Evolutionary dynamics of membrane transporter & channels: enhancing function through fusion

Benjamin J. Willson, Liam N. M. Chapman and Gavin H. Thomas

**Address**

Department of Biology, University of York, Wentworth Way, York YO10 5DD, United Kingdom.

Corresponding authors: Thomas, Gavin H. (gavin.thomas@york.ac.uk)

# Abstract

The formation of complex multidomain proteins has occurred many times during evolution. For enzymes, this often confers the addition of new functions or new routes to regulate function. Herein we review how this same process has impacted on the function of membrane transporters and channels, proteins that, due to their integral membrane location, are potentially more constrained in the fusions they can accommodate. Using examples primarily from bacterial systems, we illustrate diverse instances of functional fusions and find evidence for promiscuous fusion partners that have fused to many different classes of membrane protein. We consider the evidence that topology and stoichiometry issues might limit the range of fusions that are selected in nature and attempt to find examples where a functional benefit of direct fusion over split proteins has been demonstrated. Finally, we consider whether the reverse process of gene fission has been important in membrane transporter evolution.

# Introduction

As organisms evolve, the ability to expand the phenotypic repertoire of their cellular functions is essential to move into new niches and to compete against other similarly-evolving organisms, the so-called ‘Red-Queen’ hypothesis. In microbial systems, in particular bacteria and archaea, the major driving force for the evolution of new functions is via horizontal gene transfer (HGT) [1,2], where innovation generated by another organism is adopted by a genetically unrelated organism. Genes encoding transporters and channels can be moved in this way too, and there is clear evidence for HGT of these proteins in bacterial systems, for example in sialic acid transporters [3,4], and more recently in eukaryotes [5–8]\*\*.

In the absence of an external pool of genes to requisition, organisms can also evolve additional functionality to existing proteins through recombination events that lead to the insertion of one protein domain within another [9,10]. This could be after the direct tandem duplication of a gene where the fusion would double the size of the gene, or the insertion of a totally new sequence somewhere within the existing one. Clearly for a functional protein to emerge, the gene would have to recombine in such a way that translation can run through from the original coding sequence into the fused partners.

Membrane transport biologists will be familiar with the concept of gene fusion shaping evolution, as the pioneering work of Milton H. Saier Jnr. has demonstrated that many important transporter families, most famously the major facilitator superfamily (MFS) proteins, evolved from smaller ancestors through duplication and fusions, likely through initially forming oligomers before fusing into single proteins [11,12]. Developments in structural biology have allowed the observation of clear structural symmetry in these proteins, giving much weight to these ideas [13]. Recent additional examples of this include the drug/metabolite transporter (DMT) superfamily, which has been proposed to derive from Small Multidrug Resistance proteins, a family of 4-transmembrane helix (TMH) transporters [14], and the proteobacterial antimicrobial compound efflux (PACE) transporters, which have been suggested to derive from an ancestral duplication of the BTP (bacterial transmembrane pair) domain [15].

These fusions are exclusively between integral membrane domains, so what mechanisms are required for the fusion of normally soluble domains to these types of proteins? The technique of C-terminal fusion of the stable green fluorescent protein (GFP) to membrane protein expression targets and the use of in-gel or whole cell fluorescence as a marker of protein expression levels [16,17] is widely used by membrane protein biologists, and, as this works in many cases, it appears that the fusion can be well tolerated and not interfere with protein stability and/or function. This permissiveness to C-terminal fusions was also supported in our recent analysis of fusions present in the major facilitator superfamily, the largest family of membrane transporters [18]. In this article, we review known examples of fusions to all transporters and channels that have been experimentally characterised, finding numerous membrane transport and channel protein architectures that incorporate many different types of soluble domains.

# Fixing a contact: fusion of soluble transporter domains

The substrate binding proteins (SBPs) are the most common soluble domain of bacterial transporters. These are found in ATP-binding cassette (ABC) transporters and also selected secondary transporters such as the tripartite ATP-independent periplasmic (TRAP) transporters and tripartite tricarboxylate transporters (TTT) [19–22]. In Gram-negative organisms, SBPs are typically abundant periplasmic proteins that sequester substrate in the periplasm and deliver it to the membrane. In Gram-positive bacteria, which lack a periplasm, they are anchored to the membrane and diffuse laterally to contact the transporter after binding ligand [23].

Fusions of the SBP to the transmembrane domains (TMDs) of the transporter are now recognised as being particularly common in selected families of ABC transporters from Gram-positive bacteria [24] (***Fig. 1***). Two examples are the glycine betaine transporter (OpuA) of *Lactococcus lactis* [24–28], and the recently identified compatible solute transporter OpuF, which is widespread in the genus *Bacillus* [29]\**.* OpuF contains a TMD-SBP fusion in all observed cases, suggesting an ancestral fusion of these components; conversely, fusions of OpuA may have arisen multiple times, as a phylogenetic tree shows fused transporters clustering amongst the unfused examples [24]. The GlnPQ amino acid transporter from *L. lactis*, *Enterococcus faecalis* and *Streptococcus pneumonia* [30] is unusual as it contains two fused SBPs per TMD. While the fusion is unnecessary for transporter activity in *L. lactis*, the linker between the SBPs and TMD domain plays an important role [31]\*. SBP fusions are rare in Gram-negative bacteria [24], although a study of the transporters in the predatory δ-proteobacterium *Bdellovibrio bacteriovorus* identified a fusion of an SBP for a maltose ABC transporter to one of the TMD proteins [32], whose structure has shed light on how this fusion has occurred [33].

These represent fusions of SBPs to their cognate integral membrane components, where the physical connection presumably provides some physiological advantage over the unfused version. There are other examples of the SBP being recruited to non-cognate transporter/channel architectures to provide a possible ligand-gating mechanism. An early example of this was seen from biochemical and structural work on glutamate receptor ion channels (iGluRs) in eukaryotes that revealed glutamate-binding domains fused to the channel subunit which were involved in agonist binding and channel opening [34]; more recent work on small conductance mechanosensitive channels in bacteria have found multiple examples of SBPs fused to the channel protein [35,36], where again they might have a function in ligand-induced channel gating.



