al. 2010). Systems homologous to SiaPQM were subsequently found to be 118 involved in the uptake of sialic acid in several pathogens, such as Vibrio cholerae, 119 Fusobacterium nucleatum and Vibrio vulnificus (Severi et al. 2005), the latter being shown to transport sialic acid in 67 clinical isolates (Lubin et al. 2012). Signature-tagged mutagenesis 120 121 studies in Pasteurella multocida, an opportunistic pathogen of livestock, showed that 122 disruption of genes related to sialic acid metabolism resulted in decrease of virulence in mice 123 models (Fuller et al. 2000). A subsequent study showed that a SiaP homolog was involved in 124 the uptake of sialic acid in this bacterium (Steenbergen et al. 2005), and that disruption of 125 sialic acid uptake resulted in decreased virulence in a turkey model (Tatum et al. 2009). 126 Severi et al. (2007) provides a review of how sialic acid uptake and metabolism is used as a virulence factor in different pathogens, and Vimr et al. (2004) provides a more general 127 review about sialic acid metabolism. Thomas (2016) gives a recent overview of the different 128 129 uptake strategies and transport systems used by different pathogens for the uptake of sialic 130 acid.

131 In Bordetella pertussis, the causative agent of whooping cough, two DctP homologs are 132 encoded in the vicinity of virulence-related operons modulated by the BvgA/BvgS two-133 component system. The two proteins were crystalized by Rucktooa et al. (2007) with a 134 pyroglutamic acid bound in the substrate cleft. One of these proteins is highly expressed in *B*. 135 pertussis, although the membrane components of the system seem to be mutated and non-136 functional. Although it is unclear what physiological role pyroglutamic acid would have, this 137 amino-acid is present in the filamentous hemagglutinin produced by *B. pertussis*, and it was speculated it could serve as a glutamate reserve. In fact, BugE, an abundantly expressed SBP 138 139 from the TTT family also was shown to bind glutamate (Huvent et al. 2006), suggesting 140 glutamate metabolism might play an important role in the pathophysiology of this bacterium.

TRAP systems are very important also in environmental organisms and in biotechnologically 141 relevant processes. In Halomonas elongata, Grammann et al. (2002) showed that the 142 143 TeaABC operon was responsible for accumulation of the compatible solute ectoine, in 144 response to osmolarity stress, and that this transporter was osmoregulated. In Rhodobacter 145 capsulatus a TRAP system was shown to be involved in the import of several 146 monocarboxylic 2-oxo-acids involved in amino-acid biosynthesis (Thomas et al. 2006). Chae and Zylstra (2006) showed the involvement of TRAP transporters in the degradation of 147 148 several benzoate derivatives, including toxic chlorinated aromatics. In Rhodopseudomonas 149 palustris the TarPQM system was shown to be involved in the degradation of lignin-derived 150 aromatic compounds, in a redundant function also executed by an ABC transporter in the 151 same gene cluster (Salmon et al. 2013). GaaPQM from Agrobacterium tumefacis was 152 described to be involved in plant virulence (Zhao and Binns 2016). Maimanakos et al. (2016) showed that TRAP transporters are found in the vicinity of arylmalonate decarboxylases 153 154 (AMDases) and recently, Meinert et al. (2017) showed a TRAP system involved with the 155 uptake of five different sugars in Advenella mimigardefordensis, but only after they have 156 been converted to their respective sugar acids in the periplasm (Thomas 2017).

Vetting *et al.* (2015) published a highly significant study, which multiplied several times our understanding about substrate specificity in TRAP systems. 8240 SBP's were used to build a sequence similarity network, grouping them into several clusters. From these, 304 representatives of non-characterized groups were then screened, coupling differential scanning fluorescence, crystallography and mass spectrometry of co-purified ligands. The methodology shows the importance of using complementary methods and proposes an efficient strategy for the study of SBP's. As a result, 71 of the isofunctional clusters had a

ligand assigned; 69 high-resolution crystal structures were obtained; previously known 164 165 ligands were assigned to non-characterized clusters and several new ligands were found to be captured by TRAP transporters, such as D-glucuronate/D-galacturonate, 6-carbon aldonic 166 167 acids. cell-wall constituents, lipopolysaccharide components, glycerol-3-phosphate/ diglycerol-phosphate, 2-acetolactate, orotic acid, indole acids, pantoate/D-erythonate, and 168 ethanolamine, this last being a particular surprise due to its positive charge in contrast to the 169 170 typical negatively charged carboxylates of most other TRAP transporter substrates. This work was done as part of the Enzyme Function Initiative (EFI), a network aiming to characterize 171 172 the biochemical and physiological function of different classes of enzymes, among which are 173 soluble binding proteins, through high-throughput sequence/structure based strategies 174 (http://www.enzymefunction.org/).

175 **2.2. The neglected group: TAXI-TRAP transporters**

176 It was observed by Kelly and Thomas (2001) that, in some cases, the SBPs associated with 177 the DctQM subunits in the genome showed very limited sequence similarity to DctP, forming 178 a distinct group, TRAP associated extracytoplasmic immunogenic (TAXI) proteins, named 179 after an immunogenic protein of unknown function from the pathogen Brucella (Mayfield et 180 al. 1988). A previous study by Rabus et al. (1999) had found some similarity between TAXI 181 proteins and the E. coli glutamate binding protein, and the only structure available for a 182 TAXI protein, generated by Takahashi et al. (2004), reinforced these initial findings, as it was 183 described as a glutamate/glutamine binding protein. However, the deletion of a TAXI protein 184 from Psychrobacter arcticus was shown by Bakermans et al. (2009) to affect growth also in 185 other dicarboxylic acids such as acetate, butyrate and fumarate. TAXI-TRAP systems usually 186 have the DctQM subunits fused (Fig 1 and Fig 2A) and, because they are found also in many 187 Archaea species, it is believed that this system is an ancient form of TRAP transporter. 188 Although Mulligan et al. (2011) provided a brief speculation about potential function of 189 TAXI-TRAP systems based on their genomic context, a complete characterisation of this 190 group is still to be generated.

191 **2.3. Two is not enough: The TPAT system**

192 In addition to the classical TRAP and the TAXI-TRAP transporters, a third class of TRAP 193 system was characterised by Deka et al. (2012) in the pathogen Treponema pallidum. T. 194 *pallidum* is the causative agent of syphilis, a disease which continues to be a challenge in 195 global health. This organism is an obligatory pathogen, which lacks many vital biosynthetic 196 pathways for nucleotides, lipids and most amino-acids, relying on transport systems to obtain these vital requirements from the human host (Radolf et al. 2016). Deka et al. (2012) 197 198 observed the existence of a single operon encoding a TRAP system in T. pallidum genome, 199 composed of three genes, as shown in Fig. 2a. One dctP and one dctQM homologue, named 200 $tatP_T$ and tatQM, and a third gene of unknown function, named tatT. The biochemical and 201 crystallographic characterisation of TatT showed this soluble protein was formed by 13 a-202 helixes and one small helix, structured around a central hydrophobic pore which opened to 203 both ends of the structure. Some of these helices were homologous to a tetratricopeptide 204 motif (TPR), normally involved in protein-protein interactions (D'Andrea and Regan 2003), which gave the name for this group of TRAP transport systems as TPR-protein associated 205 206 transporters (TPAT). Using cross-linking, western blotting, analytical ultracentrifugation and computational modelling, it was shown that TatT formed a trimer, which in turn interacted 207 with three subunits of the DctP homolog TatP_T (Deka et al. 2012). A later study by 208 209 Brautigam et al. (2012) confirmed these predictions through crystallization of the TatT and 210 $TatP_T$ complex. In these structures, it was shown that the substrate cleft from $TatP_T$ was 211 aligned to the C-terminal side of the pore in TatT, with minor structural changes happening upon complexation, the main one being the displacement of one loop from TatP_T domain 2 in 212

213 contact with the binding cleft, called a "cleft-finger". The hydrophobicity observed both in 214 TatT pore and TatP_T cleft, together with the presence of a linear hydrophobic molecule crystalized in the TatT pore, suggested that this system is involved in the uptake of 215 216 hydrophobic molecules (Deka et al. 2012). As both proteins are found in vivo as lipoproteins, 217 anchored to the membrane, it was suggested as a mechanism that this interaction created a 218 chaperone environment for the transport of lipids through the periplasmic hydrophilic 219 environment, where TatT would receive the lipid from the host, anchored in the outer 220 membrane, and transfer it to TatP_T, anchored in the inner membrane, which in turn would 221 deliver it to the TatQM subunit (Brautigam et al. 2012). TPAT systems were found in 35 222 other species, among other spirochaetes and also among free-living proteobacteria. In this 223 latter group, it was mostly found in species capable of degrading hydrocarbons, reinforcing 224 the potential role in aliphatic transport this distinct group of TRAP transporters might have.

