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Review

ABC transporters: bacterial exporters-revisited five years on

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Contents

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1. Introduction

Type I secretion systems exist in monoderm (surrounded by a single membrane) Gram-positive bacteria and diderm (containing both an inner cytoplasmic membrane and an outer membrane) Gram-negative bacteria. They are also called, in the literature, adenosine triphosphate-binding cassette (ABC) secretion systems since one of the translocator protein subcomponents or subdomains is an ABC-ATPase. Type I systems are of interest for studying ABC transporters involved in an alternative transport pathway responsible for the secretion across bacterial membranes of a wide range of compounds (see Table 1). The type I system is also intriguing as a secretion pathway since it is not limited to translocation across one single bacterial membrane, but apparently achieves movement across the periplasm and two membranes simultaneously, avoiding lateral leakage. This article will review the latest aspects of ABC proteins in Bacteria with an emphasis on procaryotic ABC export systems. Recent results coming from the work of several laboratories studying the ABC secretion pathway will then be constructively analysed with comments upon the techniques and approaches used. Finally, we aim to integrate the results into a global picture of what we know about type I secretion systems.

2. ABC transporters or ABC traffic ATPases

2.1. What defines an ABC transporter/ABC traffic ATPase?

A highly conserved sequence of approximately 215 amino acids folds into a soluble protein domain known as an ABC domain or module. It is not our intention to list all the structural features one finds in an ABC domain, this being covered elsewhere in this issue. Of importance, however, are the Walker A and B motifs common to all nucleotide-binding proteins and a particular minimum signature sequence, consensus LSGGQ, displayed uniquely by and thus identifying members of the ABC superfamily [1–4].

At this point it is useful to define an *ABC protein* as distinct from an *ABC transporter*, since there is an increasing tendency to use these specific terms interchangeably, leading to considerable confusion in the literature. An ABC protein, refers only to proteins containing an ATP-binding cassette or ABC-ATPase domain which is involved in coupling the energy of ATP hydrolysis to many physiological processes, not necessarily, but usually, transport related. On the other hand, an ABC transporter, synonymous with traffic ATPase (or permease for import systems), is formed when the cytoplasmic ABC-ATPase associates with a hydrophobic membraneous domain (MD), which in most, but not all, ABC transporters is predicted to form six putative α-helical transmembrane segments. An early concept, still not definitively proven, is that ABC transporters possess a common four–domain arrangement constituted by homo- or heterodimers of co-operating MD,ABC units [5,6]. With many ABC transporters, the membrane domain (MD) is fused to the ABC protein, producing a single MD-ABC unit, which if forming a functional dimer, as is generally supposed, can be represented as $\{MD\text{-}ABC\}_2$. However, for many import and export systems, particularly in bacteria, the MD and ABC protein is formed by one or two separately encoded membrane proteins ($\{MD\}_2\{ABC\}_2$). In this case, the membrane protein might be termed an ABC-dependent transport protein, or an ABC permease but should not be indicated simply as an ABC protein. Similarly to SecA, the ATPase of the SecYEG protein translocation system, the ATP-binding domains of ABC transport-

Table 1
Examples of the roles and range of allocrites transported by ABC exporters in procaryotes

$\{MD\}_2$-$\{ABC\}_2$ configuration[a]				$\{MD\text{-}ABC\}_2$ configuration[b]			
Function	ABC transporter[c]	Allocrite	Organism	Function	ABC transporter	Allocrite	Organism
SDR	DrrAB	Antitumour antibiotic	*Streptomyces peucetius*	MDR	LmrA	Multidrugs	*Lactotococcus lactis*
Immunity self-protection	NisFEG	Nisin lantibiotic	*Lactococcus lactis*	Lantibiotic peptides	Nis T	Nisin precursor	*Lactotococcus lactis*
Peptide antibiotics	BsrABC	Bacitracin	*Bacillus licheniformis*	GG-type bacteriocins, pheromones	LcnC[d]	Lactococcins A,B and M/N	*Lactotococcus lactis*
Teichoic acid	TagHG	Poly(glycerolphosphate), poly(glucosegalactosamine phosphate)	*Bacillus subtilis*	Lipodepsipeptides, cyclic peptides	SyrD	Syringomycin	*Pseudomonas syringae*
Capsule polysaccharide	BexABC	Polyribosylribitol	*Haemophilus influenzae*	Lipopolysaccharide	MsbA	Lipid A precursor	*Escherichia coli*
Lipopolysaccharide O-antigen	RfbBA	D-Galactan I	*Klebsiella pneumonia*	Low molecular weight carbohydrates	ChvA	β-(1–2)-Glucans	*Agrobacterium tumefaciens*
Lipo-oligosaccharide	NodIJ	Nodulation factor	*Rhizobium leguminosarum*	Fatty acylated toxins	HlyB	HlyA-RTX toxin	*Escherichia coli*
Pilin	PilHI	PilA subunit	*Myxococcus xanthus*	Hydrolytic enzymes	PrtB	Protease	*Erwinia chrysanthemi*
Exoenzymes	EcsAB	Exoamylase	*Bacillus subtilis*	S-layer capsule	LipB	SlaA S-protein	*Serratia marcescens*
Haem protein biogenesis	CcmAB	?[e]	*Escherichia coli*				

[a]This configuration of ABC exporter normally associates with auxiliary proteins of the MPA-OMA family for the translocation of polymers into or across the external cell membrane.

[b]This configuration associates with auxiliary proteins of the MFP-OMF family for the secretion of allocrites destined for the outer cell surface or the extracellular medium.

[c]The four domains of these ABC transporters are expressed on separate polypeptides. In the protein abbreviation used here for the ABC transporter, the ABC domain is followed by the MD domain(s).

[d]These ABC transporters are equipped with an extra N-terminal cytoplasmic domain involved in proteolytic processing of the peptide allocrite.

[e]Recent evidence suggests that CcmC is not a subunit of the CcmAB transporter now thought to translocate some component other than haem required for cytochrome *c* maturation [26].

ers are thought to undergo complex conformational changes related to translocation events, inserting themselves into and across the membrane at least in some cases [7,8].

ABC transporters are concerned with the transport across biological membranes of many different types of compounds with most bacterial ABC systems being involved in the import of small solutes, dependent upon a specific binding protein, for example HisJ, required for histidine uptake [9,10]. A specific term covering the wide variety of molecules translocated by ABC exporters is lacking. We prefer to use the term *allocrite*, loosely derived from the Greek meaning compounds which are transported. This avoids the misuse of the word substrate since the coupling of ATP binding and hydrolysis does not involve enzymatic modification of the transported compound by the ABC transporter. In any case, the substrate of the ABC-ATPase is ATP.

2.2. Lessons we can learn from phylogenetic analyses of the ABC superfamily

Thanks to genome-sequencing projects, inventories of the complete number of ABC-ATPase domains per genome are now readily available and several independent attempts have already been made to classify ABC-ATPases encoded within each microbial genome [11–17]. With the advent of cataloging the huge ABC superfamily, it is increasingly urgent to insist upon a standard method for the classification of ABC transporters and the adoption of a systematic convention for the naming of recognised subfamilies. This will prevent later disorder and facilitate intergenome comparisons.

In Bacteria, it is clear that ABC-ATPases constitute the largest superfamily with some species encoding from 20 to more than 76 different such proteins per genome. ABC transporter systems (including the MD domains and solute binding proteins) account for at least 5% of all proteins encoded by the *Escherichia coli* and *Bacillus subtilis* genomes. In the first global analysis of the ABC domains recently described by Saurin et al. [12], the conclusion is drawn that an extremely early division occurred within the ABC-ATPase family, before the differentiation of eucaryotes from procaryotes. This primordial division separates ABC domains according to the *direction of transport*, import or export, with which the domain is concerned. This could reflect a functional difference with respect to the action of the ABC domain or the nature of its interaction with the MD, necessary for coupling the energy of ATP hydrolysis for either import or export.

In both interkingdom and single genome analyses of ABC domains, a strong correlation has been noted between the unrooted trees derived from the ABC modules and the clustering of the MD modules. The results indicate that each ABC transporter cluster involves a specific modular organisation of polypeptides associated with the transport of a given group of allocrite. Thus, despite the wide diversity of compounds transported by the ABC transporter superfamily, there must be some specificity for the association between the ATPase and a cognate MD, reflecting the nature of the corresponding allocrite. It is important to note that this concept of specificity can be considered in two ways, taking account of the highly conserved nature of the ABC domain and the relative diversity of MDs. The ultimate specificity for recognition of allocrites must presumably be found in the MD or transport domain. On the other hand, in order to understand the observed clustering of MDs with certain ABC proteins in phylogenetic studies and therefore the ability of a universal ATPase to energise a wide variety of transport domains, there must be some built-in specificity, allowing a given ABC-ATPase to recognise its cognate MD.