# *Figure 1: Diversity of fused transporter architectures. A: fusions of the ABC transporter superfamily. Represented fusions: fusions of the OTCN and PAO families to substrate-binding proteins (SBPs); fusion of NukT to a peptidase domain, which is involved in cleavage and maturation of the lantibiotic substrate of the transporter; fusion of MetN to a domain with a ferredoxin-like fold, which modifies transporter activity by binding methionine; fusion of Rv1747 to two Forkhead-associated domains, which bind phosphorylated peptides and may mediate transporter activity in response to phosphorylation; and fusion of TagH to a C-terminal extension, which is associated with tolerance to high temperatures. B: fusions of the MFS transporter superfamily. Represented fusions: fusion of ProP to an osmosensory coiled coil (Osmo\_CC) domain, which is involved in osmoregulation; fusion of LplT to PlsC, ACS and ACS C-terminal (ACS\_Cterm) domains, which are involved in lysophospholipid repair; fusion of MamZ to a YedZ domain, which may be involved in ferric iron reduction; and fusion of PepT1/PepT2 to immunoglobulin-like domains, which may recruit trypsin to the transporter. C: fusion of the aquaglyceroporin family to arsenate reductase, which reduces arsenate to arsenite, the substrate of the transporter. D: fusions of the PTS channel subunit EIIC to EIIA and EIIB. Blue highlights represent additional fused sequences; pale grey represents separate proteins which form a complex with the transporter; dashed lines represent domains which are found in some, but not all, examples of the transporter.*

# Fusions of transporters and channels to ‘regulatory’ domains

Transporters can also be fused to other ‘accessory’ domains, introducing novel functions to the transporter, either through the introduction of regulatory domains, allowing post-translational regulation of transporter activity, or of catalytic domains, combining enzymatic conversion of the transporter substrate to the process of transport. The addition of regulatory domains to ABC transporters has been studied in a number of cases [37], Barabote et al. identified a range of fusions to different transporter families [38], and in our recent review, we discuss a number of fusions to the MFS transporters [18]. However, while the increasing number of sequenced bacterial genomes reveals increasing new examples of these fusions, still only few have been studied experimentally. Examples of fusions mentioned in the text are shown schematically in ***Fig. 1***.

One mechanism of regulation is the introduction of small molecule binding domains, allowing modulation of transporter activity by intracellular metabolites. This has been observed in the *Escherichia coli* ABC transporter for L-methionine, MetNI [39–41], and the *Methanosarcina acetivorans* molybdate/tungstate importer ModBC [42], which are both inhibited by high concentrations of their respective substrates. In MetNI [40], this is mediated by the fusion of the ATPase to a domain with a ferredoxin-like fold (***Fig. 1***); in ModBC, this is mediated by a different domain, albeit with a similar mechanism [42]. Another mechanism of regulation is the introduction of regions that can be post-translationally modified, leading to a structural change. A particularly interesting example of this is Rv1747 from *Mycobacterium tuberculosis*, which has an N-terminal extension with two Forkhead-associated (FHA) domains connected by a linker. Phosphorylation of the linker leads to self-binding by the FHA domains, potentially modulating the activity of the transporter [43]\*. Finally, a third mechanism is to link activity of the transporter to a physical change inside the cell. For example, orthologs of the osmoprotectant transporter ProP have a C-terminal extension which has been proposed to interact with the membrane under low osmolarity, but is released under conditions of high osmolarity, activating the transporter [44,45]. In one group of ProP orthologs, the C terminal region also contains a coiled coil region, which is suggested to form dimers with other ProP C termini under high osmolarity, thereby stabilising the active conformation [45].

While not a regulatory fusion per se, the TagH ATPase from *Bacillus subtilis* has a C-terminal extension with a TMH that is required for protein activity at high temperatures [46]. The extension also contains a 225 amino acid extracellular region with unknown function.

## Promiscuous partners – RCK, CBS and UspA domain fusions are found in many contexts

While we have described a number of distinct experimentally studied systems, most are limited to unique combinations of particular domains with particular membrane architectures. Here we find that some domains appear to have a propensity to fuse with multiple membrane transporters and channels.

One example is the ‘regulation of conductance of K+’ (RCK) domain, which is involved in regulation of potassium transport in response to Ca2+ concentration, and has been observed in a range of different K+ channel families, including the archaeal MthK channel, the mammalian BK channel, and the *E. coli* Kch channel [47], as well as in K+/H+ antiporters such as *E. coli* KefC [48]. However, we will discuss these examples in a later section, as the interesting stoichiometry of these fusions warrants special attention.

The CBS (named after cystathionine β-synthase) domain, which is involved in nucleotide binding [49] appears to be fused to a variety of different transporters and channels. These were first seen in the human chloride channel, CLC-5 [50,51] through the elegant work of Dutzler and colleagues, where they occur in tandem pairs and are involved in channel gating (***Fig. 2A***). Recent work on the bacterial MgtE Mg2+ channel [52](TC 1.A.26 family) has elegantly demonstrated both biochemically and structurally that tandem CBS domains bind ATP which modifies the Mg2+-dependent gating of the channel [53]\* (***Fig. 2A***). Moving into classical secondary transporters, the CNNM metal transporters (TC 1.A.112), studied primarily in mice, but with bacterial homologs, contain CBS domains that bind ATP, which appears to be a prerequisite for Mg2+ binding during transport [54] (***Fig. 2A***). Likewise, we identified C-terminal fusions of CBS domains to MFS transporters in bacteria, where the role of the CBS domain is still unknown [18] (***Fig. 2A***). There are also good examples in primary transporters. In the bacterial ABC transporter OpuCA, the fusion of tandem CBS domains is required for the osmoregulatory function of the transporter [55]. There is now emerging evidence that these CBS domains, fused to similar Opu-like ABC transporters in other bacteria including pathogenic *Staphylococcus aureus* and *Listeria monocytogenes*, sense cyclic di-AMP, allowing an additional cytoplasmic regulatory signal to couple allosterically to membrane transporters [56,57]. The previously mentioned OpuF transporters have a similar fusion of one or two CBS domains, although no data is available for the function of these domains in these proteins [29]. Cyclic di-AMP has also recently been shown to regulate the Kup potassium channels, which contain a C-terminal soluble domain, suggesting that there are more examples yet to be found [58].

Another, less well understood example is the Usp (universal stress protein) domain (***Fig 2B***). The biological function of this protein family, exemplified by UspA, remains elusive even 25 years after its discovery and despite its ubiquitous presence across all domains of life [59]. Fusions have been described to the C-termini of various cyanobacterial Na+/H+ exchangers [60], to voltage gated Cl- channels and archaeal amino acid permeases [61], to the ABT (archaeal/bacterial transporter) family of APC transporters [38], and to MFS transporters, typically from Actinobacteria [18]. Interesting hybrid examples have been noted with an archaeal Usp fused to a bacterial Na+/H+ exchanger [62]\*. Usp proteins have also been noted as being located in operons with other transporters such as the TRAP transporters [63]. Despite this, their precise function is unknown; however, as the UspFG proteins, which the fused domains most resemble, are known to bind ATP, then perhaps this is another solution that Nature has stumbled across to allosterically regulate transporter and channel function by nucleotide triphosphate binding [38,61].

To conclude this section, we note the sll1864 chloride channel of *Synechocystis* sp. (strain PCC 6803 / Kazusa) is fused to both CBS and Usp domains [64], but, like the other systems, remains functionally uncharacterized (***Fig. 2C***).