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226 **2.4. Biochemical and functional studies of the DctQM subunits**

227 Unlike the ABC transporters, no crystal structures have been obtained to date regarding the 228 membrane components of TRAP systems, however some mechanistic information is 229 available particularly regarding energy-coupling. In many systems, such as SiaPQM from 230 Haemophilus influenzae, the DctM and DctQ membrane units are not expressed separately, 231 but fused in one only protein containing 17 transmembrane helices, one more than expected 232 due to an additional helix that connects the cytoplasmic C-terminal part of DctQ with the 233 periplasmic N-terminal part of DctM (Fig 2a) (Mulligan et al. 2009). Even when expressed 234 separately, DctM and DctQ were shown to form a tight complex with a 1:1 stoichiometry 235 during the folding procedure, and attempts to separate the two proteins resulted in disruption 236 of function (Mulligan et al. 2012). While DctM is believed to form a translocation channel 237 and is a member of the ion transporter superfamily (Rabus et al. 1999), the role of DctQ has 238 not been established yet and has a much more variable sequence. It is known that it is 239 essential for transporter function and it was suggested that DctQ might act to mediate 240 interactions between DctM and DctP, chaperoning DctM and stabilising it in the membrane 241 or participating in energy coupling (Wyborn et al. 2001). Mulligan et al. (2009) performed a 242 series of experiments showing that the presence of Na⁺ ions was required for sialic acid 243 transport via SiaPOM in *H. influenzae*. Replacement of Na⁺ for Li⁺ ions did not result in 244 uptake activity, and although neither ΔpH or $\Delta \psi$ alone resulted in transport in absence of Na⁺, 245 the gradients were able to promote substrate uptake when Na⁺ was present in equal 246 concentrations in both sides of the membrane. These results show that substrate uptake in 247 TRAP transporters is Na⁺ dependent and characterised as an eletrogenic process, where at 248 least two Na⁺ ions are co-transported. Not surprisingly, the TRAP family is widely found in 249 bacteria living in saline environments, using the naturally provided Na⁺ gradient to provide 250 substrate uptake, as discussed by Mulligan et al. (2007). In addition, Mulligan et al. (2009) 251 showed that in opposition to conventional secondary transporters such as the ones from the 252 MFS family, the transport in the TRAP family is unidirectional. The substrate transporter 253 exposes the binding cavities alternatively in the cytoplasm and the periplasm, but because in 254 TRAP transporters the exposure in the periplasmic side only occurs when in interaction with 255 the SBP, movement in the opposite direction is blocked, even when gradients are inverted. 256 The only condition in which contrary movement was observed was in the presence of an 257 excess of un-liganded SiaP in the periplasm, but these conditions are not physiologically 258 relevant. In addition, it was shown that replacement of the SiaP in Haemophilus influenzae 259 (HiSiaP) by an homolog from Vibrio cholerae (VcSiaP) did not complement its function, suggesting that the interactions between DctP proteins and the membrane counterparts are 260 261 specific in each case, rather than promiscuous among the family (Mulligan et al. 2009).

Mulligan *et al.* (2012) performed these same transport assays and characterisation of the SiaQM subunits in the homologous system from *Vibrio cholerae*, which comprises a true tripartite system instead of the fused subunits. The results from this study were very similar to the *H. influenzae* fused SiaQM system.

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267 **2.5 Crystal structure and dynamics of TRAP SBP's**

The first crystal structure of a TRAP SBP was the SiaP protein from Haemophilus influenzae 268 269 (Muller et al. 2006). TRAP SBP's have wings very similar to the Type II proposed structure 270 by Fukami-Kobayashi *et al.* (1999), but with a remarkably large single β -strand, which connects both domains and participates in both β-sheet domains (Fig 3A). In addition, this 271 272 family contains a long α -helix, which spans both domains and kinks upon ligand binding. These features characterise the TRAP transporters in Cluster E of the division proposed by 273 274 Scheepers et al. (2016). The hinge-bending upon ligand-binding was estimated by Muller et 275 al. (2006) to be \sim 30Å based on comparison between unliganded and ligand protein crystals.

276 Although ligand positioning inside the binding pocket is conserved, the hydrogen bonds and 277 hydrophobic interactions for each molecule coordination vary, making substrate prediction 278 difficult for this family. A conserved arginine residue in domain 2, however, turns out to be 279 crucial for ligand interaction (Fig 3C), as discussed by Fischer et al. (2010, 2015). Localised 280 in β -strand 6, which is in a stable β -sheet, the side chain of this highly conserved residue 281 (96.8% of 6142 sequences searched) points towards the binding cavity and, unusually, is 282 stabilised through a hydrophobic patch and a hydrogen bond, with a bending in CB which 283 allows the side chain to reach the pocket (Fig 3A and Fig 3C). In the presence of ligand, it 284 makes a salt bridge with the ligand carboxylate group, believed to be the first step in ligand 285 coordination. This interaction is believed to be critical for proper functioning of most SBPs 286 from the TRAP family as high-affinity binding proteins, although it is not essential for the 287 coordination of domain closure upon ligand binding (Fischer et al. 2015). The TatP_T 288 homologs, believed to be involved in the uptake of aliphatic substrates, mostly lack this 289 residue, having it substituted for an alanine (Deka et al. 2012). Mutations of this arginine 290 residue in SiaP were shown by Johnston et al. (2008) to disrupt sialic acid uptake in H. influenzae and recently Fischer et al. (2015) showed that replacing it by a lysine decreased 291 292 the binding affinity for sialic acid by SiaP from 0.14 µM to 38.7 µM, and mutating it to an 293 alanine resulted in no binding. Crystallization of these two mutant proteins, however, showed 294 minor differences in ligand coordination, where in place of the missing N atoms, coordinated 295 water molecules bridged the carboxylic group of the ligand to the protein, dissipating the 296 negative charge. Subsequent growth experiments showed that cell growth could be restored 297 in the presence of high external concentrations of sialic acid, as the higher concentration 298 would compensate for the weaker affinity; In the same study, it is shown also that this water 299 coordination enables a higher promiscuity in the binding pocket, allowing it to coordinate an 300 analogue ligand containing an amide group in place of the carboxylic acid. In addition, 301 PELDOR spectroscopy analysis recently performed by Glaenzer et al. (2017) showed that no 302 intermediate state of VcSiaP is observed in solution upon ligand binding, which can only be 303 in an open or closed conformation. Moreover, the protein does not alternate to closed 304 conformation unless the ligand is present, a fact that supports the current model in which the 305 SBP will only return to the open conformation upon interaction with the membrane 306 components, avoiding unproductive opening and closing of the binding protein (Mulligan et 307 al. 2011).