Given the high degree of conservation of the ABC-ATPase domain, phylogenetic analysis of this major superfamily can be extremely instructive, particulary in relation to the evolution of this ancient family. The segregation of ABC-ATPases into subfamilies, especially across kingdoms, is often blurred due to multiple events of gene duplication, fusion and horizontal gene transfer. The observed differences in allocrite specificity of evolutionary closely related ABC systems illustrates how gene duplication allows the modification of gene function, via the accumulation of mutations in one duplicated DNA segment, in order to provide new metabolic and physiological diversity to an organism [18]. Examination of the ABC superfamily also demonstrates how gene fusion events can contribute to the spread of a protein type. For example, one ATPase domain may have been

recruited by more than one integral membrane protein very early during evolution and these MD-ABC couples then tended to evolve as a single unit. Examples also exist of two homologous ABC domains fused together and separated by a flexible linker, which have been proposed to hijack different MD complexes for function [19]. Finally, examination of the ubiquitous ABC superfamily should be informative for comparative analysis of bacteria, archea and eucaryotes, permitting suggestions concerning evolutionary relationships. For example, different repertoires of ABC proteins are found in different genomes. This reflects in some cases the particular environment in which an organism has evolved. As a result of their analysis, Saurin et al. [12] support the theory that eucaryotes acquired ABC systems through symbiotic bacteria being the putative ancestors of organelles. Nevertheless, despite these insights, the use of the ABC family in this way for exploring the nature of the ancestral tree has been poorly developed so far.

3. Some observations and trends: what are ABC exporters doing in Bacteria?

ABC proteins of bacterial origin appear to perform functions affecting processes as diverse as cell division, regulation of translation elongation of polypeptides, to the control of cell volume [20,21]. In addition of course, ABC systems are essential for the import of a wide range of low molecular weight solutes [22]. However, for the purpose of this review, detailed discussion will be limited to bacterial ABC transporters involved in export.

Procaryotic ABC exporters display two main types of packaging of the ABC-ATPase and its cognate MD module. The domains may be fused into a single multifunctional polypeptide denoted $\{MD\text{-}ABC\}_2$, or exist as four separate proteins and denoted then as $\{MD\}_2\{ABC\}_2$. In general, each arrangement, $\{MD\text{-}ABC\}_2$ or $\{MD\}_2\{ABC\}_2$, is associated with the export of a specific set of allocrites, although alternative multidomain polypeptide fusions corresponding to a given ABC transporter and allocrite can be observed in some genomes. For example, three types of organisation of antibiotic ABC exporters have been described so far, the two arrangements described previously and an additional combination with an ATP-binding protein composed of two fused homologous nucleotide binding domains as in MsrA (for erythromycin) and OleB (for oleandomycin), from *Staphylococcus epidermidis* and *Streptomyces antibioticus* [23–25].

The allocrites transported by ABC exporters vary enormously in their size and aspect. Moreover, many of the allocrites are post-translationally modified by addition of fatty acyl or lipid groups or possess enzymatically modified rare amino acids (lanthionines) or dehydrated residues (Table 1). Ultimately, it might be hoped to equate differences in primary sequence of a given transporter related to its allocrite specificity. This will be especially exciting when further structural data emerge from crystallography or NMR studies [27,28]. Happily, arising from the power of computer-predicted sequence comparisons and alignments, several family-specific conserved sequence motifs have been identified in ABC and MD domains associated with the specific transport of similar types of compound. The conserved protein motifs identified in particular MDs are thought to be concerned with allocrite recognition, a transmembrane signalling process resulting in activation of the ABC domains or involved in ensuring the vectorial nature of the transported compound (import/export) [12,29–31]. This clustering of phylogenetically related ABC or MD domains with very similar allocrites helps the assignment of a function to uncharacterised ABC transporter orthologues from different organisms.

The following sections now represent a brief overview of the function of certain classes of bacterial ABC exporters and their distinguishing features.

3.1. Polysaccharide exporters

A cluster of ABC exporters with ABC-ATPase domains co-operating with separately encoded similar or in unusual cases, dissimilar MDs, forming homo- or hetero-oligomeric channels, have been designated as 'ABC-2 transporters' [29] (see Fig. 1), or as the subfamily ABC-A2 by Saurin et al. [12]. A signature motif unique to this cluster is present in the C-terminal section of at least one or both of the integral membrane components. In this case, conservation probably reflects the related function of

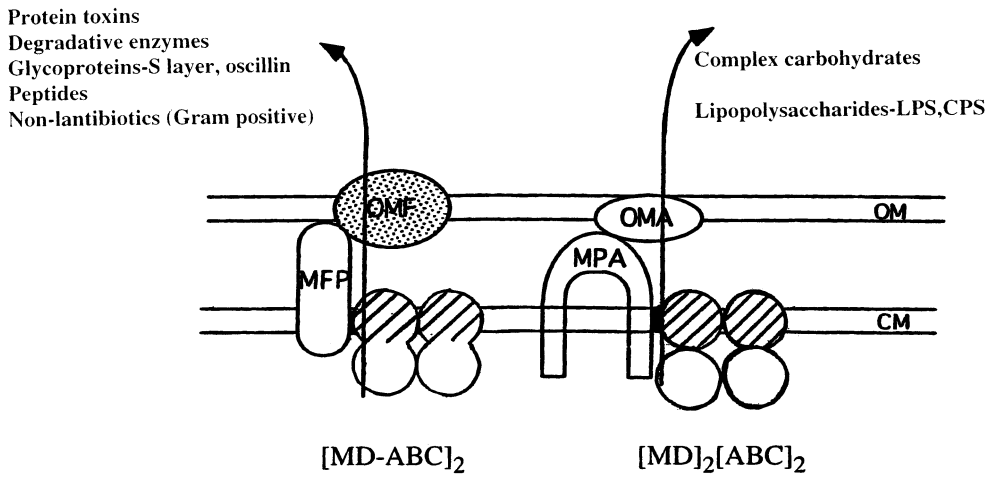

Fig. 1. Schematic representation of two types of ABC-dependent transporter complex involved in type 1 secretion (left) or ABC-2-dependent (right) in Gram-negative bacteria. Note that the MFP component which couples here with the ABC transporter and hydrolysis of ATP to drive transport, can also couple with other systems such as the MFS and RND family of transporters, driving transport using the proton motive force. Note also that in Gram-positive bacteria non-lantibiotics, such as lactococcins, are secreted by a type 1 mechanism utilising an ABC transporter plus a member of the MFP family, whilst lantibiotic peptides, such as nisin, require only the ABC transporter protein.

these transporters in the transport of a variety of compounds bearing a glycosylation modification. The hydrophilic compounds vary from small drug molecules (e.g. glycosylated oleondomycin) to complex carbohydrate polymers localised within or on the surface of Gram-positive and Gram-negative bacteria (eg. lipopolysaccharide (LPS), teichoic acids (TCA), capsular polysaccharides (CPS)). Recent experiments suggest that the polymer transport machinery in these cases may associate with the membrane bound polysaccharide biosynthetic machinery, forming a huge macromolecular complex. Amongst several advantages, this would permit the tightly coupled transport of the polysaccharide chain directly from its site of synthesis on the cytoplasmic face of the membrane [32].

3.2. ABC peptide exporters

Gram-positive bacteria produce a plethora of antimicrobial peptides. Two main classes of antagonistic peptides are the lantibiotics (class I bacteriocins) containing post-translationally modified amino acids and non-lantibiotics (class II bacteriocins), consisting of unmodified amino acids [33]. It has been shown for several of these peptides that their translocation is mediated by an ABC exporter whose ABC and MD domains are on the same polypeptide (Table 2). Unusually for compounds transported via ABC systems, class I and II bacteriocins are synthesised as precursors containing N-terminal leader peptides, which differ markedly from those of standard secretory proteins, but are, nevertheless, removed during maturation. The leader peptides of most lantibiotics (e.g. subtilin, nisin,) are removed after transport through the ATP transporter by a dedicated peptidase located extracellularly [34]. However, the non-lantibiotics (e.g. lactococcins, sakacin A) and certain lantibiotics (e.g. lacticins) with a conserved double-glycine-type leader peptide (GG-leader), are processed concomitantly with secretion by a dual-function ABC transporter (Table 2). This type of ABC transporter forms a particular subfamily called the ABC-containing maturation and secretion (AMS) protein [34,35]. The hybrid AMS proteins contain an N-terminal extension of approximately 160 amino acids forming a proteolytic domain not present in other ABC proteins. The proteolytic domain is apparently located in the cytoplasm indicating, surprisingly, that processing of the precursors into their mature form takes place at the cytosolic side of the cytoplasmic membrane [35]. In addition, AMS export mechanisms have been observed for the transport of certain competence factors and peptide pher-

Table 2
ABC peptide exporter systems of Gram-positive and Gram-negative bacteria involved in producer self-protection (immunity), processing and/or secretion

	Bacteriocin or allocrite	ABC transporter conferring immunity$\{MD\}_2$-$\{ABC\}_2$ [a]		ABC transporter of precursor peptide$\{MD\text{-}ABC\}_2$	Membrane fusion protein	Outer membrane factor
Gram-positive bacteria						
Class I lantibiotics						
FNLDV-type leader sequence	Nisin	NisFEG		NisT		
	Subtilin	SpaFG		SpaT		
	Epidermin	EpiFEG		EpiT		
G-G type leader sequence	Lacticin 481	LctFEG		LctT[b]		
	Mersacidin	MrsFEG		MrsT[b]		
Class II non-lantibiotics						
G-G type leader sequence	Sakacin A			SapT[b]	SapE	
	Sakacin P			SppT[b]	SppE	
	Leucocin A			LcaC[b]	LcaD	
	Mesentericin Y105			MesD[b]	MesE	
	Enterocin A			EntT[b]	EntD	
	Lactococcin A, B, M/N			LncC[b]	LncD	
	Lactococcin G/M			LagD[b]	LagE	
Peptide pheromones	Plantaracin A			PlnG[b]	PlnH	
	Competence peptide			ComA[b]	ComB	
Gram-negative bacteria						
G-G type leader sequence	Colicin V			CvaB[b]	CvaA	TolC
Microcins	B17		McbFE[c]		?	?
	MccJ25		McjD[c]		?[d]	TolC

[a]In the protein abbreviation used here for the ABC transporter, the ABC domain is followed by the MD domain(s).
[b]Refers to those ABC transporters that are equipped with an extra N-terminal cytoplasmic domain involved in proteolytic processing of the peptide allocrite.
[c]The microcin ABC transporters are thought to function in translocation of the precursor and play a role in self-protection.
[d]The secretion and export of MccJ25 probably involves an auxiliary membrane fusion protein which remains to be identified [39].

omones also carrying a GG-leader, and responsible for the production of the bacteriocins in lactic acid bacteria (LAB), onset of competence in *B. subtilis* and other such quorum-sensing modes [36–38].