***Figure 2.*** *Architectures of CBS (‘cystathionine β-synthase’), Usp (‘universal stress protein’) and RCK (‘regulation of conductance of K+’) fused transporters. A: fusions with CBS domains, which are able to interact with nucleotides such as ATP or cyclic-di-ATP. B: fusions with Usp domains, which are of unknown function, but may be involved in ATP binding. C: Synechocystis sp. sll1864, which is fused to both CBS and Usp domains. D: fusions to RCK domains, which regulate the transporter in response to the levels of Ca2+. Green highlights represent CBS domains; purple highlights represent Usp domains; blue highlights represent other fused regions; yellow highlights represent fused RCK domains; pale grey represents separate proteins which form a complex with the transporter; dashed lines represent domains which are found in some, but not all, examples of the transporter.*

**Fusions of transporters and channels to enzymes is relatively scarce**

There are many examples of regulatory domain fusions, but those adding full functional enzymes that would work on the substrate of the importer/exporter they are linked to are much scarcer. Some of the clearest examples are seen in bacterial ABC exporters used in lantibiotic biosynthesis [65]. A number of these peptide-based antibiotics require a proteolytic cleavage during the final stages of their synthesis, and in some cases this is mediated concomitantly with efflux through the fusion of the peptidase directly to the transporter [66,67]. These fused domains have evolved to function cooperatively during the cleavage of the lantibiotic leader peptide and subsequent transport [68], exemplifying the potential for fusions to not only co-localise catalysis and transport, but also to allow novel interactions by enabling cross-talk between domains.

Within the MFS superfamily, one of the best experimentally characterised fusions is the lysophospholipid repair system, where the flippase LplT is fused to one or both of the subsequent enzymes that work on the transported lysophospholipid [18,69,70] (**Fig. 1**). Another example is *Magnetospirillum gryphiswaldense* MamZ, which contains a ferric reductase (YedZ)-like domain. This bacterium contains a structure called the magnetosome, consisting of biosynthesised magnetite, which allows it to align with magnetic fields; the YedZ domain is important for correct magnetosome formation, likely by combining ferric iron transport with its immediate reduction [71]. The human PepT1 and PepT2 transporters are also particularly interesting as they contain two additional immunoglobulin-like domains between helices 9 and 10, rather than at the N- or C-terminus. These domains are therefore on the external side of the membrane, and were found to associate with trypsin; they may therefore have a role in recruiting the protease to the transporter, colocalising production of di- and tripeptides and subsequent uptake [72].

Fusions of phosphotransferase systems (PTS) are already well-known; the permease subunit EIIC can often be found fused to the EIIA and/or EIIB subunits in a variety of permutations [73]. Although fusions of the EI and HPr subunits to the EIIA subunit were also identified, no ‘complete fusions’ of all of the subunits were discovered. Interestingly, it has been shown that an artificial fusion protein composed of the EIIBC, EIIA, HPr and EI proteins of the *E. coli* glucose PTS system is functional and can function more efficiently than a 1:1:1:1 mixture of the component proteins [74]. This raises the question of whether such fusions occur in nature, and indeed, some complete fusions are identified in InterPro: one type 1 system (UniProt: A0A2T5CBQ6) and two type 2 systems (UniProt: A0A328F7E0 and C0QKA9). However, these would need experimental study to verify.

Another interesting example is an aquaglyceroporin from the bacterium *Salinospora tropica* that is fused to an arsenate reductase. Arsenate is highly toxic and its reduction to arsenite and subsequent efflux is a commonly employed strategy for resistance [75]. In this organism the proteins form a functional fusion [76] (**Fig. 1**), with the channel itself having evolved to increase selectivity for arsenite. The authors also note an independent fusion of a similar arsenate reductase to a different transporter family in *M. tuberculosis*, demonstrating the important potential physiological benefit of tightly coupling these two processes.

# To fuse or not to fuse?

Fusing another functional domain directly onto a membrane protein imposes topological and stoichiometric constraints that could strongly counter-select these occurring in nature. If we first consider topological problems, a fusion of a protein that is not normally secreted into an extra-cytoplasmic loop of a transporter, for example, would cause numerous problems with folding and assembly of the resulting fusion, suggesting it would be quickly selected against. Thus, the membrane proteins that are most amenable to fusion are those with NIN and/or CIN topology, which allows the fusion of additional cytoplasmic domains at the cytoplasmic termini with a simple linker sequence. This topology is common in bacterial membrane proteins; a landmark genome-wide study by Daley et al [77] has shown that 57% of the membrane proteins of *E. coli* have NIN and CIN topology. However, fusion of domains to an extracytoplasmic terminal is more difficult. From experimental manipulation of expression vectors for membrane proteins, those proteins with an NOUT topology, that could not normally be fused to GFP (which has to be in the cytoplasm to be active), can easily have an additional transmembrane helix fused to the end to locate the GFP in the cytoplasm [78], but for this to occur naturally would require an extra helix and the soluble domain to arrive at the same time. Nevertheless, there are good examples from both SWEET (‘sugars will eventually be exported transporter’) and TRAP transporters where the fusion of membrane components has required the incorporation of an additional helix to match the topology of the two individual partners [79–81]. Where the required linker transmembrane helix originated is not clear but must have been spliced between the genes.

The second problemis to match the stoichiometry of the partners. Given that most soluble enzymes do not function as monomers, this would create a problem if the fusion partner is a monomer, such as most MFS transporters are thought to be. An interesting example of a system constrained by stoichiometry is the gating of potassium transporters by RCK domains. These domains typically function as octamers, and thus need to be significantly in excess over the transporter that they regulate. A simple solution is for the RCK protein to be expressed at higher levels that then channel is by placing it as the first gene in a two gene operon, as can be seen in the *B. subtilis* KtrAB complex [82,83]. A more complex, yet extremely elegant, solution has been reached in the Ca2+-gated K+ channel MthK of *Methanobacterium* *thermoautotrophicum*, as solved by McKinnon and colleagues [84]. The channel protein is fused to a RCK encoding region and forms a tetrameric membrane complex with 4 RCK domains attached to the 4 channel domains. To achieve the final 4:8 ratio observed in the functional complex, this archaea uses an internal promoter within the structural gene for the fusion, so that it produces both the fused channel-RCK protein and stand-alone RCK domains. However, a seemingly simpler solution would be to fuse two RCK domains downstream of the channel domain, as can be seen in the eukaryotic BK channels [85]. It would be tempting to speculate that the MthK transporter represents an ‘intermediate form’ between fusion and non-fusion; however, work by Kuo et al. suggests that the activity of the channel is influenced by the oligomerisation state of the RCK domains under different conditions [86] and by the interaction of the MthK N-terminal ‘desensitisation domain’ with free RCK [87], possibly indicating a physiological role for the unfused RCK domain. With regards to the *E. coli* KefC transporter, the RCK domain lacks an internal start codon and does not contain the residues involved in dimer-dimer oligomerisation; it has been proposed to form a tetramer rather than an octamer [48], thereby circumventing the stoichiometry issue entirely.