In addition, the variable positioning of helix 3 across different proteins seem to be responsible for the adaptation of the binding pocket for different ligand sizes, given by structural changes in regions flanking this helix (Lecher *et al.* 2009). In some cases, generally for smaller TRAP ligands, cation atoms are also required for ligand coordination. However, as shown by Akiyama *et al.* (2009), these cations are usually non-specific, and have a structural role to bridge the interaction with the protein chain, and are not necessary when the ligands are capable of filling the respective space and interact directly (Fischer *et al.* 2010).

Although most TRAP SBPs are found to act as monomers, there is evidence that some of 315 316 them might require dimerization for function. Gonin et al. (2007) showed that TakP, a 317 pyruvate binding protein from Rhodobacter sphaeroides crystalized as a dimer, and the functional importance of dimerization was validated by tryptophan fluorescence quenching, 318 319 gel filtration and cross-linking experiments. The dimerization is believed to occur through a 320 kinked C-terminal helix, which swaps its position with the same portion of the dimer 321 counterpart. Additionally, Akiyama et al. (2009) crystalized a lactate binding protein from 322 Thermus thermophilus which interacts back-to-back in a dimerization process stabilised by 323 hydrophobic interactions in the C-terminal region of the protein. Finally, Cuneo et al. (2008) 324 confirmed also the dimerization state of a TRAP protein from Thermotoga maritima through 325 gel filtration analysis and X-ray scattering. It remains unclear, however, how this 326 dimerization process would promote or interfere with the transport mechanism when 327 interacting with the DctQM subunits.

3. The TTT family

329 As with the TRAP transporters, systems in the TTT family are composed of a conserved 12 330 TM protein, (TctA homologs) believed to act as a symport protein energized by an 331 electrochemical ion-gradient (although this has not been experimentally determined) and 332 poorly-conserved 4 TM protein (TctB homologs) with unknown function, in combination 333 with an SBP (TctC homologs) which binds the substrate with high affinity (Fig. 1) (Sweet et 334 al. 1979, Winnen et al. 2003). However, the TTT family has not been subject to many experimental studies and knowledge about this family is still scarce; the topic being last 335 336 reviewed by Winnen et al. (2003).

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338 3.1. Substrate diversity of the TTT family: Role of the solute binding-protein and occurrence in pathogens

340 As the prototype for the TTT family, the Tct citrate transporter was first described by Sweet 341 et al. (1979) in the pathogen Salmonella typimurium, one of the most important causative 342 agents of food-borne gastrointestinal infections and a growing problem due to the recent 343 emergence of multidrug resistant strains (Hur et al. 2012). TctC was found to be involved in 344 the uptake of the tricarboxylic acid citrate with low-µM affinity, with citrate uptake severely 345 reduced in this organism upon disruption of the *tctC* gene. This function gave the name to the family as tricarboxylate transporters (Sweet et al. 1979, Somers et al. 1981). A series of 36 346 347 tricarboxylate and di-carboxylate metabolites were later shown by Sweet et al. (1984) to 348 inhibit citrate binding to TctC to varying extents, suggesting that the substrate range for this 349 protein might not be restricted to citrate. Genetic mapping studies initiated by Somers and 350 Kay (1983) and finished by Widenhorn et al. (1988) showed that downstream of the tctC 351 locus there were two more encoded proteins, of 19 kDa and 45 kDa, corresponding, respectively, to the transmembrane proteins TctB and TctA (Fig. 1 and Fig. 2b). The gene 352 353 arrangement of *tctCBA*, is similar to that found for the majority of TRAP transporters 354 (Mulligan *et al.* 2011). Encoded in the opposite direction, a fourth gene, *tctD*, was shown by

355 Widenhorn *et al.* (1989) to encode a transcription regulator of the *tctCBA* operon, which was 356 found to be repressed when *tctD* was deleted or in the presence of glucose in the medium. Homologous systems to TctCBA are found in many bacteria, mainly Proteobacteria, and 357 358 citrate uptake is the commonest identified role for the few other TTT systems experimentally 359 characterised to date (Antoine et al. 2003, Brocker et al. 2009, Hosaka et al. 2013, Graf et al. 360 2016). Citrate has been shown to act in some cases as an iron chelator for different transport 361 systems (Yancey and Finkelstein 1981, Braun 2001, Luck et al. 2001, Banerjee et al. 2016), 362 and although the potential role of TctC acting as an iron transport protein has not been investigated to date, experiments performed with Salmonella typhimurium (Sweet et al. 1979) 363 showed that citrate binding to TctC is improved in the presence of $Na^+ Ca^{2+}$, Mn^{2+} and Fe^{2+} , 364 while partially inhibited by Mg^{2+} , Ni^{2+} , Zn^{2+} and Co^{2+} . In addition, growth experiments performed by Brocker *et al.* (2009) using a homologous TctCBA system from 365 366 367 Corynebacterium glutamicum showed that this system was able to uptake citrate in the presence of Ca^{2+} and Mg^{2+} , but not Sr^{2+} . 368

369 After the characterisation of TctC, all proteins homologous to the TctCBA systems in newly 370 released genomes were annotated either as unknown proteins or citrate uptake systems, and 371 this family was neglected for over a decade, until one TctC homologue was found by Antoine 372 et al. (2000) to be encoded upstream of the pertussis toxin (PTX) virulence island, one of the 373 most important toxins produced by the causative agent of whooping cough, Bordetella 374 pertussis. This gene was found to be conserved in this locus for different Bordetella species 375 and was named *bugT*, standing for '*Bordetella* uptake gene'. Although a relationship between 376 the BugT protein and the production of PTX was not confirmed, regions coding for other 377 virulence factors, such as the adenylate cyclase toxin (AC) and the dermonecrotic toxin 378 (DNT) also contained *bugT* homologs, while two *bug* homologs were negatively regulated by 379 the BvgAS two-component system, responsible for the activation of virulence factor 380 production (Antoine et al. 2000, Antoine et al. 2003). Recently, a single-nucleotide-381 polymorphism (SNP) in one bug gene was consistently identified in an Australian epidemic 382 strain of *B. pertussis* (Safarchi et al. 2016). As discussed in the next sections, further searches 383 in the *B. pertussis* genome found homologs of Bug proteins to be extensively 384 overrepresented, with 76 genes encoding distinct homologs (Antoine et al. 2000). In contrast, 385 only two sets of genes coding for transmembrane proteins homologous to tctAB were found 386 in *B. pertussis*, and most of the BugT homologs showed no obvious membrane counterparts 387 encoded in their genomic vicinity, hence the designation of them as "orphan" proteins.

The only complete operonic encoded TTT system in Bordetella pertussis, encoded by 388 389 bctCBA, contains bug4 as the tctC homolog, and was found to be the equivalent of tctCBA 390 from S. tvpimurium, as expression was upregulated by citrate and gene disruption resulted in 391 lower citrate uptake rates (Antoine et al. 2003). As shown by Antoine et al. (2005), upstream 392 of the *bctCBA* operon is encoded the two-component system *bctDE*, transcribed in the same 393 direction but forming a separate operon (Fig 2B), which showed a basal expression level 394 independent of citrate. When this two-component system was deleted, expression of bctCBA 395 was not detected, showing that *btcDE* was in fact activating transporter gene expression. 396 Disruption of the *bctBA* components, on the other hand, increased operon expression, due to 397 an accumulation of citrate in the periplasm to be directed to signalling purposes (Antoine et 398 al. 2005). Finally, when bctC was deleted, bctBA expression was reduced to basal levels even 399 in high citrate concentrations, inferring that the two-component system is enough to maintain 400 a basal level expression, but is not enough to enhance expression in the presence of citrate. Together, those data showed that citrate-bound BctC was required for both transport and 401 signalling, interacting either with BctE or BctA; a model confirmed in the same study by 402 403 bacterial two-hybrid assays, showing unprecedented evidence that TctC homologs can be

404 involved also in regulatory processes (Antoine et al. 2005). The presence of citrate 405 responsive regulatory genes and two-component systems adjacent to tctCBA operons is not uncommon, as shown for S. typhimurium by Widenhorn et al. (1989), for Comamonas sp. by 406 407 Hosaka et al. (2013), for Advenella mimigardefordensis by Wubbeler et al. (2014); and in the genomic searches provided by Antoine et al. (2003). Brocker et al. (2009) also characterised 408 409 a citrate-reponsive two-component system controlling *tctCBA* expression in *Corynebacterium* 410 glutamicum, although in this case the regulatory proteins were adjacent to another transport 411 system. Interestingly, some of the TTT systems are found in the genome with the *tctB* subunit 412 downstream of tctA, such as the slcHFG systems from Roseovarius nubinhibens (Denger et 413 al. 2009) and Chromohalobacter salexigens (Fig 2B) (Denger and Cook 2010). This feature 414 is also observed in some TRAP systems, and systems with this genomic organization are 415 thought to be more similar among them (Mulligan et al. 2011).