The production of peptides of this type is not exclusively a phenomenon of Gram-positive bacteria. Gram-negative Enterobacteriaceae also produce peptide antibiotics. The best characterised from *E. coli* include microcins MccB17 and MccJ25, both exported by ABC transporters [39–41]. The only known peptide of the double-glycine type in *E. coli* is colicin V which is similarly processed and exported by a hybrid ABC transporter protease. In summary, the ABC peptide exporters are suitable vehicles for secretion, rather promiscuous in fact, allowing a large variety of proteins to be translocated, provided a recognisable N-terminal region is present, acting as a secretion signal. As with the polypeptide, C-terminal signal dependent, transport mechanism for ABC transporters, essentially nothing is known of the structural requirements of peptides necessary for successful transport through the translocator [42].

3.3. ABC drug exporters

ABC drug transporters are responsible for single drug resistance (SDR) in Gram-positive bacteria, and these are specific for transporting a single or a group of closely related drugs. Nearly all SDR transporters show a variation of the $\{MD\}_2\{ABC\}_2$ arrangement [25]. Only one bacterial ABC drug transporter conferring multidrug resistance (MDR) has been experimentally characterised, namely LmrA of the Gram-positive bacterium, *Lactobacillus lactis* [43,44]. LmrA shows an $\{MD\text{-}ABC\}_2$ arrangement. However, the existence of MDR ABC transporters may be more widespread than first realised, with the recent reports of cryptic MDR systems in genera other than lactic acid bacteria [45]. Curiously, no functional ABC, single or multidrug, transporter has yet been definitively established to exist in Gram-negative bacteria. A few ABC transporters have been suggested to be putative drug exporters in Gram-negative bacteria on the basis of some sequence identity with the human multidrug P-glycoprotein, but experimental evidence demonstrating any associated drug resistance is still lacking. In fact, for certain of these ABC transporters, non-drug related functions have since been identified, for example MsbA, involved in the transport of lipids to the inner surface of the outer membrane of *E. coli* [46]. The lack of ABC antibiotic and drug resistance systems in Gram-negative bacteria is further highlighted by the relatively frequent occurrence of such ABC exporters encoded by the *B. subtilis* genome [11]. This raises interesting questions, such as why was there an expansion or retention of ABC exporters conferring drug resistance in Gram-positive bacteria? Perhaps ABC drug exporters in Gram-negative bacteria are redundant in the face of many alternative secondary transporter drug efflux systems of extremely ancient origin. In contrast, drug efflux pumps of the ABC-type appear to be recent acquisitions to genomes and have appeared only occasionally during the evolution of this superfamily [47].

In fact, the in vivo role and therefore the true physiological reason for the appearance of ABC multidrug transporters is of some dispute [48]. In both eucaryotes and procaryotes, it is still not clear whether removing toxic chemicals from the cell is the true physiological function of MDR systems. Rather, this capacity to transport drugs may reflect an adaptation from a more specific role in the transport of unidentified endogenous molecules, such as lipids. Indeed, for major MDR systems in mammals, lipids have been shown to be physiological allocrites [49–51]. Thus, MDR systems may have developed to provide a selective advantage against biocides or have evolved to provide self-resistance to toxic metabolites produced by the microorganism itself. In this context, it is important to analyse bacterial ABC exporters known to be involved in self-resistance to small peptide bacteriocins.

3.4. ABC exporters involved in producer self-protection

Bacteria producing antimicrobial peptides also have to be protected from the lethal effects of these compounds. In some cases, as for the colicins produced by Gram-negative bacteria, this can be achieved by a specific immunity protein, synthesised by the colicin producer, as an integral membrane protein possessing three to four transmembrane domains. The immunity protein binds to the pore-forming domains of the corresponding colicin to block the action of either outgoing or incoming colicin picked

up from the medium [52]. Amongst several mechanisms proposed to operate for immunity to class I lantibiotics (e.g. subtilin, nisin, lacticin 481) produced by Gram-positive bacteria, one is a quite different mechanism appearing to involve active extrusion [53]. In this case, another ABC exporter is required for the re-translocation of those exported peptides that have penetrated the membrane from the external medium. The vast majority of such immunity ABC exporters are encoded by two (spaFG) or three (nisEFG, lctEFG) subunits constituting homo- or heterodimers of the membrane spanning unit. Whilst the ABC-ATPase in these systems are highly homologous, the MDs show few sequence identities (about 20–30%) [54–56].

The ABC peptide exporters involved in producer self-protection have the same $\{MD\}_2\{ABC\}_2$ organisation as certain other ABC transporters that provide resistance to peptide antibiotics and drugs, for example, the Bcr bacitracin transporter of *Bacillus licheniformis* and the Drr system for export of daunorubicin by *Streptomyces peucetius* [57,58] (Table 1). Almost nothing is known about the mechanism of immunity conferred by ABC peptide exporters to membrane damaging peptide antibiotics. This is presumed to involve removal of the bacteriocin from its target within the bilayer and its expulsion to the surrounding medium, thus preventing accumulation in the membrane [54,57]. This mechanism of interception of hydrophobic compounds in the bilayer and their elimination from the inner leaflet by a 'flippase' or a 'vacuum cleaner' mechanism, is of course the favoured mechanism for the action of multi-drug resistance transporters. Much of the evidence for this mechanism is based on studies using LmrA of *L. lactis*, which has an $\{MD\text{-}ABC\}_2$ transporter organisation [59].

The ABC transporters of the lantibiotic immunity systems are encoded within the same operon as the genes involved in the biosynthesis and export of lantibiotics (see Section 3.2). That two specialised ABC transporters are involved, respectively, in secretion of the precursor or in immunity to the mature form of the exported allocrite, raises questions concerning the specificity and capacity of each transporter to recognise its particular form of the allocrite. There is no homology between these respective transporters which in addition are configured differently, the MD and ABC domains of the ABC exporter involved in transport of the lantibiotic precursor being constituted from a single $\{MD\text{-}ABC\}_2$ polypeptide. Surprisingly, in Gram-negative producers of microcins B17 and J25, the same ABC transporter is thought to function both in export and immunity [39,41].

4. Further transport across procaryotic membranes

At least some ABC exporters may expel drugs and peptide lantibiotics across a single membrane, the cytoplasmic membrane, and therefore no additional proteins are involved (see Table 2). However, ABC exporters transporting allocrites destined for the outer membrane of Gram-negative bacteria, the cell surface of Gram-positive and Gram-negative bacteria or the extracellular medium, often if not invariably require the presence of additional auxiliary components to complete the transport pathway. In some cases, the allocrites may be passed on to separate transport systems independently of the ABC transporter. In other cases, however, the auxiliary proteins (sometimes called helper or accessory proteins) are indispensable to the function of the ABC transporter and together form an integral export unit. Two distinct types of auxiliary protein units have been characterised and designated (sometimes unfortunately misleading) family names by Saier and colleagues [60–63]. Each type is considered distinct through their relative topology and through their association with a particular subclass of ABC exporter and therefore different allocrite. In other words, each ABC-dependent exporter unit appears to be tailor made to suit a defined allocrite as illustrated in Fig. 1.

Thus, subunits of ABC exporters encoded by separate genes ($\{MD\}_2\{ABC\}_2$) and belonging to the aforementioned ABC-2 (ABC-A2) transporter family, associate with inner membrane auxiliary proteins designated the membrane periplasmic auxiliary (MPA) family. An additional subclass of outer membrane protein, the outer membrane auxiliary (OMA) family, is required by these systems in Gram-negative bacteria. The $\{MD\}_2\{ABC\}_2$-MPA-OMA secretion systems are largely involved in the biogenesis of the bacterial cell surface, transporting cell wall compo-

nents and complex carbohydrate polymers of the cell surface, such as LPS and CPS (Fig. 1). In contrast, fused {MD-ABC}$_2$ ABC transporters associate with inner membrane auxiliary proteins of the membrane fusion protein (MFP) family, with an additional outer membrane factor (OMF) auxiliary protein required to complete transport across the outer membrane in Gram-negative bacteria. This multicomponent ATP driven transport system, {MD-ABC}$_2$-MFP-OMF, forms a transenvelope structure known as the type I or ABC-dependent secretion system (Fig. 1). The genetic determinants of type I systems are frequently plasmid-borne and often form part of an operon. Type I secretion systems are particularly relevant to the pathogenic properties of strains, since this is also used for secretion of toxins, degradative enzymes and various cell surface protective structures, as will be described in the next section.