There is an additional question of whether the size of the fusion partner is important. Many of the examples we have seen are relatively small regulatory domains (<100 amino acids) that are being fused and this was noted in earlier studies about fusions more generally by Anantharamana, Koonin and Aravind [64]. While this seems an attractive hypothesis, we have seen examples of larger fusions like the lysophospholipid recycling MFS fusions in *E. coli*, which suggests that small size is not an essential feature. Also, there could be good reasons why fusions may be evolutionarily disfavoured. Some ABC transporters clearly exploit the fact that they can couple to multiple SBPs that each bind different ligands and so fusing to one particular partner would limit this [88]. However, it is still possible for a transporter to fuse to multiple different SBPs, as can be seen in the *L. lactis* GlnPQ transporter, to at least partly retain this desired property [31].

# Limited evidence for gene fission in membrane transporter evolution in bacteria

Gene fission describes the evolutionary process by which fused domains become independent. Fission is achieved either by the splitting of one open reading frame into two or more (type 1) or the degradation of one or more domains (type 2) [89]. Within bacteria, protein fission occurs 5x less frequently than fusion, possibly reflecting the disadvantageous nature of the initial domain loss [36] or that the low frequency of tandem duplications in bacteria limits the creation of extra copies of genes where fission can occur without altering the function of the original gene (89). The propensity of membrane protein fissions specifically has not been fully studied; however, some limited evidence has been observed within the membrane components only.

An example of a type 1 fission comes from the SecDF membrane proteins involved in protein transport across the cytoplasmic membrane. These proteins are not-fused in the model organism *E. coli*, being present as a heterodimer of two 6 TMH-containing proteins, SecD and SecF [90]; however, within many Gram +ve bacteria, including *B. subtilis* and *S. aureus*, a single 12 TMH containing polypeptide, SecDF, has been formed as a result of gene fusion [91,92] and the structure of the fused protein from *Thermus thermophilus* has been solved [93,94]. Interestingly the model actinobacterium *Streptomyces coelicolor* contains two variants of the SecDF system, both a fused and heterodimeric version [95]. In the same study, a heterodimeric system in *Dehalobacter sp.* FTH1 is identified that is buried within a larger clade of fused homologues, suggesting that a gene fission event has taken place [95], reverting this back to the heterodimeric system. Clearly the structural and topological relationship between these two membrane proteins is permissive to their sequential fusion and subsequent fission during evolution.

Type 1 fission has been suggested for the *M. tuberculosis* MmpL13 protein, an 11-12 TMH transporter that has split producing a 4 and 6 TMH protein respectively [96,97]\*; however, while this is proposed to be a genuine fission, experimental determination of the function of the ‘split’ system is required. There is also evidence of a type 1 fission returning a previously fused transporter back into an ancestral unfused state in the long-chain chromate ion transporter (LCHR) protein of *Geobacter metallireducens*, which underwent a late fission to produce two short-chain CHR proteins [98].

Type 2 fission can involve both soluble and intermembrane domains. The nucleotide transport proteins (NTTs) have a fusion to a HEAT (‘Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1’) repeat domain. However, multiple subsequent NTT-HEAT fissions have been recorded within several bacteria, forming a clade [99]\*\*. An even more evolutionarily labile fusion between HEAT and downstream cyclic nucleotide binding domains has also been recognised [99]. Truncation of a transmembrane protein can also be achieved by type 2 fission, as the 6 TMH Cation Diffusion Facilitator (CDF) family has been identified as undergoing fission to produce 4 TMH calcium release activated channels (CRACs), homologous to TMH 3-6 of the CDF [100]. Finally, an example from Usp fusions, the unfused *Haloarcula marismortui* ABT transporter ‘Hma4’, sits phylogenetically within a cluster of fused transporters [38], suggesting that the encoding gene has undergone a fission event resulting in loss of the Usp domain.

# Conclusions

We have seen that there are multiple clear examples of transporters and channels that during evolutionary history have acquired additional domains through recombination which have been selected for and maintained. Given that a random fusion of a soluble domain into an integral membrane protein will likely produce something non-functional, the persistence of these examples suggests that there are benefits to host fitness mediated through these fusions. This could be through more efficient transporter by coupling directly to the enzyme working on the transported substrate, or having a regulatory domain that allows the cell to more fine-tune activity of a particular transporter, for example. However, there is little direct evidence to support this from controlled studies comparing fused to non-fused partners. One striking example was the discovery using experimental evolution in the bacterium *Pseudomonas fluorescens* strain SBW25 that a wrinkly-spreader phenotype evolved multiple times in different experiments from a range of deletions in an operon which resulted in a signalling protein now being directly fused to the integral membrane protein [101]\*\*. In this case the membrane protein was only required to sequester the signalling domain to the membrane and its own function was lost, but this beautiful experiment demonstrates a massive advantage of working with bacterial systems in that experimental approaches to study the evolution of gene function can be undertaken successfully. From an evolutionary stand point it also highlights that while fusion to a membrane protein has been selected for in this work, It also highlight how it is easy to fuse to a membrane protein, but not in a way which retains its function.

There is still little hard experimental data on the rates of diffusion of membrane proteins and their soluble partners, although in some cases it has been suggested that diffusion is limiting, which would support a function for fusions [102]\*. We have seen that regulatory domains appear to be the most common type of fusions, usually added to the C-terminus of an existing functional transporter/channel that can over time allow the membrane protein domain to be regulated allosterically through small molecule binding to the regulatory domain. Perhaps there are common metabolic or physiological cues that these domains can sense that can be advantageous for regulating the activity of many different transporter proteins to ingrate intracellular signals into the allosteric regulation of transporter activity. These examples appear to be more ancient and are core features of some ubiquitous channels. Examples of complete enzyme fusions are much rarer and both topological and stoichiometic reasons may strongly select against this occurring. Finally, our review have pulled together as many known examples of fusions and fissions from mainly bacterial systems; what is really needed now are comprehensive genome-wide surveys of these events to assess their evolutionary importance and diversity. Also the few systems where fusions/fissions have occurred naturally need much deeper study to elucidate what benefit(s) they confers to the organisms and then how these rules could be used for designing novel artificial fusions for use in biotechnology.

**Conflict of Interest**

None declared

**Acknowledgements**

We acknowledge BBSRC for funding to Benjamin Willson (BB/N01040X/1) for Project DeTOX and also BBSRC for funding Liam Chapman funded through the White Rose DTP studentship (BB/M011151/1).

**References**

1. Ochman H, Lawrence JG, Groisman EA: **Lateral gene transfer and the nature of bacterial innovation**. *Nature* 2000, **405**:299–304.

2. Koonin E V., Wolf YI: **Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world**. *Nucleic Acids Res* 2008, **36**:6688–6719.

3. Almagro-Moreno S, Boyd EF: **Insights into the evolution of sialic acid catabolism among bacteria**. *BMC Evol Biol* 2009, **9**:118.

