Society for General Microbiology

Symposium 58

Edited by
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Transport of molecules across microbial membranes

TRANSPORT OF MOLECULES ACROSS MICROBIAL MEMBRANES

EDITED BY

J. K. BROOME-SMITH, S. BAUMBERG, C. J. STIRLING AND F. B. WARD

FIFTY-EIGHTH SYMPOSIUM OF THE SOCIETY FOR GENERAL MICROBIOLOGY HELD AT THE UNIVERSITY OF LEEDS SEPTEMBER 1999

Published for the Society for General Microbiology

PUBLISHED BY THE PRESS SYNDICATE OF THE UNIVERSITY OF CAMBRIDGE
The Pitt Building, Trumpington Street, Cambridge, United Kingdom

CAMBRIDGE UNIVERSITY PRESS
The Edinburgh Building, Cambridge CB2 2RU, UK www.cup.cam.ac.uk
40 West 20th Street, New York, NY 10011-4211, USA www.cup.org
10 Stamford Road, Oakleigh, Melbourne 3166, Australia
Ruiz de Alarcón 13, 28014 Madrid, Spain

First published 1999

Printed in the United Kingdom at the University Press, Cambridge

Typeset in Monotype Times 10/12pt, in 3B2 [PN]

A catalogue record for this book is available from the British Library

ISBN 0 521 77270 2 hardback

TRANSPORT OF MOLECULES ACROSS MICROBIAL MEMBRANES

This volume considers the transport of molecules, large and small, across the membranes of prokaryotic and eukaryotic microbial cells. A diverse range of related phenomena are covered, but the unifying themes are the signal peptides that target proteins to particular destinations, and the role of chaperonins. Topics covered include: secretion of proteins out of the bacterial cell by Type I, II and III mechanisms, including the newly recognized bacterial signal recognition pathway in Type II; passage across internal membranes of eukaryotic proteins, whether destined for secretion or en route to internal organelles such as chloroplasts and peroxisomes; how bacteria obtain the energy required for solute uptake, the role of phosphorylation, and evolutionary relationships of some of the proteins involved; and efflux pumps for toxic substances in bacterial, animal and plant cells.

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SYMPOSIA OF THE SOCIETY FOR GENERAL MICROBIOLOGY

Series editor (1996–2001): Dr D. Roberts, Zoology Department, The Natural History Museum, London

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INTRODUCTION

Our understanding of how molecules are transported across microbial membranes has lagged far behind our understanding of processes that occur within the aqueous compartments of these cells. There is little doubt that this is because it is so difficult to analyse the structures of the membrane proteins that mediate, or play central roles in, these processes. Membrane proteins are inherently difficult to purify and crystallize in (active) forms suitable for high-resolution analysis, because they are amphipathic molecules. The problem is exacerbated by the fact that most are non-abundant, and cannot be successfully overproduced without aggregating within, or even killing, the producing cell. Indeed, it was not until 1985 that Michel's group, applying a novel amphiphile-coating approach, which rendered the surfaces of photosynthetic reaction centre molecules uniformly polar, provided us with the first atomic resolution structure of a membrane protein (Deisenhoffer *et al.*, 1985). Even now, with the structures of soluble proteins being solved at the rate of one or more a day, the number of membrane proteins whose structures have been solved is only just into double figures. In each case ingenious strategies have had to be deployed to get crystals that are suitable for high-resolution analysis – the membrane proteins have been coated with amphiphiles and had their polar surfaces expanded with monoclonal antibodies, or crystallized in two-dimensional lattices (within phospholipid bilayers) or within custom-built three-dimensional lattices (reviewed by Ostermeier & Michel, 1997).