4.1. ABC exporters of type I systems transport polypeptides and non-proteinaceous material

ABC transporters of type I secretion systems provide an energised ATP-dependent transport pathway for proteins localised to the surface of the bacterial outer membrane or released as soluble proteins into the extracellular medium. Proteins remaining associated with the cell surface include certain glycanases and the protective S-layer protein. Soluble extracellular proteins include examples, such as hydrolytic enzymes (metallo-proteases, lipases), haem-binding scavenger proteins (HasA, FrpA) and pore-forming toxins (haemolysins, leukotoxins) [64].

Two distinctive features unify these diverse allocrites of type I secretion systems. Most, but not all, the allocrites secreted via this pathway are classified as repeats in toxin (RTX) Ca^{2+}-binding proteins. This is attributable to a variable number of glycine-rich RTX nonapeptide motifs which fold to form a β-roll structure which binds calcium ions [65]. In some cases, the RTX repeats appear to be required for efficient secretion through the transport apparatus, especially for large, heterologous proteins [65–67]. Secondly, with few exceptions, secretion is dependent upon a non-cleaved secretion signal located at the C-terminus of the polypeptide. Comparison of the C-terminii reveals poor conservation at the primary sequence level except for a 4–6 residue motif at the extreme end which allows division of the allocrites into three subfamilies: HlyA-like, metalloprotease-like and others [68,69]. Exceptionally HasA, a comparatively small type I allocrite (19 kDa), lacks the RTX-Ca^{2+}-binding repeats and possesses a C-terminal signal sequence which is cleaved after secretion [70].

Although, type I export systems usually involve the ATPase fused to the C-terminal of the membrane domain, notation {MD-ABC}$_2$, there may be exceptions to this, such as the recently discovered DevBCA exporter, encoding an ABC transporter with an apparent {MD}$_2${ABC}$_2$ arrangement, associated with an MFP/OMF [71]. The ABC exporters of the membrane-targeted HlyA-related toxins (HlyB, CyaB, LktB) constitute a sub-group distinct from the ABC metalloprotease exporters. Surprisingly, in this subclass the ABC component at least is more closely related to ABC-ATPases of exporters concerned with the transport of lipophilic drugs (e.g. LmrA), lipids (e.g. MsbA), polysaccharides (e.g. HetA) and the highly substituted carbohydrate rich β-1,2-glucans (e.g. NdvA and ChvA) [12]. This last allocrite is found almost exclusively in bacteria of the Rhizobiaceae family, being important for symbiosis and nodule invasion. The common factor that may link these ABC exporters of which HlyB is the prototype, is that their function all involve transport of allocrites of a lipidic nature. HlyA-like toxins are post translationally modified with two fatty acid additions, β-glucans for example are non-proteinaceous allocrites. However, at least in the case of HlyA, acylation of the toxin is not apparently involved in any way in the secretion mechanism [72]. Type I secretion transporters are intriguing in another way, with their ABC domain phylogenetically the closest amongst the bacterial ABC proteins to the TAP1/2 (peptide transporter) and Mdr (for example P-glycoprotein) found in mammals, including humans [12,72].

4.2. What does the integral transmembrane channel of the ABC polypeptide transporters look like?

Considerable reference has been made to the ABC exporters of type I systems in connection with their similarity to the two extensively studied eucaryotic

ABC transporters: the heterodimeric TAP1/2 transporter associated with antigen presentation and human Mdr1 (Pgp), responsible for the resistance of cells to several unrelated drugs, but also implicated in lipid transport. This similarity is based on the surprisingly close relationship of the ABC-ATPase of HlyB to these proteins in phylogenetic analyses [12], but also extends to significant similarity detected between the distal regions of the MD of HlyB and Pgp [31,71–73].

ABC polypeptide transporters also share with the TAP and Mdr ABC exporters a limited ability to recognise and transport heterologous allocrites. This includes other RTX-proteins but also colicin V in the case of HlyB. For Pgp a variety of transported allocrites have been reported, including the yeast mating pheromone [74]. Attempts to map allocrite recognition sites in each of these ABC transporters has given rise, as with other ABC transporters, to the idea of an allocrite binding site located within the membrane domain and a translocation mechanism, involving in this case peptide or polypeptide induced intramolecular signalling to activate the ATPase, with consequent release of energy for transport. Efforts to define the steric limits of the binding site of these transporters and residues involved in allocrite selection has had limited reward so far [75,76].

The arrangement of the α-helical transmembrane segments (TMS) of Pgp, TAP and HlyB have been difficult to analyse and the possibility of the presence of β-sheet structures cannot be excluded [77]. Current algorithms appear to predict transmembrane segments of ABC transporters less well than for other membrane proteins, such as bacteriorhodopsin, emphasising the need for experimental measurement of the topology of these ABC proteins. The predicted topology of TAP1 indicates a model which spans the membrane eight times, with several large loops exposed in the lumen of the endoplasmic reticulum [78]. At least two conformations with 'two times four' or 'two times six' TMS have been described for Pgp, where the four domains are fused {MD-ABC-MD-ABC} to form a single polypeptide [79,80]. Based on β-lactamase fusions, a topology for HlyB indicated eight TMS [81] which deviated significantly from that predicted simply by algorithms, both with respect to number (6) and positioning of some TMS. A subsequent study [82] using β-galactosidase and alkaline phosphatase also gave eight TMS with some deviation in their precise positioning. Finally, a unified model combining this data was devised [83], which also maximises the use of hydrophobic residues to determine the limits (21 residues) of specific TMS. The overall result gives a model with eight TMS, but still with significant deviation of the position of TM4 and TM6 from that predicted from the hydropathy. TM1 and 2 in HlyB are not predicted by most algorithms and reside in an N-terminal which is extended compared with other type I ABC transporters. Moreover, TM1 at least can be deleted without loss of function [84].

Electron microscopic (EM) images of Pgp 2D crystals are still the only structural information available for any intact ABC transporter [85]. The EM images resolved to 2.5 nm reveal a possible four-domain MD-ABC-MD-ABC assembly, but a clear structural model for Pgp remains elusive. At this resolution it was not possible to resolve many features of the MDs and the two asymmetric lobes on the cytoplasmic side of the molecule could only tentatively be labelled as the ATP-binding domains. A strikingly evident feature of the structure, however, is the existence of a large crater with a diameter of 2.5 nm, apparently narrowing to finally close within the lipid bilayer, presumably formed by the MD domains. This proposed translocation 'pore' or chamber is a much larger structure than should be required for the transport of small drug molecules across the membrane and the importance of this feature for transport function has yet to be demonstrated. It is generally anticipated that ABC transporters, with similarly organised membrane domains and a highly conserved ABC-ATPase domain, will function in a similar way, with the binding of the allocrite stimulating ATPase action. This in turn is coupled to movement of the allocrite across the membrane, involving conformational changes in the membrane domain. This similarity in function might therefore be expected to be reflected in the overall modular design exemplified so far by the Pgp structure. Despite this, however, is it really possible to make a functional comparison between multidrug and protein transporters, given that each transporter deals with extremely different allocrites, unrelated in size? This is discussed in the next section.

4.3. Can the molecular mechanism for the secretion of drugs and proteins be the same?

Functional studies with ABC type I exporters at the molecular level have been slow in developing, most likely due to technical difficulties in purification of the component parts. For the haemolysin system, this has in part also been due to difficulties in overexpression and even detection of the HlyB protein by Western blotting of SDS-PAGE gels. Thus, the addition of other detergents in the SDS-sample buffer is necessary to prevent aggregation of the protein, allowing its entry into the acrylamide gel (J. Young, 1999, Thesis, unpublished data). ATPase activity has been demonstrated for an HlyB-ABC-GST fusion [86] and for the ABC domains of intact PrtD [87] and HlyB (this laboratory unpublished), indicating that the ATPase can function separately from the auxiliary proteins that form the rest of the secretion complex. Limited functional studies of PrtD in membrane vesicles or after purification have met with moderate success so far. Surprisingly, addition of the secretion signal peptide for the PrtD allocrite inhibited ATPase activity, which may reflect an abortive reaction in the absence of the auxiliary proteins [87].

Although studies of the ATP cycle and the mechanism of allocrite recognition and binding sites have been limited with ABC polypeptide transporters of bacterial systems, these important aspects have been extensively studied for cystic fibrosis transmembrane conductance regulator protein (CFTR), Pgp and its homologues. The use of sophisticated tools such as labelled drug analogues that display changes in fluorescence when in a lipid membrane or aqueous environment, have resulted in several detailed proposals for how drugs are recognised, acquired and passed through the transporter [61,88,89]. In relation to attempts to extrapolate these results to the transport of proteins by type I ABC exporters, it is necessary to emphasise two main differences between these systems. Small hydrophobic drugs in general partition spontaneously into lipid bilayers and so probably approach the transporter 'laterally' from the inner leaflet of the bilayer. Large proteinaceous allocrites like HlyA probably approach the transporter 'horizontally' from the cytoplasm. Thus, such different allocrites must traverse the membrane by two quite different transport pathways despite the presumed similar basic design of the respective ABC transporters involved. The proposal that Pgp acquires its allocrites from the membrane is supported from data using fluorescent analogues and interestingly the EM structure described by Rosenberg et al. indicates the presence of a cleft open laterally to the membrane bilayer [85]. Finally, it is not out of the question that drugs are transported from the inner leaflet of the lipid bilayer to the outer leaflet or the exterior by transport along the outside of the MD via the TMS-lipid interfaces, thus avoiding completely the proposed interior aqueous pore of the Pgp [89,90].