4. Thomas GH: **Sialic acid acquisition in bacteria-one substrate, many transporters**. *Biochem Soc Trans* 2016, **44**:760–765.

5. Milner DS, Attah V, Cook E, Maguire F, Savory FR, Morrison M, Müller CA, Foster PG, Talbot NJ, Leonard G, et al.: **Environment-dependent fitness gains can be driven by horizontal gene transfer of transporter-encoding genes**. *Proc Natl Acad Sci* 2019, **116**:5613–5622.

6. Savory FR, Milner DS, Miles DC, Richards TA: **Ancestral Function and Diversification of a Horizontally Acquired Oomycete Carboxylic Acid Transporter**. *Mol Biol Evol* 2018, **35**:1887–1900.

7. Marsit S, Sanchez I, Galeote V, Dequin S: **Horizontally acquired oligopeptide transporters favour adaptation of *Saccharomyces cerevisiae* wine yeast to oenological environment**. *Environ Microbiol* 2016, **18**:1148–1161.

8. Schönknecht G, Chen W-H, Ternes CM, Barbier GG, Shrestha RP, Stanke M, Bräutigam A, Baker BJ, Banfield JF, Garavito RM, et al.: **Gene transfer from bacteria and archaea facilitated evolution of an extremophilic eukaryote.** *Science* 2013, **339**:1207–10.

9. Pasek S, Risler J-L, Brezellec P: **Gene fusion/fission is a major contributor to evolution of multi-domain bacterial proteins**. *Bioinformatics* 2006, **22**:1418–1423.

10. Doolittle RF: **The Multiplicity of Domains in Proteins**. *Annu Rev Biochem* 1995, **64**:287–314.

11. Saier MH: **Tracing pathways of transport protein evolution**. *Mol Microbiol* 2003, **48**:1145–1156.

12. Saier MH: **Transport protein evolution deduced from analysis of sequence, topology and structure**. *Curr Opin Struct Biol* 2016, **38**:9–17.

13. Forrest LR: **Structural Symmetry in Membrane Proteins.** *Annu Rev Biophys* 2015, **44**:311–37.

14. Bay DC, Turner RJ: **Diversity and evolution of the small multidrug resistance protein family**. *BMC Evol Biol* 2009, **9**:140.

15. Hassan KA, Liu Q, Elbourne LDH, Ahmad I, Sharples D, Naidu V, Chan CL, Li L, Harborne SPD, Pokhrel A, et al.: **Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens.** *Res Microbiol* 2018, **169**:450–454.

16. Drew DE, von Heijne G, Nordlund P, de Gier J-WL: **Green fluorescent protein as an indicator to monitor membrane protein overexpression in *Escherichia coli***. *FEBS Lett* 2001, **507**:220–224.

17. Drew D, Lerch M, Kunji E, Slotboom D-J, de Gier J-W: **Optimization of membrane protein overexpression and purification using GFP fusions**. *Nat Methods* 2006, **3**:303–313.

18. Willson BJ, Dalzell L, Chapman LNM, Thomas GH: **Enhanced functionalisation of major facilitator superfamily transporters via fusion of C-terminal protein domains is both extensive and varied in bacteria**. *Microbiology* 2019, doi:10.1099/mic.0.000771.

19. Davidson AL, Dassa E, Orelle C, Chen J: **Structure, Function, and Evolution of Bacterial ATP-Binding Cassette Systems**. *Microbiol Mol Biol Rev* 2008, **72**:317–364.

20. Mulligan C, Fischer M, Thomas GH: **Tripartite ATP-independent periplasmic (TRAP) transporters in bacteria and archaea**. *FEMS Microbiol Rev* 2011, **35**:68–86.

21. Maqbool A, Horler RSP, Muller A, Wilkinson AJ, Wilson KS, Thomas GH: **The substrate-binding protein in bacterial ABC transporters: dissecting roles in the evolution of substrate specificity**. *Biochem Soc Trans* 2015, **43**:1011–1017.

22. Rosa LT, Bianconi ME, Thomas GH, Kelly DJ: **Tripartite ATP-Independent Periplasmic (TRAP) Transporters and Tripartite Tricarboxylate Transporters (TTT): From Uptake to Pathogenicity**. *Front Cell Infect Microbiol* 2018, **8**:33.

23. Bosdriesz E, Magnúsdóttir S, Bruggeman FJ, Teusink B, Molenaar D: **Binding proteins enhance specific uptake rate by increasing the substrate-transporter encounter rate**. *FEBS J* 2015, **282**:2394–2407.

24. van der Heide T, Poolman B: **ABC transporters: one, two or four extracytoplasmic substrate-binding sites?** *EMBO Rep* 2002, **3**:938–43.

25. Obis D, Guillot A, Gripon JC, Renault P, Bolotin A, Mistou MY: **Genetic and biochemical characterization of a high-affinity betaine uptake system (BusA) in Lactococcus lactis reveals a new functional organization within bacterial ABC transporters.** *J Bacteriol* 1999, **181**:6238–46.

26. Bouvier J, Bordes P, Romeo Y, Fourçans A, Bouvier I, Gutierrez C: **Characterization of OpuA, a glycine-betaine uptake system of Lactococcus lactis.** *J Mol Microbiol Biotechnol* 2000, **2**:199–205.

27. Patzlaff JS, van der Heide T, Poolman B: **The ATP/substrate stoichiometry of the ATP-binding cassette (ABC) transporter OpuA.** *J Biol Chem* 2003, **278**:29546–51.

28. Gul N, Schuurman-Wolters G, Karasawa A, Poolman B: **Functional characterization of amphipathic α-helix in the osmoregulatory ABC transporter OpuA.** *Biochemistry* 2012, **51**:5142–52.

29. Teichmann L, Kümmel H, Warmbold B, Bremer E: **OpuF, a New Bacillus Compatible Solute ABC Transporter with a Substrate-Binding Protein Fused to the Transmembrane Domain.** *Appl Environ Microbiol* 2018, **84**.

30. Fulyani F, Schuurman-Wolters GK, Žagar AV, Guskov A, Slotboom D-J, Poolman B: **Functional Diversity of Tandem Substrate-Binding Domains in ABC Transporters from Pathogenic Bacteria**. *Structure* 2013, **21**:1879–1888.

31. Schuurman-Wolters GK, de Boer M, Pietrzyk MK, Poolman B: **Protein Linkers Provide Limits on the Domain Interactions in the ABC Importer GlnPQ and Determine the Rate of Transport.** *J Mol Biol* 2018, **430**:1249–1262.

32. Barabote RD, Rendulic S, Schuster SC, Saier MH: **Comprehensive analysis of transport proteins encoded within the genome of Bdellovibrio bacteriovorus.** *Genomics* 2007, **90**:424–46.