416 The study of two abundantly expressed Bug proteins led to the first crystal structures for TTT 417 family SBPs. These proteins, discussed in detail in the next sections, were fortuitously crystalized with substrates in their binding pocket. BugD contained an aspartate molecule 418 419 (Huvent et al. 2006) and BugE contained a glutamate molecule (Huvent et al. 2006). Amino-420 acids are the most important carbon and nitrogen sources for *B. pertussis*, which is incapable 421 of metabolism of substrates through the glycolytic pathway (Huvent et al. 2006); As the Bug 422 proteins are highly expressed, they might play a crucial role in uptake of core metabolic 423 pathways. Herrou et al. (2007) characterised one of the Bug proteins (Bug27), found to be 424 overexpressed in the presence of nicotinic acid, an essential vitamin and a negative modulator 425 of *B. pertussis* virulence. It was shown that this protein binds, with an affinity lower than 1 426 µM, not only to nicotinate, but also nicotinamide, citrate, benzoate and quinaldic acid. This 427 protein generated also the first TTT SBP crystal structure in an unliganded conformation 428 (Herrou et al. 2007). The binding of Bug27 to nicotinic acid/nicotinamide might suggest it 429 plays a role in virulence modulation, either by interacting with a membrane signal protein or 430 simply transporting nicotinic acid to the cytoplasm. Interestingly, Brickman et al. (2017) 431 suggested that another Bug protein, Bug69, might also be related to the uptake of nicotinic acid and related compounds. 432

433 Although not the focus of this review, the potential of the TTT family as a new source for 434 biotechnology relevant uptake systems was also exposed by the genomic analysis performed 435 by Antoine et al. (2003), where it was observed that in many organisms, the bug homologs 436 were located near operons that conferred specific abilities to each strain, such as catechol degradation, showing that the importance of this family is wider than the suggested so far and 437 438 that its diversity might correlate with the metabolic versatility and adaptability of an 439 organism. In addition, a genomic search regarding arylmalonate decarboxylases (AMDases) 440 by Maimanakos et al. (2016) found several members of the TTT family in the vicinity of 441 these enzymes for five of the eight predicted AMDase clusters, either as orphan proteins, in 442 the case of β -proteobacteria; or complete systems, in the case of α -proteobacteria, suggesting 443 the TTT proteins might act to import the carboxylated substrates for subsequent catalysis by 444 the AMDases. Other biotechnologically relevant discoveries include sulfolactate metabolic pathways in Roseovarius nubinhibens (Denger et al. 2009) and Chromohalobacter 445 446 salexigens, which contain a TTT uptake system for this substrate (Denger and Cook 2010) 447 named *slcHFG*; a TTT system from *Comamonas sp.*, TpiBA and TphC, able to uptake terephthalate (Hosaka et al. 2013); the TctCBA from Advenella mimigardefordensis able to 448 449 uptake the synthetic molecule disulfide 3,3'-dithiodipropionic acid (DTDP), a precursor for synthetic polythioesters (Fig 2B) (Wubbeler et al. 2014); a tctA homolog genetically 450 proximal to genes coding to esterase enzymes that degrade organophosphates and potentially 451 452 related to aromatic compound degradation (Batista-Garcia et al. 2014); the TctABC system 453 from *Halomonas* involved in galactarate/glucarate metabolism (Leyn *et al.* 2017); and a 454 recent discovery from our group of AdpC, an "orphan" SBP from Rhodopseudomonas *palustris* which binds medium chain-length dicarboxylic acids ranging from adipate (C6) to 455 456 azelate (C9) (Rosa et al. 2017). Searching the Enzyme Function initiative (EFI) database, it was observed that 19 homologs of TctC were in their library, however only one of them, a 457 458 TctC homolog from Polaromonas sp. was crystalized in the open apo conformation (PDB 459 accession code 4X9T). Table 1 summarises the known range of characterised TTT systems 460 with their respective ligands. Initially believed to bind exclusively to citrate, the substrate range for the TTT family is clearly much broader and new substrates are continually being 461 462 found. With the exception of nicotinic acid in Bug27 (Herrou et al. 2007), all substrates 463 characterised so far seem to have two carboxylic groups, or other functional groups such as 464 sulphate and amide, and further studies will show if this is indeed a required property for 465 substrates in the TTT family.

466

3.1.Properties and function of the TctAB subunits

467 The TTT systems are predicted to contain two membrane proteins, homologous to TctA and 468 TctB. Although crystal structures of these proteins have not been elucidated, information 469 from the primary and secondary sequences of these subunits were studied, in addition to 470 some physiological characterisations, in an attempt to understand the energetic and structural 471 mechanisms of the TTT family.

472 Winnen et al. (2003) showed that while TctB and TctC showed only 27% and 31% identity 473 on average between family members, respectively, TctA orthologues suggested 42% identity 474 and 53% similarity in similar comparisons. Topology predictions suggested the number of 475 transmembrane helices in TctA homologs might vary in different systems, ranging from 9 to 476 12 in bacteria, and 7 to 11 in archaea. In either group, the N-terminal side was predicted to be 477 in the cytoplasm, and large hydrophilic loops between helices 2 and 3 are conserved among 478 all organisms analysed, suggesting this region must have an important role in protein 479 function. The motif G-Hy₃-*G-Hy₃-*G-Hy₂-*P-G-Hy-G, where Hy is an aliphatic 480 hydrophobic residue and * means a fully conserved residue, are found to be highly conserved 481 both in TM1 and TM7, suggesting the 12TM protein originated from a duplication in a 6TM 482 ancestor. TctB homologs were predicted to have between 4 and 5 transmembrane domains, 483 also with predicted cytoplasmic N-termini, and are very poorly conserved among bacteria and 484 were not observed in archaeal sequences (Winnen et al. 2003). In a systems biology study 485 comparing a wild type and a multi-drug resistant strain of Salmonella enterica, Ricci et al. 486 (2012) observed a G109S SNP in TctA. Although shown to be unrelated to the antimicrobial 487 resistance, this mutation compromised growth on several carbon sources, and curiously 488 seemed to confer a delay in the production of Reactive Oxygen Species (ROS) under stressful 489 conditions. Our genome searches revealed for the first time, that at least one case, in 490 Paraburkholderia caribensis, a fusion between the two membrane subunits can also be 491 observed, forming a single TctAB protein (Fig 2b), similar to what was described for some 492 DctQM proteins from the TRAP family, in particular the ones constituting TAXI-TRAP 493 systems (Mulligan et al. 2011).