The current hypothesis for the transport of large, relatively hydrophilic proteins, such as HlyA, envisages that they may traverse an interior, aqueous chamber of the transporter complex. If this is true, the molecular mechanism and the transport pathway for the secretion of drugs and proteins will ultimately be shown to be different. Integral to this issue is the problem of allocrite recognition by the ABC transporter. It is still probable that the initial binding site(s) for both proteins and drugs resides within the membrane domain, albeit at topologically distinct sites. However, evidence also exists to suggest that the ABC domain may also (directly or indirectly) participate in interactions with the allocrite [23,91]. With respect to drug transporters, a longstanding problem has been what single feature of the exporter complex might recognise such a wide variety of allocrites. This is still unclear; however, a comparable situation with the transcriptional activator, BrmR from *B. subtilis*, which also responds to a variety of drug molecules, has recently been illuminated with publication of the crystal structure [92]. The structure deduced also in the presence of a drug molecule indicates a mechanism for drug induced local unfolding of the protein to allow the drugs to enter a hydrophobic pocket, apparently capable of accommodating different molecules.

5. The allocrites of type I systems

Novel classes of allocrite and their corresponding secretion systems have recently come to light in diverse genera (see Fig. 1). Thus, the bifunctional S-layer-RTX-toxins secreted by *Caulobacter* sp. con-

sist of an N-terminal S-layer protein fused to a C-terminal domain containing the secretion signal and RTX motif [93–95]. Other newly defined sets of type I allocrites are cell-associated exopolysaccharide (EPS) processing enzymes named as ExsH, PglA and PglB of *R. meliloti*, SpsR of *Sphingomonas* sp. and Egl of *Azorhizobium caulinodans* [96,97]. PglA, PglB and SpsR do not contain RTX repeats, possessing instead novel heptapeptide repeats that may be involved in the formation of a β-helix structure. Egl has yet another type of repeat motif thought to form a different fold to that formed by the RTX motif but still involved in the binding of calcium. Thus, a recurring theme of allocrites passing through type I systems is the existence of internal repeats. It remains to be determined if the different classes of the repeat motif contribute to secretion through the transport apparatus and/or re-folding of the allocrite as it emerges on the cell surface.

6. The diverse roles of ABC-dependent secretion systems

The best characterised type I systems are the prototype *Erwinia chrysanthemi* protease and the *E. coli* haemolysin secretion systems, typified by the transport complexes, PrDEF and HlyBDTolC respectively [98,99]. Type I systems are not, however, only involved in the secretion of virulence factors, but extend to much wider roles related, for example, to the phenomena of development, gliding and swimming in diverse Eubacteria. Examples of the latter are filamentous cyanobacteria are Gram-negative bacteria with a complex life cycle characterised by behavioural manifestations, such as social motility. The DevBCA exporter was recently identified in *Anabaena* sp. which form nitrogen fixing, heterocysts. This ABC-exporter complex secretes a component that is essential for the assembly of the thick laminated layer formed on the cell surface of this Gram-negative bacteria [71]. In relation to motility in the cyanobacterium, *Phormidium uncinatum*, a type I system is probably involved in the secretion of oscillin, a Ca^{2+} binding RTX glycoprotein that has an important role in the locomotion of this organism. Oscillin forms an array of parallel fibrils on top of the S-layer and acts as a platform for the assembly and attachment of carbohydrate fibrils that guide the rotation of the bacterium [100]. Based on homology searches of the non-redundant protein database, type I systems can be predicted to be present in several species including the fresh water cyanobacterium *Synechocystis* sp., the soil bacterium *Sphingomonas* sp., the hydrogenobacterium *Aquifex aeolicus* and the spirochete *Treponema pallidum* [101]. Thus, type I secretion pathways are polyphyletic and more widespread than originally thought.

The presence of type I secretion systems in Gram-positive bacteria further demonstrates the universality of this mechanism of transport which in Gram-negative bacteria is adapted to span the periplasm between the inner and outer membrane. One can consider that secretion across the thick negatively charged peptidoglycan outer layer of Gram-positive bacteria will pose equally difficult problems to those generated by the double membrane of Gram-negative bacteria. Type I exporters are in fact prevalent in LAB, Gram-positive bacteria adapted to dairy environments. Interestingly, this is the same genera in which complex ABC multidrug transporters have been identified such as LmrA. LAB type I systems are concerned with the secretion of non-lantibiotics and competence stimulating peptides (Table 2).

It is interesting to ask why the secretion of non-lantibiotics (e.g. lactococcins, plantaricins) in LAB involves an ABC transporter requiring an MFP accessory protein, while the lantibiotics (e.g. subtilin, nisin, lacticins) are transported by a single ABC transporter? The need for an accessory protein in the case of the non-lantibiotics suggests that the MFP proteins could have a special role in relation to the transport of these particular peptides. These alternative pathways may be the consequence of the different mode of action and accompanying immunity systems, associated with the two classes of bacteriocins [53,56]. Thus, lantibiotics are proteolytically activated after secretion by a membrane associated protease and act by forming pores in the cytoplasmic membrane of target cells, without the need for a membrane-associated receptor. LAB, which produce lantibiotics, possess a lantibiotic host protection mechanism that is based on an ABC transporter (see Section 3.4). This acts at the level of the cytoplasmic membrane, presumably keeping the concentration of lantibiotic below a critical level necessary

for the appearance of pores. Non-lantibiotics, (e.g. lactococcins), however, are proteolytically activated during their transport through the cytoplasmic membrane and rely on a specific membrane receptor in target cells for their pore-forming action. Other mechanisms of self-protection appear to operate in these examples, although detailed information is not available. It is thought that the immunity protein interacts with the non-lantibiotic before it can reach the membrane or acts indirectly, binding to the membrane receptor used by the antibiotic [102]. The type I transport system is perhaps crucial for directing the active non-lantibiotics through and away from the cytoplasmic membrane, contributing towards a protection function in the susceptible LAB producer, since this also produces the receptors.

Type I secretion systems (ABC-dependent) have also been identified in Gram-positive genera other than LAB. These are responsible for bacteriocin export in *Enterococcus faecalis*, and in the organisation of fimbrial tufts in *Strepotococcus crista*. Moreover, several examples are encoded in the *B. subtilus* genome, but are of unknown function [101].

Presumably, association of two protein partners MD-ABC, MFP and an OMF in Gram-negative bacteria is the optimum architectural answer for obtaining efficient transport across a protective barrier with minimum lateral leakage into the periplasm [103,104]. We will now take a brief look at the auxiliary proteins associated with the ABC transporter of type I secretion systems, noting that both the MFPs and OMFs are sets of highly divergent proteins united by a common function.

7. The auxiliary proteins of type I ABC secretion systems

7.1. MFPs

The members of the MFP (membrane fusion protein) family of proteins were initially identified based on similar size and predicted structural features, together with some weak sequence similarities [62] (see Fig. 2). The MFP proteins can be subdivided into different classes, clustering according to the type of inner membrane component with which they associate and then according to the allocrite transported [63]. MFPs of ABC-dependent secretion systems are absolutely essential for the secretion of allocrites and in their absence, the allocrite remains in the cytoplasm and does not accumulate in the periplasm. This suggests that the MFP is an integral component of the transporter complex intimately linked to the

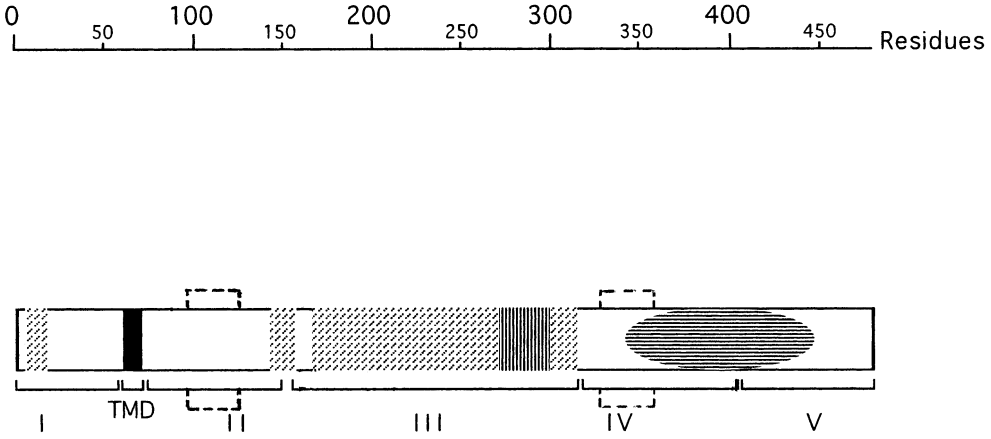

Fig. 2. Schematic representation of the major features of HlyD, a prototype MFP protein. Above are amino acid residue numbers from the N-terminal (left). The transmembrane domain is represented as a black rectangle; segments predicted (Pred Prot) to form α-helices of 10-residues or more, hatched rectangles; probable β-sheet domain, horizontal lined oval; coiled-coil motif [105], vertical striped box. The split motif of the conserved lipoyl/biotin 'swinging arm' [138] is represented by the two broken line boxes. The roman numerals below identify different domains of HlyD deduced on the basis of similarity plots with other members of the HlyD family (A. Pimenta, M.A. Blight, I.B. Holland, unpublished); regions II, IV and V are the most conserved. Residues from the TMD to the C-terminal are periplasmic.