33. Licht A, Bommer M, Werther T, Neumann K, Hobe C, Schneider E: **Structural and functional characterization of a maltose/maltodextrin ABC transporter comprising a single solute binding domain (MalE) fused to the transmembrane subunit MalF.** *Res Microbiol* 2019, **170**:1–12.

34. Madden DR: **The structure and function of glutamate receptor ion channels**. *Nat Rev Neurosci* 2002, **3**:91–101.

35. Malcolm HR, Maurer JA: **The mechanosensitive channel of small conductance (MscS) superfamily: not just mechanosensitive channels anymore.** *Chembiochem* 2012, **13**:2037–43.

36. Booth IR, Miller S, Müller A, Lehtovirta-Morley L: **The evolution of bacterial mechanosensitive channels**. *Cell Calcium* 2015, **57**:140–150.

37. Biemans-Oldehinkel E, Doeven MK, Poolman B: **ABC transporter architecture and regulatory roles of accessory domains**. *FEBS Lett* 2006, **580**:1023–1035.

38. Barabote RD, Tamang DG, Abeywardena SN, Fallah NS, Fu JYC, Lio JK, Mirhosseini P, Pezeshk R, Podell S, Salampessy ML, et al.: **Extra domains in secondary transport carriers and channel proteins**. *Biochim Biophys Acta - Biomembr* 2006, **1758**:1557–1579.

39. Kadner RJ: **Regulation of methionine transport activity in Escherichia coli.** *J Bacteriol* 1975, **122**:110–9.

40. Kadaba NS, Kaiser JT, Johnson E, Lee A, Rees DC: **The high-affinity E. coli methionine ABC transporter: structure and allosteric regulation.** *Science* 2008, **321**:250–3.

41. Yang JG, Rees DC: **The allosteric regulatory mechanism of the Escherichia coli MetNI methionine ATP binding cassette (ABC) transporter.** *J Biol Chem* 2015, **290**:9135–40.

42. Gerber S, Comellas-Bigler M, Goetz BA, Locher KP: **Structural Basis of Trans-Inhibition in a Molybdate/Tungstate ABC Transporter**. *Science (80- )* 2008, **321**:246–250.

43. Heinkel F, Shen L, Richard-Greenblatt M, Okon M, Bui JM, Gee CL, Gay LM, Alber T, Av-Gay Y, Gsponer J, et al.: **Biophysical Characterization of the Tandem FHA Domain Regulatory Module from the Mycobacterium tuberculosis ABC Transporter Rv1747**. *Structure* 2018, **26**:972-986.e6.

44. Tsatskis Y, Khambati J, Dobson M, Bogdanov M, Dowhan W, Wood JM: **The Osmotic Activation of Transporter ProP Is Tuned by Both Its C-terminal Coiled-coil and Osmotically Induced Changes in Phospholipid Composition**. *J Biol Chem* 2005, **280**:41387–41394.

45. Culham DE, Marom D, Boutin R, Garner J, Ozturk TN, Sahtout N, Tempelhagen L, Lamoureux G, Wood JM: **Dual Role of the C-Terminal Domain in Osmosensing by Bacterial Osmolyte Transporter ProP**. *Biophys J* 2018, **115**:2152–2166.

46. Yamada T, Miyashita M, Kasahara J, Tanaka T, Hashimoto M, Yamamoto H: **The transmembrane segment of TagH is required for wall teichoic acid transport under heat stress in Bacillus subtilis**. *Microbiology* 2018, **164**:935–945.

47. Jiang Y, Pico A, Cadene M, Chait BT, MacKinnon R: **Structure of the RCK domain from the E. coli K+ channel and demonstration of its presence in the human BK channel.** *Neuron* 2001, **29**:593–601.

48. Roosild TP, Castronovo S, Miller S, Li C, Rasmussen T, Bartlett W, Gunasekera B, Choe S, Booth IR: **KTN (RCK) Domains Regulate K+ Channels and Transporters by Controlling the Dimer-Hinge Conformation**. *Structure* 2009, **17**:893–903.

49. Baykov AA, Tuominen HK, Lahti R: **The CBS Domain: A Protein Module with an Emerging Prominent Role in Regulation**. *ACS Chem Biol* 2011, **6**:1156–1163.

50. Meyer S, Dutzler R: **Crystal Structure of the Cytoplasmic Domain of the Chloride Channel ClC-0**. *Structure* 2006, **14**:299–307.

51. Meyer S, Savaresi S, Forster IC, Dutzler R: **Nucleotide recognition by the cytoplasmic domain of the human chloride transporter ClC-5**. *Nat Struct Mol Biol* 2007, **14**:60–67.

52. Hattori M, Tanaka Y, Fukai S, Ishitani R, Nureki O: **Crystal structure of the MgtE Mg2+ transporter**. *Nature* 2007, **448**:1072–1075.

53. Tomita A, Zhang M, Jin F, Zhuang W, Takeda H, Maruyama T, Osawa M, Hashimoto K-I, Kawasaki H, Ito K, et al.: **ATP-dependent modulation of MgtE in Mg2+ homeostasis.** *Nat Commun* 2017, **8**:148.

54. Hirata Y, Funato Y, Takano Y, Miki H: **Mg 2+ -dependent Interactions of ATP with the Cystathionine-β-Synthase (CBS) Domains of a Magnesium Transporter**. *J Biol Chem* 2014, **289**:14731–14739.

55. Chen C, Beattie GA: **Characterization of the osmoprotectant transporter OpuC from Pseudomonas syringae and demonstration that cystathionine-beta-synthase domains are required for its osmoregulatory function.** *J Bacteriol* 2007, **189**:6901–12.

56. Huynh TN, Choi PH, Sureka K, Ledvina HE, Campillo J, Tong L, Woodward JJ: **Cyclic di-AMP targets the cystathionine beta-synthase domain of the osmolyte transporter OpuC**. *Mol Microbiol* 2016, **102**:233–243.

57. Schuster CF, Bellows LE, Tosi T, Campeotto I, Corrigan RM, Freemont P, Gründling A: **The second messenger c-di-AMP inhibits the osmolyte uptake system OpuC in Staphylococcus aureus.** *Sci Signal* 2016, **9**:ra81.

58. Quintana IM, Gibhardt J, Turdiev A, Hammer E, Commichau FM, Lee VT, Magni C, Stülke J: **The KupA and KupB Proteins of Lactococcus lactis IL1403 Are Novel c-di-AMP Receptor Proteins Responsible for Potassium Uptake.** *J Bacteriol* 2019, **201**:e00028-19.

59. Vollmer AC, Bark SJ: **Twenty-Five Years of Investigating the Universal Stress Protein: Function, Structure, and Applications**. In *Advances in applied microbiology*. . 2018:1–36.

60. Jangir MM, Vani B, Chowdhury S: **Analysis of seven putative Na+/H+ antiporters of Arthrospira platensis NIES-39 using transcription profiling and In-silico studies: an indication towards alkaline pH acclimation**. *bioRxiv* 2018, doi:10.1101/344416.