Against this background it is worth reflecting on the considerable importance of membrane transport processes. Eukaryotic microbes have numerous different subcellular compartments, and the proteins they synthesize must be efficiently transported to their correct subcellular destinations. Small molecules (nutrients, ions, drugs, metabolites) are transported into or out of the cell and its organelles, and specialized protein complexes within the

membranes mediate energy transduction and transmembrane signal transduction processes. Even in the relatively simple bacterial microbes a substantial proportion of the proteins synthesized in the cytoplasm (around 25–30%) are destined for extracytoplasmic locations. In the Gram-negative bacteria, which have an extra, outer, membrane surrounding the plasma membrane, extracytoplasmic proteins must be correctly localized to one of four compartments – the inner membrane, the periplasm, the outer membrane or the exterior. One major question that several articles in this symposium address is: how do large hydrophilic polypeptide substrates pass through hydrophobic membranes? Another recurring question is: how are polypeptide substrates recognized as being destined for different subcellular locations and correctly targeted to them? Many of the micro-organisms that have been most intensively studied are human, animal or plant pathogens. They make contact with their hosts via their external surfaces and appendages. Protein secretion is often of special importance for delivering virulence factors into the host cell. Finally, we are now in the age of genomics, and it is clear that amino acid sequence similarity comparisons are hugely impacting on our insight into protein evolution and biological processes. Such comparisons are of special value where membrane proteins are concerned, since structural studies lag so far behind those on soluble proteins.

TRANSPORT PROCESSES

Membrane proteins fulfil a variety of crucial cellular functions, and as Saier & Tseng remind us (this volume): 'These transporters are essential for virtually all aspects of life as we know it on Earth.' Thus, whilst it has so far proved impossible to purify, crystallize and obtain high-resolution structural data for all but a few membrane proteins, there is a very strong impetus to continue to explore and develop novel approaches that may help shed light on their structure and function. In the first few chapters of this symposium we are brought up to date on our knowledge of several different classes of membrane transport proteins. In an article that reads like a good detective novel, Kim Lewis describes the proteins that cause multidrug resistance by catalysing drug efflux. The MDR proteins are ubiquitous and occupy four different superfamilies of membrane proteins. Clinically significant drug resistance is caused by increased expression of *mdr* genes. Perhaps the most taxing question here is: how can MDRs bind and extrude a wide variety of different substrates? In fact, amino acid sequence comparisons reveal that MDRs have evolved multiple times from efflux proteins of much narrower substrate specificity. (Amino acid substitutions in the ancestral proteins have caused the switch to a broader substrate specificity.) Moreover, although MDRs extrude a variety of unrelated compounds, their preferred artificial substrates are almost invariably amphipathic cations. As these substances are able to partition into the membrane, the possibility that MDRs only

'consider' substances within the membrane as their ligands has been raised. It is now clear that LmrA, a functional bacterial homologue of mammalian P-glycoprotein, can pump ligands from the inner leaflet of the membrane to the exterior. Maybe mammalian P-glycoprotein has evolved its exceptional ability to flip drugs from the inner to the outer leaflet of the plasma membrane, because here they can then be detoxified, whereas extrusion would simply be followed by their re-entry into the cell. As it seemed likely that MDRs could have evolved to protect microbes from the potentially damaging effects of amphipathic cations, Lewis and colleagues searched for natural compounds of this type. They found that a group of plant alkaloids – the isoquinoline alkaloids, such as berberine and palmatine – fitted the bill, and that these had potent antimicrobial activity in the presence of MDR inhibitors. Moreover, they established that a berberine-producing plant also made two different MDR inhibitors. Multidrug resistance is a severe clinical problem, so there is real hope that these natural MDR inhibitors can be used in conjunction with conventional antimicrobials to overcome it.