494 Evidence of cation dependence for transport in the TTT family was first provided by Sweet *et* 495 *al.* (1979), showing that binding of citrate to TctC was enhanced in the presence of Na⁺ Ca²⁺, 496 Mn²⁺ and Fe²⁺, while Mg²⁺, Ni²⁺, Zn²⁺ and Co²⁺ inhibited uptake. Similarly, Brocker *et al.* 497 (2009) demonstrated that citrate transport by a TctCBA system of *Corynebacterium* 498 *glutamicum* was enhanced by Ca²⁺ and Mg²⁺, but not Sr²⁺. Later studies by Hosaka *et al.* 499 (2013), however, showed that both the addition of protonophores and an alkaline pH 497 disrupted terephthalate uptake by the TpiBA system in *Comamonas sp*, while deletion 501 experiments in the same study showed that both subunits were essential for substrate uptake. 502 Furthermore, terephthalate uptake was not disrupted when *Comamonas sp* was grown in 503 absence of Na⁺, suggesting that at least this particular process was more dependent on the 504 proton-motive force rather than a sodium gradient, distinct from SiaPQM sialic acid TRAP 505 transporters (Mulligan *et al.* 2009).

506 Discovering a *tctA* homolog next to esterase genes in metagenomics searches, named *tctA* ar, 507 Batista-Garcia et al. (2014) attempted to build a structural model for TctA ar based on its primary sequence, given that this protein showed no considerable homology to any other 508 509 secondary transporter available in the database. The TctA homolog of Comamonas sp., 510 characterised in previous studies (Hosaka et al. 2013), was used as a control and called 511 TctA ct. Two templates were used against each sequence (PDB codes 3VVN and 4K1C), 512 resulting in 4 models in total. In addition to the already described duplicated motif, a high 513 degree of identity was observed between residues 60 and 110 in TctA homologs. The 514 computational models agreed with the predicted 12TM domain protein, and it was proposed 515 in the 3VVN-based models that the 20 residue conserved motif were in the vicinity of the predicted binding pocket of the protein. In addition, G106, mutated in previous studies (Ricci 516 517 et al. 2012), would be located between the two repeats, being involved in the beginning of the 518 translocation pathway. In the 4K1C model, the two copies of the domain would be in contact 519 with each other in a helix, which would facilitate conformational changes during the transport 520 cycle. Moreover, given that the TTT transporters were initially known for the transport of 521 citrate, docking of this molecule into the two models was attempted, in addition to the 522 modelling of a Na⁺ binding pocket, suggested to be necessary for citrate transport (Sweet et 523 al. 1979). It was shown in the 3VVN models that the potential Na⁺ binding pockets were 524 located in C-terminal variable regions, while the citrate-binding pocket differs in each of the 525 two proteins. The TctA ar showed a unique pocket for citrate, while TctA-ct showed several 526 along the predicted channel, which might act as different steps in the translocation pathway. 527 In the 4K1C models, several binding pockets were predicted for citrate in both proteins, with 528 at least one positively charged residue to interact with the ligand in each of them. These 529 models are an important step towards understanding of translocation mechanisms in the TTT 530 family, although biological confirmation of the transport mechanisms in this family are 531 clearly essential.

532 Evolutionary studies on the TTT family performed by Winnen et al. (2003) suggested that the 533 TctA protein is the original core transport protein, and that the TctB homolog worked as an 534 accessory protein. This claim is supported by the presence of TctA homologs in Archaea, 535 added to the absence of TctB or TctC homologs, and reinforced by the model studies performed by Batista-Garcia et al. (2014). However, the findings on the TRAP transporters, 536 where the DctQ subunit is also observed in archaea, and was found essential to function 537 538 challenge this hypothesis (Mulligan et al. 2011, Mulligan et al. 2012). Because the archaea 539 harbouring TetA homologs were found in extreme environments, amongst groups of 540 methanogens, hyperthermophiles and halophiles, it was suggested that the TctA homologs 541 would be involved in a range of different specific metabolic niches (Winnen et al. 2003), but experimental evidence is still lacking to show if this is the case. 542

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544 **3.2.**Crystal structure and substrate coordination in TctC homologs

The binding proteins from the TTT family, homologous to TctC (Sweet et al. 1979), show a conserved size (ranging from 29kDa to 33kDa), topology and secondary structure organization, but differ considerably in the primary sequence, where among them an identity around 30% is observed. Consequently, a big difference in overall pI is also seen, ranging 549 from 5 to 9.6 (Antoine et al. 2000). At the time of writing, only six structures of TTT SBPs 550 have been deposited in the Protein Data Bank (PDB), four of them with a substrate in the binding pocket; it is already possible to identify, however, some common features among 551 552 them. The average of 300 amino-acid residues comprises the mature form of the proteins (without signal peptide), separated into two globular domains. Domain one is usually formed 553 554 by residues $1 \sim 100$ and $230 \sim 300$ from the N and C termini, forming a β -sheet of five strands, 555 with topological arrangement β_2 - β_1 - β_3 - β_9 - β_4 , surrounded by ~6 α -helices. Domain 2 is comprised of residues 100~229, forming also a central β -sheet of 5 strands with topological 556 arrangement β 6- β 5- β 7- β 4- β 8, surrounded by ~4 helices. Domain 2 sometimes contains a 557 558 disulphide bridge between cysteine residues located in $\alpha 5$ and $\beta 7$, but this is not a feature 559 common to all proteins (Huvent et al. 2006, Rosa et al. 2017). The junction of the two domains is formed by two β -strands, S4 and S9, which are part of domain 1 but extend up to 560 domain 2, and hydrogen bonds between the two domains are scarce. All these features show 561 562 that TTT SBP characterized so far can be classified into the Type II binding protein group, 563 according to the scheme of Fukami-Kobayashi et al. (1999), or cluster E-II, accordingly to 564 the new division proposed by Scheepers et al. (2016). Fig. 3B shows the crystal structure of Bug D as a representative for the TctC homologs. Upon binding to the substrate, it was 565 estimated that the two domains close in an angle of 24.7°, based on the structure of the 566 567 unliganded nicotinic acid binding protein Bug27 (Herrou et al. 2007). Although TctC 568 proteins most commonly seem to bind to molecules containing carboxylic groups, curiously there is usually a slight overall negative charge in the binding pocket, likely dissipated by the 569 570 water molecules or dipole effects of the surrounding helices (Rosa *et al.* 2017). Two β-turns, between $\beta 1$ and $\alpha 1$; and $\beta 7$ and $\alpha 7$, form a "pincer-like" structure important in substrate 571 coordination, closing around one carboxylic group of the ligand, while the remainder of it is 572 buried in the pocket (Fig 3). The residues present in the loops characterise distinguishing 573 574 signatures for proteins of this family, with the motif [P*-F-X-A-G*-G*-X-X-D*] in domain 1 575 being almost ubiquitous among the protein sequences, where X means any residue and * 576 means a very conserved residue. The backbone atoms of residues in this region seem to make 577 hydrogen bonds with two water molecules, present in all substrate-containing structures and 578 also very well conserved in position. These water molecules bridge hydrogen bonds between 579 the protein main chain and the proximal carboxylic group in the substrate. This pattern is 580 observed in the coordination of adipate by AdpC (Rosa et al. 2017), aspartate in BugD 581 (Huvent et al. 2006) and glutamate in BugE (Huvent et al. 2006). In some Bug protein 582 sequences, although these residues are not conserved, they are substituted by others where the 583 side-chain would contain a hydroxyl group, potentially maintaining hydrogen bonds in 584 similar position to what would be expected of the water molecules (Huvent et al. 2006). As 585 shown by Herrou *et al.* (2007), the two β-loops which form the "pincer-like" structure and the 586 two water molecules are not well defined when the ligand is not present. The coordination of 587 the ligand's distal carboxylate group, buried in the pocket, is much less conserved, with $\alpha 3$ 588 and α 5 helices apparently varying in position to accommodate each substrate (Huvent *et al.*) 589 2006, Rosa et al. 2017). Although not conserved in sequence or topology, the involvement of 590 water molecules in the coordination of the distal carboxylate groups was observed in all 591 cases. In some proteins, hydroxyl groups from threonine and serine residues also form 592 hydrogen bonds with the carboxylate in the substrate, but their positions vary. The carbon 593 chain of the substrate, on the other hand, is stabilized by much more conserved hydrophobic 594 interactions, such as Phe14 in AdpC, which seem to act as a docking site for the substrate, 595 and two glycines (Gly18 and Gly163 in AdpC). As a dynamic model, Herrou et al. (2007) 596 suggested that the unliganded form of the SBP would be in an open conformation, with the "pincer-like" structures flexible. Substrate would then bind to domain 1, which would cause a 597 598 conformational change that would bring the water molecules and domain 2 together. A