61. Kvint K, Nachin L, Diez A, Nyström T: **The bacterial universal stress protein: function and regulation**. *Curr Opin Microbiol* 2003, **6**:140–145.

62. Méheust R, Watson AK, Lapointe F-J, Papke RT, Lopez P, Bapteste E: **Hundreds of novel composite genes and chimeric genes with bacterial origins contributed to haloarchaeal evolution.** *Genome Biol* 2018, **19**:75.

63. Mulligan C, Kelly DJ, Thomas GH: **Tripartite ATP-independent periplasmic transporters: application of a relational database for genome-wide analysis of transporter gene frequency and organization.** *J Mol Microbiol Biotechnol* 2007, **12**:218–26.

64. Anantharaman V, Koonin E V, Aravind L: **Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains**. *J Mol Biol* 2001, **307**:1271–1292.

65. Severi E, Thomas GH: **Antibiotic export: transporters involved in the final step of natural product production**. *Microbiology* 2019, doi:10.1099/mic.0.000794.

66. Håvarstein LS, Diep DB, Nes IF: **A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export.** *Mol Microbiol* 1995, **16**:229–40.

67. Nishie M, Shioya K, Nagao J, Jikuya H, Sonomoto K: **ATP-dependent leader peptide cleavage by NukT, a bifunctional ABC transporter, during lantibiotic biosynthesis**. *J Biosci Bioeng* 2009, **108**:460–464.

68. Zheng S, Nagao J, Nishie M, Zendo T, Sonomoto K: **ATPase activity regulation by leader peptide processing of ABC transporter maturation and secretion protein, NukT, for lantibiotic nukacin ISK-1**. *Appl Microbiol Biotechnol* 2018, **102**:763–772.

69. Harvat EM, Zhang Y-M, Tran C V, Zhang Z, Frank MW, Rock CO, Saier MH: **Lysophospholipid flipping across the Escherichia coli inner membrane catalyzed by a transporter (LplT) belonging to the major facilitator superfamily.** *J Biol Chem* 2005, **280**:12028–34.

70. Zheng L, Lin Y, Lu S, Zhang J, Bogdanov M: **Biogenesis, transport and remodeling of lysophospholipids in Gram-negative bacteria.** *Biochim Biophys acta Mol cell Biol lipids* 2017, **1862**:1404–1413.

71. Raschdorf O, Müller FD, Pósfai M, Plitzko JM, Schüler D: **The magnetosome proteins MamX, MamZ and MamH are involved in redox control of magnetite biomineralization in Magnetospirillum gryphiswaldense.** *Mol Microbiol* 2013, **89**:872–86.

72. Beale JH, Parker JL, Samsudin F, Barrett AL, Senan A, Bird LE, Scott D, Owens RJ, Sansom MSP, Tucker SJ, et al.: **Crystal Structures of the Extracellular Domain from PepT1 and PepT2 Provide Novel Insights into Mammalian Peptide Transport**. *Structure* 2015, **23**:1889–1899.

73. Barabote RD, Saier MH, Jr.: **Comparative genomic analyses of the bacterial phosphotransferase system.** *Microbiol Mol Biol Rev* 2005, **69**:608–34.

74. Mao Q, Schunk T, Gerber B, Erni B: **A String of Enzymes, Purification and Characterization of a Fusion Protein Comprising the Four Subunits of the Glucose Phosphotransferase System of *Escherichia coli***. *J Biol Chem* 1995, **270**:18295–18300.

75. Ben Fekih I, Zhang C, Li YP, Zhao Y, Alwathnani HA, Saquib Q, Rensing C, Cervantes C: **Distribution of Arsenic Resistance Genes in Prokaryotes.** *Front Microbiol* 2018, **9**:2473.

76. Wu B, Song J, Beitz E: **Novel channel enzyme fusion proteins confer arsenate resistance.** *J Biol Chem* 2010, **285**:40081–7.

77. Daley DO, Rapp M, Granseth E, Melén K, Drew D, von Heijne G: **Global topology analysis of the Escherichia coli inner membrane proteome.** *Science* 2005, **308**:1321–3.

78. Hsieh JM, Besserer GM, Madej MG, Bui H-Q, Kwon S, Abramson J: **Bridging the gap: A GFP-based strategy for overexpression and purification of membrane proteins with intra and extracellular C-termini**. *Protein Sci* 2010, **19**:868–880.

79. Severi E, Randle G, Kivlin P, Whitfield K, Young R, Moxon R, Kelly D, Hood D, Thomas GH: **Sialic acid transport in *Haemophilus influenzae* is essential for lipopolysaccharide sialylation and serum resistance and is dependent on a novel tripartite ATP-independent periplasmic transporter**. *Mol Microbiol* 2005, **58**:1173–1185.

80. Mulligan C, Geertsma ER, Severi E, Kelly DJ, Poolman B, Thomas GH: **The substrate-binding protein imposes directionality on an electrochemical sodium gradient-driven TRAP transporter.** *Proc Natl Acad Sci U S A* 2009, **106**:1778–83.

81. Hu Y-B, Sosso D, Qu X-Q, Chen L-Q, Ma L, Chermak D, Zhang D-C, Frommer WB: **Phylogenetic evidence for a fusion of archaeal and bacterial SemiSWEETs to form eukaryotic SWEETs and identification of SWEET hexose transporters in the amphibian chytrid pathogen *Batrachochytrium dendrobatidis***. *FASEB J* 2016, **30**:3644–3654.

82. Holtmann G, Bakker EP, Uozumi N, Bremer E: **KtrAB and KtrCD: two K+ uptake systems in Bacillus subtilis and their role in adaptation to hypertonicity.** *J Bacteriol* 2003, **185**:1289–98.

83. Albright RA, Ibar J-LV, Kim CU, Gruner SM, Morais-Cabral JH: **The RCK Domain of the KtrAB K+ Transporter: Multiple Conformations of an Octameric Ring**. *Cell* 2006, **126**:1147–1159.

84. Jiang Y, Lee A, Chen J, Cadene M, Chait BT, MacKinnon R: **Crystal structure and mechanism of a calcium-gated potassium channel**. *Nature* 2002, **417**:515–522.

85. Giraldez T, Rothberg BS: **Understanding the conformational motions of RCK gating rings**. *J Gen Physiol* 2017, **149**:431–441.

86. Martinac B, Buechner M, Delcour AH, Adler J, Kung C: **Pressure-sensitive ion channel in Escherichia coli.** *Proc Natl Acad Sci U S A* 1987, **84**:2297–301.

87. Kuo MM-C, Maslennikov I, Molden B, Choe S: **The Desensitization Gating of the MthK K+ Channel Is Governed by Its Cytoplasmic Amino Terminus**. *PLoS Biol* 2008, **6**:e223.