Arsenic resistance genes are found in nearly all organisms, perhaps because the primordial soup was rich in dissolved metals, and therefore resistance to toxic metals was important to all early life forms. In the article by Bhattacharjee *et al.* we learn that membrane proteins with the ability to extrude arsenicals have evolved at least three times. In bacteria ArsB acts as a secondary transporter, catalysing the extrusion of arsenite coupled to the membrane potential. However, in some organisms the ArsA ATPase is also produced and it binds to ArsB, converting it to a primary transporter that extrudes arsenite at the expense of ATP hydrolysis. Interestingly, the ArsB membrane protein has a topological arrangement [N-in C-in with 12 membrane-spanning segments (MSSs)] that is more reminiscent of secondary rather than primary transporters. (ArsA homologues are found in bacteria through to man, but so far the physiological function of the eukaryotic ArsA homologues remains unknown.) Recently another family of membrane proteins that confer arsenite resistance has been identified in both bacteria and yeasts. One of these 10 MSS proteins, Acr3p of *Saccharomyces cerevisiae*, has now been shown to be a plasma membrane arsenite efflux protein. However, *Sacch. cerevisiae* also harbours the protein Ycf1p, a vacuolar membrane ABC transporter, which is known to confer cadmium resistance by pumping $Cd(GS)_2$ conjugates into the yeast vacuole. Recently it has become clear that Ycf1p also pumps arsenite into the vacuole. Homologues of Ycf1p and Acr3p are likely to exist in all eukaryotes.

Poolman highlights the fact that transporters do not accumulate solutes to such high levels as are predicted from the driving forces for these processes. In fact, leak pathways rarely make a significant contribution, at least in primary (ATP-driven) transport processes, and product inhibition is a major player. When cells are starved of energy and the ion motive force

drops, then solutes would be expected to leak out via their secondary transporters. However, in some microbes the solutes are retained because the transporters themselves are highly sensitive to changes in the internal pH, and as the pH value falls below the physiological level they lose activity. Other mechanisms such as inducer exclusion in Gram-negative bacteria, osmosensing and catabolite repression all act to regulate transport activity. This article serves as a salutary reminder that transporters are sophisticated devices, and even when we understand their basic mode of action, we can only meaningfully relate this to actual cellular physiology if we take into account mechanisms for modulating their activity to prevent catastrophically high solute accumulation.

Given the dearth of high-resolution structural information on membrane proteins, and the current explosion in genomic sequencing, molecular archaeological studies are particularly pertinent to the analysis of transmembrane transport systems (see Saier & Tseng, this volume). The considerable effort of Saier and co-workers has led to the identification of over 200 different families of transporters. These studies reveal that transporter families have arisen continuously over the last 4 billion years and some, for example the major facilitator superfamily, are ancient and ubiquitous, whilst others, for example the mitochondrial carrier family of anion exchangers, arose much later and are confined to particular eukaryotic organelles. We also learn that many permeases arose by tandem intragenic duplication and that a 6 TMS module is, for currently unknown reasons, particularly popular. Phylogenetic analysis is now sufficiently refined that virtually every newly sequenced transporter can be classified with respect to its structure, function and mechanism just by considering how similar it is in amino acid sequence to previously identified transporters.

The other contributions to this symposium are concerned specifically with the translocation of polypeptides across microbial membranes. No one chapter deals exclusively with the process by which polypeptides are translocated across the bacterial cytoplasmic membrane using the Sec machinery. This process is, however, briefly described by Filloux and alluded to by Soto & Hultgren, in their descriptions of two different pathways for the translocation of polypeptides from the periplasm to the exterior of Gram-negative bacteria, the substrates for which are Sec-dependent periplasmic proteins. However, Young *et al.* review our current knowledge of protein translocation across the endoplasmic reticulum (ER) membrane, and it is clear that the translocon – the proteinaceous membrane channel through which the polypeptide exits the cytosol – as well as various features of the translocation process are fundamentally similar in bacterial and eukaryotic microbes. In recent years it has proved possible to complement the elegant genetic analysis of protein export in yeast with sophisticated *in vitro* studies, most notably involving the identification of cross-linking partners of translocating polypeptides, and fluorescence quenching studies.