ABC transporter, probably in order to provide a continuous transport pathway across the periplasm or peptidoglycan to the cell surface.

MFP proteins are anchored in the cytoplasmic membrane by a single N-terminal transmembrane region or for one subclass of MFP, via a lipid moiety. All MFPs are likely to form coiled-coils, characterised by the presence of several heptad repeat sequences (abcdefg)*n* in the extracellular/periplasmic domain [105]. The role of this structure in relation to the function of MFPs remains speculative, but could include an extensible/retractory capacity similar to that of contractile proteins. Coiled-coil domains are not only implicated in protein dynamics, however, they can also be regions for subunit oligomerisation as in the fibrous proteins. Interestingly, in silico analysis of the coiled-coil segments of MFP proteins using the multimeric prediction program MultiCore (http://ostrich.lcs.mit.edu/cgi-bin/multicoil) predicts the formation of a two- rather than a three-stranded intra- or intermolecular coiled-coil multimerisation unit (unpublished observation).

A subfamily of the MFP family is represented by HlyD and its homologues present in both Gram-negative and Gram-positive bacteria. The HlyD subfamily is defined by a consensus protein signature located at the extreme C-terminal end of these proteins (Prosite entry PS00543; [106]). Although speculation as to the possible role of this motif includes involvement in an interaction with the OMF protein, its presence in HlyD-like proteins of Gram-positive bacteria, which lack an outer membrane discourages this view. Pertinently, HlyD-like proteins of Gram-positive bacteria possess extracytoplasmic domains of a similar length to those of the larger MFPs of Gram-negative bacteria. The peptidoglycan-traversing domain of MFPs do not contain recognised consensus sequences involved in protein–cell-wall anchoring or association, unlike proteins such as MotB of the flagellar motor [107]. This could suggest that the trans-envelope MFPs need to be flexible rather than rigid in structure. Despite the fact that several MFP proteins of Gram-negative bacteria function with the same OMF, TolC, it is not evident from simple sequence gazing that a common sequence, involved in an interaction with TolC, can be identified. Thus, the interaction, if it exists, probably relies on conformation rather than a specific amino acid sequence.

7.2. OMFs

In Gram-negative bacteria, an additional auxiliary protein is required for the transfer of allocrites across the outer membrane [61]. These OMFs probably work in conjunction with the MFPs (Table 2). Two dimensional crystallography of the best characterised representative, TolC, showed two globular modules, a membrane-embedded moiety and a substantial extra-membrane domain proposed to extend down into the periplasm [108]. Two different models have been proposed for the topology of TolC. One model based on a particular algorithm favours the formation of an 18-stranded β-barrel transmembrane domain with a C-terminal periplasmic domain [108]. However, specialist prediction methods involving intelligent neural networks trained and tested on multiple alignment sets make a strong case for transmembrane β-strands dominated by substantial amounts of α-helical structure in TolC [101]. This makes it unlikely that the OMFs adopt a β-barrel fold, rather a mixed α/β fold unlike any other OM protein of known structure. At least six α-helices adjacent to the predicted β-strands are predicted, all with high coiled-coil probability. In this second model, the coiled-coil domains self-associate into a helical bundle predicted to correspond to the periplasmic domain. Both models agree that the OMFs are quite distinct from the porins bearing no sequence similarity and an extra-membrane domain that could contribute towards forming a trans-periplasmic channel and/or be involved in interactions with the MFP or ABC translocator components. Based on gel filtration experiments and 2D images of TolC, it appears that the OMFs trimerise [108]. However, it was not possible from the 2D images at 1.2 nm resolution to deduce whether three TolC monomers form a central pore or alternatively whether each subunit forms a pore.

A further general feature of the OMFs includes a tandem symmetry of the N- and C-terminal halves of these proteins [101,109]. This suggests that the OMFs arose by an intragenic duplication event and that the two halves of these proteins show a similar conformation. In most of the type I secretion systems, the genes encoding the ABC-MFP exporter are linked to the gene encoding a specific OMF constituent. In cases such as the haemolysin secretion system, the gene for the OMF is not linked and tends to be

multifunctional, participating in a variety of interactions and receiving numerous translocating allocrites for both import and export [39,110–112]. Presumably, therefore, the OMF is not involved in allocrite specificity with respect to the type I system.

8. The logistics of type I transport

There are many fascinating questions to be posed concerning the type I secretion mechanism to which a brief allusion is made here. In Gram-negative bacteria, the allocrite has never been detected in the periplasm. For this reason, the transport pathway is referred to as a one-step secretion process, indicating transport of the allocrite directly from the cytosol to the external environment (Fig. 1). This could be achieved in two ways depending on the action of the MFP. MFPs may reduce the spatial separation between the extracellular surface and cytoplasmic membrane by bringing the two into close proximity. In Gram-negative bacteria, this could occur through interactions of the MFP with the inner and outer membrane protein components of the secretion apparatus. It is not clear how a comparable effect could be achieved in the case of the rigid external peptidoglycan layer of Gram-positive bacteria. Alternatively, MFPs may provide a channel across the periplasm and the peptidoglycan allowing molecules to reach the cell surface directly.

The periplasmic compartment of Gram-negative bacteria has an estimated depth ranging from 13 to 25 nm and contains a thin single-layered peptidoglycan [104]. Biophysical measurements of a functional version of the MFP protein, AcrA, consisting of just the periplasmic domain, predicted its length to be about 17 nm, agreeing with the prediction that these proteins can form extended molecules capable of spanning the periplasm [113]. If the MFP proteins do form a transenvelope pipeline, what could be the nature of the structure? Is it closed or permeable to the non-reducing redox environment of the periplasm. Two observations in fact suggest that the allocrite is not completely isolated from the periplasm. The operons of type I systems that secrete proteases also encode a specific protease inhibitor, presumably for protection against intracellular proteolytic activity. Surprisingly, the inhibitor, unlike its cognate protease is exported via the Sec system and N-terminal signal sequence, to the periplasm. Consequently, its ability to prevent the action of the protease, if this is secreted via an independent, physically separate route is rendered redundant, unless the inhibitor can access the allocrite during transit. This paradox remains unresolved [114]. Secondly, the 9-kDa allocrite, Colicin V, is di-sulphide bonded, while most other allocrites of type I systems are cysteine-less, as are the periplasmic domains of the MFPs. In the case of colicin V, the formation of di-sulphide bridges also implies that this protein passes through the periplasm accessible to enzymes like DsbA.

In relation to possible discrete translocation pathways for type I proteins, it is interesting to contemplate what volume, for example, the 107-kDa HlyA molecule would occupy within the periplasm or within a transenvelope channel? The answer depends on whether the allocrite is transported in a completely or partially folded or unfolded state during translocation. In the cytoplasm, it is presumed that the allocrites, characterised in most cases by a C-terminal signal sequence, are presented to the transporter post-translationally but 'unfolded'. Free calcium concentrations available in the cytoplasm are too low to allow folding of the RTX Ca^{2+}-binding repeat motif. Cytosolic chaperones have been reported to participate in the type I secretion process, for example in HlyA transport (J. Whitehead and J.M. Pratt, personal communication) and HasA, although notably not containing a glycine-rich repeat, appears to require SecB for transport [115]. Furthermore, two members of the bacteriocin family can be transported by the core Sec machinery of *E. coil* when fused to the appropriate signal peptide, suggesting that the peptides remain sufficiently unfolded for acceptance by the Sec-machinery prior to translocation [116,117]. Participation of a chaperone is evidence that the allocrite is maintained unfolded prior to transport.

Several type I C-terminal secretion signal peptides have been subjected to CD and NMR spectra and shown to be highly flexible and unstructured in aqueous solution and sometimes even in membrane mimetic environments [70,115,118,119]. Although some secondary structural features are predicted in the C-terminal secretion signals of type I allocrites, their importance to the transport process is questionable

since secretion levels are unaffected by even major mutational changes predicted to disrupt secondary structure. Thus, the secretion motif recognised by the apparatus is probably contained within an unstructured sequence dependent on a few dispersed key residues [120]. A relatively unstructured secretion sequence, whose specificity is determined by the pattern of a few side chains, is reminiscent of the proposed mechanism for the recognition of the major histocompatibility (MHC) class I peptides by the HlyB homologue, TAP1/2 [75]. Such a mechanism could explain the lack of strong conservation of the type I secretion signal and the relative promiscuity of the secretion apparatus. This is particulary well illustrated by the versatile *E. coli* α-haemolysin (HlyA) secretion apparatus HlyBD,TolC, recognising more than 400 heterologous proteins fused to the C-terminal signal sequence, but otherwise unrelated to HlyA [121]. It is this property that permits its potential use for the presentation and delivery of antigens to the immune system in bacterial vaccine carriers [122].