599 comparison between the five available TctC homologs available in the PDB (only one AdpC 600 structure was used) is shown in Table 2. The root mean square deviations (RMSD), as expected, show a bigger difference when comparing closed and Apo structures, and smaller 601 602 RMSDs when comparisons were made between two liganded structures. Taken together, the crystal structures presented to date give a good general mechanism for ligand coordination in 603 604 the TetC homologs, and further studies will enable us to validate this model and detail the 605 potential differences for substrates containing different functional groups, such as 606 nicotinamide (Herrou et al. 2007) and sulfolactate (Denger and Cook 2010).

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608 **3.3. Present in abundance: The overrepresentation of TctC homologs in some bacteria**

The limited number of sequences available at the time resulted in a bias in the studies 609 610 performed by Winnen et al. (2003), which suggested that the TTT systems were mostly present in α -proteobacteria, and that most other bacterial groups had few or no homologs of 611 these proteins. Genomic searches following the discovery of BugT by Antoine et al. (2000). 612 613 however, revealed the bug genes to be very overrepresented in Bordetella pertussis, with 79 614 BugT homologs, making this family the most abundant in the genome. Following this discovery, Antoine et al. (2003) performed a wider genome analysis, showing that this 615 616 overrepresentation was extended to several Bordetella species, and that some of the Bug 617 proteins were also among the most abundant in cell protein extracts in B. pertussis. As stated 618 in previous sections, the numbers of TTT transmembrane components did not follow the 619 same process, being found in small numbers and consequently configuring most BugT homologs as "orphan proteins", with no obvious transmembrane counterparts. The existence 620 621 of orphan bug homologs was also observed by Antoine et al. (2003) in the genomes of several other bacteria genera, although in that study the only *bacterium* shown to have as 622 623 many representatives as the *Bordetella* species was the β -proteobacterial relative *Cupriavidus* 624 metallidurans. At the time that search was performed, there were around 200 complete 625 bacterial genomes available in the databases, and more recent genome releases showed that at 626 least two other β-proteobacteria genera, Advenella and Cupriavidus also showed an overrepresentation of tctC homologs (Wubbeler et al. 2014). For this review, we reassessed 627 the distribution of TTT systems using the 8049 fully assembled genomes in Genbank, of 628 629 2323 different species, to provide an updated analysis of the presence of TTT systems in 630 bacterial genomes.

A total of 2,323 complete bacterial genomes retrieved from the NCBI database were screened 631 632 for TctA and TctC homologs using the TBLASTN tool. Searches were performed against the coding sequence (CDS) database of each species using lists of protein sequences of either 633 TctA or TctC homologs as queries rather than single sequences, in order to avoid query bias. 634 635 For the TctC search, the queries were: TctC from S. enterica (Sweet et al. 1979); BugD, BugE and Bug27, from B. pertussis (Herrou et al. 2007), TphC fom Comamonas sp. (Hosaka 636 637 et al. 2013) and AdpC from R. palustris (Rosa et al. 2017). For the TctA search, we used the TctA from S. enterica (Sweet et al. 1979), A. mimigardefordensis (Wubbeler et al. 2014), and 638 639 C. glutamicum (Brocker et al. 2009); the BctA from B. pertussis (Antoine et al. 2005); the 640 TpiA from Comamonas sp. (Hosaka et al. 2013) and the SlcF from R. nubinhibens (Denger et al. 2009). Due to the poor sequence conservation among the TctB proteins, our searches with 641 642 this subunit proved to be unsuccessful. TBLASTN reports were obtained for a range of Evalues from 1 to 10⁻¹⁵ in order to determine the best threshold to avoid spurious hits, while 643 644 still retaining distant paralogs. The complete table with number of hits for both proteins with an e-value of 10^{-15} is presented in supplementary Table S1. 645

647 Our searches revealed that, in accordance with the findings of Antoine et al. (2003) and 648 further reinforced by Wubbeler et al. (2014), the most extreme examples of 649 overrepresentation of this group of proteins are found among β-proteobacteria, especially 650 among Bordetella species, as shown in Table 3. However, this phenomenon is not restricted 651 to this group, but extends also to species in the α -proteobacteria phyla (Table 3). In fact, the genome of the environmental a-proteobacterium Rhodoplanes sp. encodes 434 TctC 652 653 homologs in its 8.2 Mbp genome, more than double that of some *Bordetella* species. A more detailed investigation of the expansion in Rhodoplanes will be reported elsewhere 654 (manuscript in preparation). Although this analysis shows that the overrepresentation of TctC 655 656 homologs is mostly found in proteobacteria, a deeper phylogenetic analysis is still required in 657 order to clarify whether this feature found in different subgroups originates from duplications 658 in a common ancestor or were independent events resulting from convergent evolution and 659 independent multiplication events. A search for TctA homologs, on the other hand, as shown 660 in Table 4, suggests that the genomes containing the largest numbers of homologs are found 661 among α and γ -proteobacteria, with only one β -proteobacteria showing 8 or more homologs. 662 In this search, the top hits are no higher than 21 per genome, and are usually associated with a 663 similar number of TctC homologs, possibly forming complete tripartite systems. In this case, organisms outside of the class of proteobacteria, such as clostridia, spirochaetes and bacilli 664 665 are also observed to harbour these homologs. An overview of the number of genomes encoding different numbers of TctC and TctA homologs are shown in Fig. 4, and the full 666 667 complement of genomes analysed is shown in Supplementary Table S1.

At an e-value of 10⁻¹⁵ in BLAST searches, the TctC homologs outnumber the TctA homologs 668 669 in 176 genomes, as shown in Table 5. As already discussed, in these cases it might be that one TctA interacts with more than one TctC, or that the latter are involved in processes other 670 671 than transport, such as signalling and chemotaxis (Antoine et al. 2003, Piepenbreier et al. 672 2017). These latter suggestions are reinforced by the fact that in 36 genomes, one tctC 673 homolog was found, but no *tctA* homologs, although the hypothesis that the binding proteins 674 might interact with transmembrane domains of other transporter classes cannot be excluded. 675 In our initial searches, we found that 210 genomes showed an excess of *tctA* homologs in 676 relation to *tctC*, an unprecedented observation to the best of our knowledge. In order to see whether these observations were due to too strict threshold, our searches were repeated with 677 678 different e-values, shown in table 5. As shown, using an e-value of 10⁻⁹, the number of genomes where this situation occurs is reduced to 66, and to 39 in 10^{-6} . At the latter 679 680 threshold, 6 genomes indicated the presence of TctA homologs, but no TctC homologs. Investigating these 6 genomes individually, we found that 4 of them contained a truncated 681 682 *tctC* homolog in the vicinity of the *tctA* gene and in one the *tctA* gene was clearly mutated. 683 The single remaining genome, *Mageeibacillus indolicus*, indeed seems to have no indication of any periplasmic binding proteins in the vicinity of the *tctA* gene. If the existence of a *tctA* 684 gene without any *tctC* is not a search artefact, one possible explanation would be that SBPs of 685 other types of transport systems could be capable of interacting with the TTT transmembrane 686 687 subunits. An alternative is that such rare orphan TctA proteins in bacteria are, like in archaea, capable of functioning without the involvement of an SBP (Winnen et al. 2003), although 688 both the TTT systems characterised so far and the experiments with the TRAP transporters 689 690 suggest otherwise (Brocker et al. 2009, Mulligan et al. 2009, Hosaka et al. 2013). In another 691 316 genomes, the numbers of *tctC* and *tctA* genes match exactly, suggesting all proteins 692 would be involved in transport through tripartite systems. Finally, about half of the genomes 693 searched (1621) contained no homologous proteins to any of the queries used, suggesting, 694 given TctA homologs are also found in archaea (Winnen et al. 2003), that TTT systems were 695 lost during evolution in these phylogenetic branches.