Such studies are either impossible or extremely difficult to conduct on bacteria, largely because of the technical complications that result from having to turn the membrane vesicles derived from the bacterial cells inside-out in order to bring the cytoplasmic contents to the outside. Just as we had settled into thinking of the translocon as an environment for the one-way transport of unfolded polypeptides, the application of this barrage of elegant techniques has yielded some big surprises. These recent studies have revealed that the translocon is wider than required for linear extrusion of polypeptides, so have led us to consider that maybe polypeptides start to fold even within the translocon. We have also learnt that translocation will apparently run in reverse if the polypeptide is not properly modified or fails to fold, enabling its degradation via the cytosolic ubiquitin-proteasome pathway. There is a growing awareness of the importance of the gating at both ends of the translocon. The ribosome makes intimate contacts with the translocon and it has been suggested very recently that the ribosome controls translocon gating by a conformational mechanism. During translation, the ribosome undergoes conformational changes, which then induce conformational changes in the translocon to control gating.

Proteins destined for translocation across the bacterial cytoplasmic membrane or the eukaryotic ER membrane are made with hydrophobic N-terminal signal peptides that are essential for translocation, and, in the case of soluble proteins, are eventually proteolytically cleaved from the translocated protein. It has been known for over two decades that higher eukaryotes contain a ribonucleoprotein particle, termed signal recognition particle, or SRP, that recognizes signal peptides and binds and delivers nascent preproteins to the ER membrane, by docking with the SRP receptor. Although genetic screens failed to reveal a bacterial SRP, sequence comparisons eventually revealed that bacteria do contain an SRP, albeit of a rather more primitive form than in higher eukaryotes. For a long time no role in protein targeting could be positively ascribed to bacterial SRP, and it was argued that bacterial SRP could have a different function to mammalian SRP. Valent *et al.* provide us with a historical perspective on the discovery of bacterial SRP and the eventual acceptance of a role for it in targeting membrane proteins, in particular, to the cytoplasmic membrane. Since signal peptides differ considerably in amino acid sequence, a key question concerning the targeting of signal-peptide-containing proteins is: how can such diverse ligands be recognized by a single receptor (SRP)? The structure of the signal-peptide-binding domain of the P48 SRP component of *Thermus aquaticus* reveals that, as predicted more than 10 years ago, this highly hydrophobic methionine-rich domain forms a hydrophobic groove that is lined with flexible amino acid side chains. It is thus sufficiently large and pliable to be able to accommodate signal peptides of different shapes and sizes. Finally, SRP is proving to be ubiquitous – it is present in all bacteria

and eukaryotes so far examined, and it is found in the stroma of chloroplasts as well as the cytosol.

In the Gram-negative bacteria secretion of proteins to the medium can occur in two stages, with proteins being exported in a Sec-dependent fashion to the periplasm, and then being translocated across the outer membrane. Alternatively it can occur in a single step, with the exoproteins being transported from the cytoplasm across both the inner and outer membranes, without the involvement of the Sec machinery and a periplasmic intermediate. Type I secretion systems are the simplest and, perhaps for this reason, currently the best understood systems for the direct secretion of exoproteins from the cytoplasm to the exterior of Gram-negative bacteria. Most type I systems are responsible for the secretion of just one or a few closely related exoprotein substrates, belonging to the toxin, protease or lipase families. The first type I secretion system to be characterized, and the most extensively studied, is the system responsible for the secretion of α-haemolysin (HlyA) by haemolytic *Escherichia coli*. However, related systems have since been found in a wide variety of bacteria. They are responsible for the secretion of metalloproteases (*Erwinia chrysanthemi*), lipases (*Pseudomonas fluorescens*), S-layer proteins (*Campylobacter fetus* and *Caulobacter crescentus*) and, in some bacteria, several unrelated proteins (a metalloprotease, a lipase, a haem-binding protein and an S-layer protein in *Serratia marcescens*, and glycanases and a nodulation protein in *Rhizobium leguminosarum*) (Binet *et al.*, 1997; Awram & Smit, 1998; Thompson *et al.*, 1998; Kawai *et al.*, 1998; Finnie *et al.*, 1998). Type I secretion systems are relatively simple. Just three proteins form the substrate-specific channel and drive exoprotein transport through it to the exterior. As shown in Fig. 1(a), they are an ATP-binding cassette (ABC) protein exporter (e.g. HlyB), a membrane-fusion protein, or MFP (e.g. HlyD), and an outer membrane protein, or OMP (e.g. TolC). The ABC protein exporter is a polytopic inner membrane protein which recognizes the exoprotein substrate(s) and which binds and hydrolyses ATP. The MFP is an N-in C-out inner membrane protein. It interacts both with the ABC protein exporter and, via its extended C-terminal domain, with the periplasmic domain of the β-barrel OMP. Usually the three genes encoding the 'ABC exporter' are linked to those encoding the exoprotein substrates. However, NodO, one of four or more substrates for the chromosomally encoded type I exporter of *R. leguminosarum*, is plasmid-encoded (Finnie *et al.*, 1997). Likewise, the gene encoding TolC, the OMP of the α-haemolysin secretion system, is unlinked to *hlyABD*. But TolC is also used by another ABC transporter, the colicin V transporter, and it has additional roles in colicin E1 permeation and chromosome segregation.