88. Thomas GH: **Homes for the orphans: utilization of multiple substrate-binding proteins by ABC transporters**. *Mol Microbiol* 2010, **75**:6–9.

89. Leonard G, Richards TA: **Genome-scale comparative analysis of gene fusions, gene fissions, and the fungal tree of life.** *Proc Natl Acad Sci U S A* 2012, **109**:21402–7.

90. Pogliano JA, Beckwith J: **SecD and SecF facilitate protein export in Escherichia coli.** *EMBO J* 1994, **13**:554–61.

91. Bolhuis A, Broekhuizen CP, Sorokin A, van Roosmalen ML, Venema G, Bron S, Quax WJ, van Dijl JM: **SecDF of Bacillus subtilis, a molecular Siamese twin required for the efficient secretion of proteins.** *J Biol Chem* 1998, **273**:21217–24.

92. Quiblier C, Zinkernagel AS, Schuepbach RA, Berger-Bächi B, Senn MM: **Contribution of SecDF to Staphylococcus aureus resistance and expression of virulence factors**. *BMC Microbiol* 2011, **11**:72.

93. Furukawa A, Yoshikaie K, Mori T, Mori H, Morimoto Y V., Sugano Y, Iwaki S, Minamino T, Sugita Y, Tanaka Y, et al.: **Tunnel Formation Inferred from the I-Form Structures of the Proton-Driven Protein Secretion Motor SecDF**. *Cell Rep* 2017, **19**:895–901.

94. Tsukazaki T, Mori H, Echizen Y, Ishitani R, Fukai S, Tanaka T, Perederina A, Vassylyev DG, Kohno T, Maturana AD, et al.: **Structure and function of a membrane component SecDF that enhances protein export**. *Nature* 2011, **474**:235–238.

95. Zhou Z, Li Y, Sun N, Sun Z, Lv L: **Function and Evolution of Two Forms of SecDF Homologs in Streptomyces coelicolor**. *PLoS One* 2014, **9**:105237.

96. Sandhu P, Akhter Y: **The internal gene duplication and interrupted coding sequences in the MmpL genes of Mycobacterium tuberculosis: Towards understanding the multidrug transport in an evolutionary perspective**. *Int J Med Microbiol* 2015, **305**:413–423.

97. Sandhu P, Akhter Y: **Evolution of structural fitness and multifunctional aspects of mycobacterial RND family transporters**. *Arch Microbiol* 2018, **200**:19–31.

98. Díaz-Pérez C, Cervantes C, Campos-García J, Julián-Sánchez A, Riveros-Rosas H: **Phylogenetic analysis of the chromate ion transporter (CHR) superfamily**. *FEBS J* 2007, **274**:6215–6227.

99. Major P, Embley TM, Williams TA: **Phylogenetic Diversity of NTT Nucleotide Transport Proteins in Free-Living and Parasitic Bacteria and Eukaryotes**. *Genome Biol Evol* 2017, **9**:480.

100. Matias MG, Gomolplitinant KM, Tamang DG, Saier MH: **Animal Ca2+ release-activated Ca2+ (CRAC) channels appear to be homologous to and derived from the ubiquitous cation diffusion facilitators**. *BMC Res Notes* 2010, **3**:158.

101. Farr AD, Remigi P, Rainey PB: **Adaptive evolution by spontaneous domain fusion and protein relocalization**. *Nat Ecol Evol* 2017, **1**:1562–1568.

102. Schavemaker PE, Boersma AJ, Poolman B: **How Important Is Protein Diffusion in Prokaryotes?** *Front Mol Biosci* 2018, **5**:93.

Paper descriptions

5. Milner et al 2019 \*\*

An important paper on HGT in eukaryotes with a particular focus on transporters. A "transporter-gene acquisition ratchet" is proposed where the transportome is continually evolving via methods of duplication, HGT and differential gene loss.

29: Teichmann et al 2018 \*

This paper describes the characterisation of a novel family of compatible solute transporters. The distribution, architecture and substrate specificity are all considered. Interestingly, all members of this family are fused, suggesting that they derive from a single ancestral fusion. Furthermore, the paper contains a phylogenetic tree of compatible solute transporters in the genus *Bacillus*, which shows several clusters of fused OpuA transporters.

31: Schuurman-Wolters et al 2018 \*\*

This paper examines the role of the inter-domain linkers of the fused SBPs in the amino acid transporter GlnPQ. The study demonstrates some of the potential constraints for the linker sequences, and would therefore be especially interesting for those studying the effects of artificial linkers in fusion proteins. If the linker is too short, the interactions of the SBPs with the transporter are impaired; conversely, if the linkers are too long, the Vmax for transport decreases as the SBP takes more time to bind the transporter, and the Km for transport of weakly binding substrates increases as the rate of transport decreases relative to the rate of release into the supernatant.

43: Heinkel *et al.* 2018 \*

This study describes the interesting potential regulatory module of Rv1747, where the fused FHA domains are able to bind to the phosphorylated linker peptide. Although the effect on transporter activity was not experimentally confirmed, the results of the paper suggest that this allows the activity of the trasnporter to be fine-tuned through the alteration of the phosphorylation state of the linker regions.

53: Tomita *et al.* 2017 \*\*

The study describes a unique regulatory role for the CBS domains of the magnesium channel MgtE, where the binding of ATP sensitises the channel to intracellular magnesium, thus promoting the closure of the channel at physiological magnesium concentrations. The uptake of magnesium by this channel is therefore connected to both the concentrations of internal magnesium and ATP.

62: Méheust et al 2018 \*

The study provides evidence that gene fusion, including fusion of domains acquired from bacteria, has resulted in the formation of numerous chimeric genes in *Haloarchaea*, and identifies that many of these are involved in core metabolism and adaptation to high salt concentrations, suggesting that they have been important in the adaptation of the *Haloarchaea* to their environmental niche.

97. Sandhu & Akhter, 2018. \*

This paper is a focussed study on the molecular evolution of the Mycobacterial MmpL RND efflux pump. It introduces the role of fusion and gene duplication, exploring the importance of this event in producing internal symmetry. It also suggests examples of fissions, although these examples need experimental characterisation

99: Major et al 2017. \*\*

This paper offers an interesting insight into the fusion partners of NTTs. Phylogenetic analysis suggests a single, bacterial origin for the NTT-HEAT fusion, and offers multiple theories about the uptake of this fusion in the bacterial and eukaryotic lines containing it.

101: Farr et al 2017. \*\*

This beautiful experimental evolution approach demonstrated real adaptation of *Pseudomonas fluorescens* to a new phenotype, finding multiple genetics changes that bring a di-guanylate cyclase domain to the membrane through fusion with an integral membrane protein.

102: Schavemaker, Boersma & Poolman 2018 \*

An interesting review on the importance of protein diffusion in prokaryotes which poses questions in the field that need answers that are critical to help elucidate when membrane transporter/enzyme fusions would be functionally beneficial.