What happens to the allocrite during the secretion process remains a black box of possibilities, for the moment limited only by the imagination. For example, can the RTX repeats, characteristic of type I allocrites, bind calcium during transit through the aqueous periplasmic compartment, thought to be rich in divalent cations, since this communicates with the cell exterior [123]. How can the relatively hydrophilic allocrite access the hydrophilic cell surface for final release? The energetics of the translocation process across one or two membranes are also not well understood. Are the allocrites pushed and/or pulled directly to the extracellular space only by the energy of nucleotide hydrolysis carried out by the ABC transporter? The one relevant study performed using the haemolysin secretion system suggests secretion is both ATP and pmf dependent, but occurs through consecutive steps that differ in energetic demand [124]. One possibility if the kinetics of secretion are very fast [125], is that unfolding, followed by refolding of the allocrite is promoted by the transporter itself in conjunction with, for example LPS and Ca^{2+}. In addition, from recent results involving both HlyA mutants with an altered secretion signal, and mutations in HlyD, indicate that correct folding of the HlyA molecule is affected ([126], A. Pimenta and J. Young, unpublished), we conclude that HlyA must at least be partially unfolded during translocation. In that case, the re-folding reaction itself, presumably catalysed by surface Ca^{2+} binding to and stabilisation of the C-terminal RTX-β strand structure, could contribute to the final stages of translocation coupled to the release of the toxin. Interestingly, similar re-folding steps involving Ca^{2+} appear to be implicated in secretion of subtilisin from *B. subtilis* and in the secretion to the endoplasmic reticulum of several proteins [127,128].

Recently, the attention of several laboratories has focused on the structural organisation of the type I translocator complexes and its relationship to the allocrite. This is a sensible prerequisite to understanding the mechanism of how ABC secretion systems work. The approach used has concentrated on the identification of protein–protein interactions, using a variety of methods, reaching so far some different, even conflicting conclusions. The aim of this last section is to review these new data and summarize how they either support or contradict the relevant transport concepts. Much of this work has concentrated on the prototype Hly and Prt transporters as model systems.

9. Identifying protein–protein interactions

9.1. Molecular genetic approaches

9.1.1. Hybrid transporters

Exploiting the difference in the broad spectrum of allocrites secreted by two type I exporters, Wandersman and colleagues attempted to identify which protein element was involved in initial recognition of the allocrite [129]. Different combinations of ABC-MFP-OMF constituents from two translocators were expressed and the flexibility of the hybrid exporter tested for secretion of given allocrites in vivo. It was found that co-expression of the allocrite and its own ABC transporter, dictated whether the allocrite was secreted or not, thus providing evidence that at least some specificity for initial recognition of preferred allocrites resides in the ABC-ATPase containing component. However, the interpretation of such experiments is rather limited, since the actual step (early or late) which is blocked in translocation is unknown. Moreover, these studies do not exclude

the possibility that the MFP could be playing a role in conjunction with the ABC transporter in initial recognition of the allocrite. In a related paper, Akatsuka et al. [130] describe mutations in an ABC transporter, capable of secreting two different allocrites, which abolished the secretion of one but not the other allocrite. Unfortunately, these mutations were not characterised. Further pair-wise swapping of heterologous type I exporter subunits showed that the various components are not universally interchangeable. The results clearly demonstrated that the MFP can associate with a certain ABC transporter, whilst the OMF is specific for a certain MFP in order to produce a functional transporter. Importantly, the deduced, preferred protein interactions, generally agree with phylogenetic analyses. The next logical step is to make use of chimaeric molecules of a single translocator component and determine the region within the chimera that allows secretion from one heterologous translocator, but not another. This type of experiment would help assignment of specific functions to protein domains and indicate more precisely areas of interaction between protein components. In this way, we may hope to solve the puzzle of allocrite specificity combined with appropriate structural studies.

9.1.2. Suppressor studies

Vancomycin is an antibiotic which affects the biogenesis of the peptidoglycan in Gram-positive bacteria through binding to a cytoplasmically synthesised peptidoglycan precursor [131]. With a molecular weight of 1.4 kDa, vancomycin is too large to penetrate efficiently the outer membrane barrier of *E. coli* through the porins. However, cells expressing the Hly translocator are sensitive to vancomycin and even more so in the presence of HlyA [84,132]. Schlor et al. [133] exploited the toxic effects of vancomycin to classify secretion defective HlyD mutants as conferring increased or decreased sensitivity to vancomycin. Since the sensitivity to vancomycin associated with various combinations of HlyB and mutants of HlyD was dependent on the presence of TolC, the authors concluded that HlyD can directly interact with TolC, but only in the presence of HlyB. The change in the sensitivity of *E. coli* to vancomycin caused by specific mutations in HlyD, was not dependent on HlyA. This suggested an interaction of the HlyBD,TolC complex even in the absence of the allocrite. Next, these authors selected for strains that simultaneously restored HlyA transport and suppressed vancomycin hypersensitivity, caused by a single missense mutation at the extreme C-terminal of HlyD (residue 477 out of 478). Three suppressors were localised to the periplasmic domain of HlyD (residues 362, 392, 441) and one suppressor mutation changed Pro-313 to Ser or Lys in HlyB. Although none of the mutations were shown to be allele specific, the isolation of intra- and intergenic suppressor mutations provides evidence for regions of HlyD and HlyB which interact. Based on their results, Schlor et al. [133] proposed an original model for HlyD interacting with both the ABC transporter and the OMF, showing the MFP folding back on itself at a potential gap between the coiled-coil regions, thus placing the C-terminal end of HlyD near to the inner membrane.

9.2. Biochemical approaches

9.2.1. Affinity chromatography

Co-affinity chromatography is an increasingly popular method for demonstrating protein–protein interactions; however, results should be interpreted with extreme caution, especially when dealing with hydrophobic membrane proteins. The technique assumes that detergent solubilised proteins retain their overall structure and moreover maintain normal associations throughout the solubilisation, wash and elution steps. Rigorous controls must be included to ensure that the putative interactions identified are not artifacts of the procedure itself. Létoffé et al. [134] describe the extraction of the entire metalloprotease (PrtDEF) exporter dependent upon association with two non-translocatable allocrites: affinity-tagged GST-PrtC which can be bound to glutathione-sepharose and HasA, retained by haem-agarose. Subsequent co-affinity purification experiments performed with incomplete or hybrid translocator components, led the authors to favour an ordered, sequential assembly of the translocator, i.e. the ABC transporter binds the MFP which then interacts with the OMF. The rationale behind these experiments is that the strength of the interaction of the translocator components is increased by the presence of the allocrite, presumed to be stuck in the translocator,

since it is not secreted. Unfortunately the value of the study is somewhat undermined by the absence of physical evidence to authenticate the presence of a stuck intermediate. Although the conclusions reached by the authors are not necessarily wrong, it seems improbable that three layers of non-covalent protein interactions could be maintained during isolation of envelopes followed by solubilisation from the envelopes. The negative control used in these experiments, simply the absence of allocrite, is insufficient and a more prudent control would have been a dummy co-purification of the translocator using the allocrite minus the affinity tag, thus keeping the same ionic charge and bulk conditions. As pointed out by the authors themselves, a higher proportion of the translocator proteins remained unbound than was retained by the affinity matrix via the non-translocatable allocrite, present in excess. In addition, when the final extract bound to the glutathione beads was analysed by SDS-PAGE and Coomassie blue staining, other contaminating proteins were visible while the translocator proteins themselves were detected by immunoblotting. In summary, in the absence of more controls can we be sure that authentic complexes were isolated in this study.

9.2.2. Measurements of protein stability

In the colicin V secretion system (CvaBA,TolC), the stability of the ABC transporter and the MFP was studied in the absence of the other partner, or in the absence of TolC [135]. The results indicated a mutual stabilisation of the ABC transporter and MFP. Using the same procedure for measuring protein half-life, comparable results were obtained for HlyD, the MFP of the Hly translocator [136]. However, in the experiments of Hwang et al. [135], the stability of the MFP in the absence of the ABC transporter and TolC was not tested. Under these conditions, Pimenta et al. [136] showed that the instability of HlyD, induced by the absence of TolC, was greatly reduced when HlyB was also absent. In other words, the absence of TolC per se does not affect the stability of the MFP component, unless the ABC transporter, HlyB is also present. This suggested that the ABC transporter is a determinant of HlyD stability and moreover the results provided evidence for at least two topological or organisational states of the MFP, dependent upon the presence or absence of the ABC transporter, as reflected by protease sensitivity. Thus, HlyB labilised HlyD to endogenous proteases (relative to its stability alone or in the presence of TolC), while the combined presence of HlyB and TolC rendered HlyD extremely stable to proteases. Both states of HlyD were shown to be independent of the presence of the allocrite. Interestingly, none of these data indicated an interaction between the MFP and TolC, except again in the presence of the ABC transporter.

9.2.3. Cross-linking

Hwang et al. [135] also published the first in vivo cross-linking results involving components of a type I secretion system. Using formaldehyde as a cross-linking reagent, putative interactions were proposed, based on the recognition of the cross-linked complexes with antibodies directed against specific translocator components. An MFP:MFP dimer interaction was substantiated by further experimentation and this multimerisation was shown to be independent of the presence of the allocrite. In this laboratory, recent studies have demonstrated co-affinity purification of HlyB and HlyD and HlyB with HlyA. In addition, both genetic studies and cross-linking with DSP has clearly shown the presence of higher molecular weight homo- oligomers of HlyD, at least up to trimers, whose formation depends upon the presence of a fully assembled translocator but not HlyA (J. Young, 1999, Thesis; J. Young et al., in preparation).