The reason for the overrepresentation of the Bug proteins in *Bordetella* species and other 697 698 Proteobacteria remains unclear. Antoine et al. (2003) suggested that the few transmembrane 699 domains of TTT systems evolved to be poorly specific, being able to interact with several 700 TctC homologs and thus be required for the uptake of different substrates. This hypothesis 701 was also suggested by Hosaka et al. (2013), but no evidence for this mechanism is yet 702 available. One could hypothesize that perhaps many of these proteins have similar binding 703 functions, but are expressed differentially during the infection cycle in Bordetella species in 704 order to evade the immune system more efficiently. However, the fact that many 705 environmental Proteobacteria also have this expansion of Bug proteins suggests instead that it 706 is an earlier evolutionary trait. The genome of *Ralstonia eutropha* containing 154 homologs 707 of *tctC*, reveals that the majority of them (64.1%) have in their vicinity a regulatory protein, 708 suggesting that most of these proteins are associated with regulatory mechanisms rather than 709 transport (Pohlmann et al. 2006). In this sense, the nomenclature of these SBPs as "uptake 710 genes" might not reflect their actual role in the cell. Piepenbreier et al. (2017) provides a good review of how transporters from different classes can act as first agents in signalling 711 712 pathways, and further studies will enrich our understanding to whether this is the case for the 713 TTT family.

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715

4. Concluding Remarks

716 In this review, we showed how transport systems from the TRAP and TTT families can play 717 important roles in bacteria with a focus on pathogenicity and colonisation. Recent high-718 throughput studies increased substantially the range of substrates known for the TRAP 719 family, while the TTT family is still understudied with a more limited known substrate range, 720 being unravelled in individual studies. In addition, while a lot has been elucidated regarding 721 the binding mechanisms, energetics and kinetics of the TRAP family, very few equivalent 722 studies exist for the TTT family, where especially the energy-coupling mechanisms are yet to 723 be elucidated properly. For both families, a crystal structure of the complete tripartite systems would greatly increase our understanding of the transport process across the membrane, 724 perhaps with potential applications as new drug targets in pathogenic bacteria, given the 725 726 absence of these transporters in eukarvotic cells.

727

728 Non-standard abbreviations

SBP – Solute Binding-Proteins; TTT – Tripartite Tricarboxylate Transporters; TRAP –
 Tripartite ATP-independent periplasmic transporter

731 **Conflicts of interest**

- The research was conducted in the absence of any commercial or financial relationships thatcould be construed as a potential conflict of interest.
- Author contributions
 LR reviewed the literature, co-wrote the manuscript and co-analysed the bioinformatic data.
- 736 MB generated the bioinformatics data and co-analysed it. GT edited and commented on the
- drafts. DK Conceived the idea and focus of the review, co-wrote and edited the paper, and
- 738 provided supervisory guidance.

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1019	Table 1. Experimentally characterised TTT transporters and SBPs
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Name(s)	Organism(s)	Ligand		PDB code	Reference
TetCBA BetCBA TetCBA TetCBA TetCBA	Salmonella typimurium Bordetella pertussis Corynebacterium glutamicum Commamonas sp. Geobacillus thermodenitrificans	Citrate	о Основности основновности основности основновности основности основности основности основности основности		Sweet <i>et al.</i> (1979) Antoine <i>et al.</i> (2003) Brocker <i>et al.</i> (2009) Hosaka <i>et al.</i> (2013) Graf <i>et al.</i> (2016)
BugD	Bordetella pertussis	Aspartate	°−−−− °−−− 0−−	2F5X	Huvent <i>et al.</i> (2006)
BugE	Bordetella pertussis	Glutamate	0	2DVZ	Huvent et al. (2006)
Bug27	Bordetella pertussis	Nicotinic acid <i>et al</i> .		2QPQ	Herrou et al. (2007)
SlcHFG SlcHFG	Roseovarius nubinhibens Chromohalobacter salexigens	Sulfolactate			Denger <i>et al.</i> (2009) Denger and Cook (2010)
TpiBa/ TphC	Commamonas sp.	Terephtalate			Hosaka et al. (2013)
TctCBA	Advenella mimigardefordensis	disulfide 3,3'- dithiodipropionic acid (DTDP)	HO Jo S S S	.0H	Wubbeler et al. (2014)
TetC	Polaromonas sp.	Unknown		4X9T	
TctABC	Halomonas sp.	Galactarate/ glucarate	но н		Leyn <i>et al.</i> (2017)
AdpC	Rhodopseudomonas palustris	Adipate <i>et al</i> .		50EI	Rosa et al. (2017)

Table 1. Comparison between the TctC homolog structures available in the PDB. Root Mean
 Square Deviations (RMSD) and Sequence Identity (SI) were generated using EMBL 3D
 alignment tool available at <u>http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver</u>. PDB codes
 for each of the structures is shown below the protein names in the horizontal line.

RMSDs (Å)	BugD (B)	BugE	Bug27 (B)	TctC	AdpC
SI (%)	2F5X	2DVZ	2QPQ	4X9T	50EI
BugD (B)	NA	1.33	3.00	3.89	1.75
BugE	34	NA	2.62	3.56	1.54
Bug27 (B)	25	32	NA	2.2	2.75
TctC	15	19	28	NA	3.96
AdpC	30	33	27	18	NA

Table 2. Number of TctC and TctA homologs per accession. Only accessions with more than103150 TctC homologs are presented. An e-value of 10^{-15} was used.

Species	Phyla	<i>tctC</i> homologs	tctA homologs
Achromobacter denitrificans	β-proteobacteria	99	6
Achromobacter insolitus	β-proteobacteria	177	4
Achromobacter xylosoxidans	β-proteobacteria	202	5
Acidovorax avenae	β-proteobacteria	66	4
Acidovorax citrulli	β-proteobacteria	55	4
Advenella kashmirensis	β-proteobacteria	116	1
Advenella mimigardefordensis	β-proteobacteria	129	7
Alicycliphilus denitrificans	β-proteobacteria	143	3
Bordetella bronchialis	β-proteobacteria	195	5
Bordetella bronchiseptica	β-proteobacteria	182	4
Bordetella flabilis	β-proteobacteria	214	6
Bordetella genomosp	β-proteobacteria	139	4
Bordetella hinzii	β-proteobacteria	105	5
Bordetella holmesii	β-proteobacteria	56	6
Bordetella parapertussis	β-proteobacteria	142	4
Bordetella pertussis	β-proteobacteria	81	2
Bordetella petrii	β-proteobacteria	107	5
Bordetella pseudohinzii	β-proteobacteria	86	5
Bordetella trematum	β-proteobacteria	100	5
Comamonas serinivorans	β-proteobacteria	87	1
Comamonas testosteroni	β-proteobacteria	100	2
Cupriavidus basilensis	β-proteobacteria	155	5
Cupriavidus gilardii	β-proteobacteria	98	6
Cupriavidus metallidurans	β-proteobacteria	122	5
Cupriavidus necator	β-proteobacteria	190	3
Delftia acidovorans	β-proteobacteria	157	3
Delftia tsuruhatensis	β-proteobacteria	145	3
Hydrogenophaga sp. PBC	β-proteobacteria	64	4
Polaromonas sp. JS666	β-proteobacteria	96	4
Pseudorhodoplanes sinuspersici	α-proteobacteria	99	8
Pusillimonas sp. T7-7	β-proteobacteria	52	3
Ralstonia eutropha	β-proteobacteria	156	5
Ramlibacter tataouinensis	β-proteobacteria	75	3
Rhodoferax sp. DCY110	β-proteobacteria	90	3
Rhodoplanes sp. Z2	α-proteobacteria	434	9
Variovorax paradoxus	β-proteobacteria	135	8
Verminephrobacter eiseniae	β-proteobacteria	130	3

1034	Table 3. Number of TctC and TctA homolog per accession. Only accessions with 8 or more
1035	TctA homologs are presented. An e-value of 10^{-15} was used.