The exoprotein substrates do not have N-terminal signal peptides but instead they contain short C-terminal secretion signals. Their other striking characteristic is that many contain glycine-rich repeated motifs that are

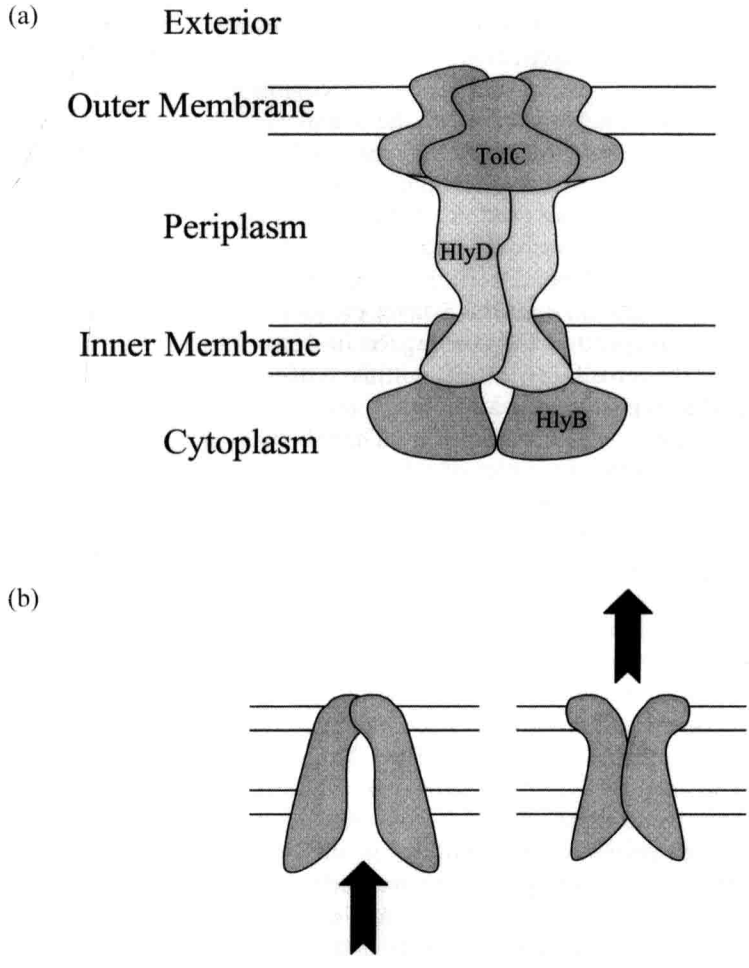

Fig. 1. Type I HlyA secretion system. (a) The three protein components of the secretion machinery are depicted. The polytopic inner membrane ABC protein exporter, HlyB, has a cytoplasmic ATPase domain. The MFP, HlyD, is a bitopic inner membrane protein with an extended C-terminal periplasmic domain. Its N-terminus contacts HlyB and its C-terminus interacts with the periplasmic domain of the outer membrane pore (the OMP), TolC. (b) The outward movement of HlyA is depicted by the filled arrow. According to current data, ATP and HlyA binding are believed to promote opening of the channel entrance (left-hand panel), whereas ATP hydrolysis is required to close the channel entrance and open the channel exit.