Using a combination of cross-linking with DSP and co-affinity purification, Thanabalu et al. [137] isolated and dissected protein interactions in the HlyA system. In this case, the ABC transporter and MFP of the Hly translocator were affinity-tagged, rather than the allocrite, as in the experiments of Létoffé et al. [134], using the Prt system. Thanabalu and colleagues reinforce the findings of the stability experiments confirming the presence of ABC transporter:MFP inner membrane complexes, independent of TolC and the allocrite. New associations of proteins were also identified including an intriguing interaction of the MFP with the allocrite, in the absence of the ABC transporter. This is in contradiction to the idea of sequential interactions induced in response to allocrite binding to the ABC transporter [134]. However, the former result could

be attributed to the fact that cross-linking was used, thus stabilising weaker interactions that might have gone undetected by other methods. This result moreover is consistent with results from this laboratory (A. de Lima Pimenta, PhD thesis, 1995, unpublished) that the MFP may play a role from the earliest point in the translocation pathway. Again, in this study, no evidence was found to suggest an interaction of the OMF with the MFP, except in the presence of the ABC transporter.

Notably, the presence of the allocrite was required for isolation, after DSP cross-linking, of the three protein translocator complex, as previously suggested by the Wandersman laboratory [134]. Interestingly, the three components of the translocator could still be cross-linked and co-purified in the presence of an ABC transporter that possessed a mutation rendering the ABC transporter defective in ATP hydrolysis, allocrite secretion, but obviously not allocrite recognition. This suggests that the type I secretion apparatus is a self-assembling structure, not requiring hydrolysis of ATP. Increased protease accessibility of the exporter components during secretion was also noted by Thanabalu et al., suggesting specific conformational changes accompanying transport of the allocrite. The conclusion was also reached that the translocator complex was apparently transient, disassembling after translocation. The authors in addition investigated the subunit stoichiometry of the MFP component using a different cross-linker, DSG. From this they provide evidence that HlyD could apparently self-associate to form trimers in the absence of its cognate translocator components.

Based on their large number of findings, Thanabalu et al. [137] favour the idea that the haemolysin translocator consists of a contiguous rather than a continuous channel. They present their view of the translocator with an interesting 'shuttle' mechanism whereby the transenvelope translocator is capable of opening at one end or the other but not simultaneously. The allocrite can accumulate in the first chamber before being moved to a second chamber imagined to be closed to the cytoplasm but open to the external medium. An attractive feature of the model is that it provides a solution to the problem of gating an open channel which would otherwise lead to leakage of cell contents. TolC is thought not to be a gating protein since it forms ion-channels in planar lipid bilayer membranes, although the size of the channels was reported to be reduced upon addition of a soluble periplasmic domain of HlyD in vitro [133]. In the discontinuous transenvelope channel model, gating is apparently provided by controlled opening of a chamber formed by HlyB and HlyD combined and regulated by ATP hydrolysis in response to allocrite binding. Interestingly, from our own genetic analysis of HlyD we have come to similar conclusions, with, however, HlyD itself possibly capable of forming two contiguous chambers (A. de Lima Pimenta, J. Young, M.A. Blight, I.B. Holland, unpublished).

9.3. In silico analysis

Complementary to the biochemical and genetical experiments described above, it is useful to include within this review some insights gained from bioinformatics, concerning the possible structure and function of the MFP component of the translocator.

As part of an ongoing genetic analysis of the function of HlyD we have included a detailed analysis of the structure of HlyD and its homologues (Fig. 2). One novel idea arising from this analysis and from published and unpublished data is that MFPs are flexible dynamic proteins, with a global conformational change or reorganisation of the protein with respect to multimerisation, being required for allocrite transport. This implies radical upgrading of the MFP's involvement during secretion. This is based on the presence of a coiled-coil helical domain centrally located in the primary sequence [105], conceivably involved in the formation of the oligomers of HlyD described above. In addition, we have identified a motif on each side of the coiled-coil pattern with homology to a half-set of a lipoyl and biotin swinging arm module [101,138]. Lipoyl/biotin swinging arm domains are present in certain multienzyme complexes and are involved in a 'swinging arm' action that contributes to the passing of a bound cofactor between catalytic sites. Some NMR structures have been solved for several lipoyl and biotin domains and CD spectroscopy has been performed on a peptide corresponding to this domain from the MFP, MexC, that associates with a different class of integral membrane protein other than an ABC transporter and is involved in the efflux of drugs.

Interestingly, this peptide was shown to adopt the expected fold under certain conditions [101]. This encouraged the hypothesis that the distantly conserved domain to the swinging arm present in MFPs, provides the possibility of intramolecular movement within these molecules. Thus, the role of the MFPs in transport may be more active than first imagined. Such movement could contribute to the formation of a trans-periplasmic channel. In addition, the MFP could simply harness the movement for actual binding and passing of the allocrite from the ABC transporter to the exterior. This active role for the MFP is supported by the fact that HlyD can influence the final conformation of the secreted HlyA as shown by the fact that mutations in HlyD result in the secretion of a largely inactive toxin which appears unfolded, (A. Pimenta, K. Racher, J. Young, this laboratory unpublished).

10. Outlook

The data gathered here from a number of laboratories agree on several points. All provide persuasive evidence to postulate that the ABC transporter, MFP and OMF function together as a structural complex, as indicated in Fig. 1 (left). Intimate association of the ABC transporter and the MFP in the absence of the OMF, during and even prior to interaction of the allocrite has been documented. The MFP component apparently interacts with the OMF, but only in the presence of an ABC transporter. Cross-linking experiments show that the MFP oligomerises to form at least a trimer. One of the most pertinent points to come out of all the data is an influence of the ABC transporter upon MFP stability and conformation. It is not clear whether this reflects the main role of the ABC transporter in energising transport or whether this is an additional role. There are now well documented examples of ABC transporters regulating the activity of a quite distinct membrane protein. Thus, the human SUR protein, involved in insulin regulation of K^+ transport, forms a specific complex with a K^+ channel whose activity is controlled by the ATPase action of SUR [139,140]. There are also reported bacterial examples of ABC transporter 'colateral' interactions with or regulation of other membrane proteins. These include AbcA of *Aeromonas salmonicida* that apparently possesses both regulatory and transport activities. SapABCDF, an ATP-driven peptide importer, regulates the activity of a potassium ion channel in *Salmonella typhimurium* and Aap, an active importer of L-amino acids, appears to influence the rate of amino acid effux in *Rhizobium leguminosarum* [141–143]. Extrapolating these findings to the HlyB-HlyD couple in type I secretion leads to a novel view of HlyB controlling (by an ATP-dependent mechanism?) the opening–closing of a multimeric HlyD transport channel connected directly both to the cytoplasm and TolC. This alternative to the HlyB membrane domain itself constituting a transport pathway across the inner membrane has some attractions.

There still exist contradictions between laboratories (or perhaps secretion systems?) concerning the nature of the assembly of the type I translocator and the necessity or not of the presence of the allocrite for assembly. Nevertheless, there is now accumulating evidence that the type I secretion systems do involve specific complexes between an ABC protein, an MFP and an OMF, with a possible stoichiometry of 2, at least 3 and 3, respectively. Moreover, some steps in the translocation pathway, involving initial recognition by the allocrite and subsequent distinct events in the translocation pathway, do seem to becoming clearer. These studies and their implications discussed here should, we hope, provide stimulation and guidance for further ongoing research.

Elucidation of the architecture and the dynamics of secretion via the ABC pathway presents a formidable obstacle to practical experimentation, particularly in the case of the transenvelope type I structures of Gram-negative bacteria. The usefulness of semi in vitro systems involving isolating osmotic or pressure shocked vesicles on sucrose density gradients is limited, since they disrupt the cell envelope together with any transenvelope structures. Unfortunately, in vitro systems which would allow reconstitution of the whole pathway across the two membranes of *E. coli*, as has been achieved for the Sec-translocation system, have so far proved impossible to develop. For this reason, it is perhaps better to apply the immunofluorescence microscopy approaches used recently in studies of supramolecular transenvelope structures, such as the type III protein secretion system and the flagellar motor [144–146]. Understanding the ultrastructure of the ABC secretion pathway could

be obtained by locating the translocator proteins on the structure by cryoelectron or immuno-electron microscopy. Copy numbers of the components could be determined by immunoblot and 2D gel electrophoretic analysis, confirming subunit stoichiometry. The absence of a known structure for any of the membrane proteins involved in the translocator is particulary frustrating and crystallisation or NMR solution structure of isolated protein domains should be instructive. Indications are that transport is fast and cross-linkers and spectroscopic probes could help document conformational changes during translocation. It should also be possible to apply other techniques, such as cysteine scanning mutagenesis to the study of the structure and function of components of the type I secretion machinery. The development and detailed characterisation of stuck intermediates, involving modified allocrites is still not being fully exploited to dissect the type I pathway. Finally, in the absence of a fully reconstituted in vitro system, the purification, solubilisation and reconstitution of the ABC and MFP components in liposomes, in order to analyse at least the initial steps in translocation is now urgently required.

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