		<i>tctA</i>	<i>tctC</i>
Specie	Class	homologs	homologs
Antarctobacter heliothermus	α-proteobacteria	12	9
Bradyrhizobium icense	α-proteobacteria	8	43
Chelativorans sp. BNC1	α-proteobacteria	13	15
Chelatococcus sp. CO-6	Chlorophyceae	11	10
Chromohalobacter salexigens	γ-proteobacteria	8	9
Defluviimonas alba	α-proteobacteria	9	9
Desulfovibrio fairfieldensis	δ-proteobacteria	9	9
Ensifer sojae	α-proteobacteria	9	6
Geosporobacter ferrireducens	Clostridia	8	9
Granulosicoccus antarcticus	γ-proteobacteria	13	13
Halomonas chromatireducens	γ-proteobacteria	8	8
Halomonas huangheensis	γ-proteobacteria	17	14
Kushneria konosiri	γ-proteobacteria	9	9
Kushneria marisflavi	γ-proteobacteria	9	9
Lachnoclostridium sp. YL32	Clostridia	8	5
Marinobacterium aestuarii	γ-proteobacteria	13	11
Marinomonas sp. MWYL1	γ-proteobacteria	9	7
Marinovum algicola	α-proteobacteria	14	12
Martelella mediterranea	α-proteobacteria	21	18
Martelella sp. AD-3	α-proteobacteria	10	9
Oligotropha carboxidovorans	α-proteobacteria	8	8
Paenibacillus			
naphthalenovorans	Bacilli	16	19
Pelagibacterium halotolerans	α-proteobacteria	8	7
Pseudorhodoplanes sinuspersici	α-proteobacteria	8	99
Rhodoplanes sp. Z2	α-proteobacteria	9	434
Sediminispirochaeta	Quine also at a s	0	0
smaragainae	Spirochaetes	9	9
Sinorhizobium fredii	α-proteobacteria	8	1
Sphaerochaeta globosa	Spirochaetes	9	6
Sphaerochaeta pleomorpha	Spirochaetes	9	17
Starkeya novella	α-proteobacteria	15	17
Sulfitobacter pseudonitzschiae	α-proteobacteria	8	10-
Variovorax paradoxus	β-proteobacteria	8	135
Yangia sp. CCB-MM3	α-proteobacteria	8	6

Table 4. Number of genomes showing different patterns in terms of numbers of tctC and tctA1040homologs, using different e-values as thresholds. ^{a2} is also counted inside of ^{a1}, and ^{b2} is also1041counted inside ^{b1}.

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e-value	10 ⁻¹⁵	10 ⁻¹²	10 ⁻⁹	10 ⁻⁶	10 ⁻³	10 ⁰
tctC > tctA ^{a1}	176	196	212	220	255	2157
tctA > tctC ^{b1}	210	100	66	47	39	64
<i>tctC</i> without <i>tctA</i> ^{a2}	36	43	54	56	79	338
<i>tctA</i> without <i>tctC</i> ^{b2}	115	33	13	6	8	9
tctC = tctA ≠ 0	316	415	444	457	456	97
No homologs	1620	1612	1601	1599	1573	5
Total Number of <i>tctA</i> homologs	1633	1637	1639	1641	1649	5705
Total number of <i>tctC</i> homologs	7213	7405	7495	7552	7632	25225

1045 Figures legends

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1047 Figure 1. Overall topologies and structures of the different Solute Binding Protein (SBP 1048 dependant) transporter families. The ABC transporters are represented by the E. coli 1049 maltose transporter MalEFGK2 (PDB 2R6G). It is composed of a solute binding protein (red), two Transmembrane (TM) domains (blue) and two Nucleotide binding domains (NBD) 1050 (orange); The secondary Tripartite ATP-independent periplasmic (TRAP) Transporters are 1051 1052 composed of a 12 TM domain channel DctM (blue) and a 4 TM domain protein DctQ 1053 (green), which can be fused together by an additional TM domain (yellow) in a DctQM protein, and a DctP or TAXI SBP protein, represented respectively by the SiaP from H. 1054 influenza (PDB 2CEY) (light red) and the TT1099 from T. thermophilus (PDB 1US4) (dark 1055 1056 red). TAXI-TRAP were always found associated with fused DctQM proteins; The Tripartite Tricarboxylate Transporter (TTT), is formed also by a 12 TM channel TctA (purple), a 4 TM 1057 1058 protein TctB (cyan) and a TctC solute binding protein, represented by Bug27 from B. 1059 pertussis (PDB 2QPQ) (Brown). In some rare cases, TctAB proteins may be also fused. Although sharing similar topology, the TRAP and TTT systems share no sequence similarity. 1060

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Figure 2. Examples of genetic organisation for different secondary SBP dependant
 transporters. (A) Gene organisations for TRAP systems. (B) Gene organisation for TTT
 systems. * represents an amido-hydrolase gene.

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1066 Figure 3. Comparison between TRAP and TTT crystal structures. (A) Overall structure of SiaP, a sialic acid binding SBP from the TRAP family in H. influenzae (PDB 3B50). 1067 1068 Domain 1 is represented in cyan (α -helix) and purple (β -sheet), and domain 2 is represented 1069 in blue (α -helix) and green (β -sheet). A sialic acid molecule is shown in the binding pocket. 1070 Arg147, important to perform a salt bridge with the carboxylic group of the substrate in most SiaP homologs is shown in red. (B) Overall structure of BugD, a aspartate binding SBP 1071 from the TTT family in B. pertussis (PDB 2F5X). Domain 1 is represented in green (a-1072 1073 helix) and orange (β -sheet), and domain 2 is represented in blue (α -helix) and brown (β -1074 sheet). An aspartate molecule is shown in the binding pocket. Two loops, between $\beta 1$ and $\alpha 1$ and between β 7 and α 6, are involved in the conserved coordination of two water molecules, 1075 1076 which bridge hydrogen bonds with the proximal carboxylic group in the substrate. These 1077 loops and waters are shown in red (C) Binding pocket of SiaP, showing the coordination of the sialic acid molecule. Arg147 perform a salt bridge with the carboxylic group in the 1078 1079 substrate. The remaining of the molecule is coordinated by hydrogen bonds and water 1080 molecules which are variable inside the TRAP family. (D) Binding pocket of BugD, showing the coordination of the aspartate molecule. The residues Ala-15, glv-16, glv-17 1081 1082 and Asp-20 participate in the loop between $\beta 1$ and $\alpha 1$, coordinating two water molecules which bridge hydrogen bonds with the proximal carboxylic group in the substrate. This 1083 1084 coordination is very conserved among the TTT family. The remaining substrate coordination 1085 occur through non-conserved hydrogen bonds and water bridging.

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1087Figure 4. Numbers of genomes containing different ranges of homologs for tctC and1088tctA.



Figure 1.TIF







Number of homologs per genome