implicated in Ca^{2+}-binding, and, hence, rapid and stable folding of the proteins following their secretion. The precise nature of the C-terminal secretion signals currently remains elusive. Often the extreme C-terminus of the exoprotein consists of a negatively charged amino acid followed by several hydrophobic amino acids, and in some exoproteins an α-helical structure is believed to exist just N-terminal to this motif. In some exopro-

teins the glycine-rich repeats may play an additional role in helping to keep the targeting signal exposed on the surface of the protein, and hence visible to its receptor, the ABC protein. Signal recognition by the ABC protein is usually limited to exoproteins of the same type. But foreign proteins can often be recognized and secreted by an ABC exporter if they are fused to a cognate C-terminal signal. For example, when a C-terminal portion of HlyA was fused to β-lactamase (minus its N-terminal signal peptide) this normally periplasmic enzyme was efficiently and specifically secreted by *E. coli* in an HlyB- and HlyD-dependent fashion (Chervaux *et al.*, 1995). It has been noted that the *Caul. crescentus* S-layer protein is particularly abundant for a type I secretion product (accounting for 10–12% of the total cell protein) and therefore the possibility of using this ABC exporter to secrete foreign proteins looks particularly attractive.

Very recently, elegant studies by Thanabalu *et al.* (1998) have revealed some details of the dynamics of α-haemolysin export. Their strategy was to express HlyA, B, D and TolC in different combinations in *E. coli* and to analyse the complexes that formed (the components that could be cross-linked to one another) *in vivo*. They found that the ABC protein and the MFP formed a complex to which the OMP was recruited only when HlyA engaged the complex, and from which it separated after HlyA had been secreted. TolC was previously found to be a trimeric pore and in this study HlyD was also found to be trimeric and to form the primary inner membrane–outer membrane bridge. Intriguingly, ATP binding and substrate binding both promoted opening of the channel entrance, but ATP hydrolysis was required for HlyA to exit the channel. It is tempting to speculate that ATP hydrolysis is required to close the channel entrance and open the channel exit, thus ensuring gating of the channel, which is presumably necessary if leakage of cytoplasmic proteins to the exterior is to be prevented (see Fig. 1b). Finally, other studies, and in particular the work reported by Delepelaire & Wandersman (1998), highlight the possibility that some, perhaps all, exoproteins may have to be prevented from folding, or even actively unfolded, in order for them to be efficiently secreted by type I systems.

In comparison to type I export, the type III export process, which is responsible for the delivery of Yops (*Yersinia* outer proteins) from the cytosol of pathogenic *Yersinia* species to its outer surface, to the external medium, and into the cytosol of the eukaryotic host cell, is poorly understood. As discussed by Anderson *et al.*, some 25 genes are involved in specifying the type III machinery. Contact with eukaryotic cells at 37 °C induces the type III machinery and the programmed secretion of some 14 different Yops to their specific extracellular destinations. Intriguingly, the secretion signals of Yops, which lie within the first 15 or so codons of the *yop* genes, are of a distinctly different nature to all other targeting signals, in that they are tolerant of frame-shift mutations. Presumably these nucleotide-

encoded signals ensure that *yop* mRNAs are only translated when the ribosomes attached to them have docked onto the type III machinery. For some Yops, cytoplasmic chaperones are additionally required for their successful secretion. A comparison of the genes required for type III secretion in other Gram-negative pathogens reveals that homologues of nine proteins are found in all known type III machines, and that eight of these are homologous to products needed for the assembly of the flagellar basal body hook complex. The ninth is a multimeric outer membrane 'secretin' protein. Secretins form gated channels in the outer membrane and function in the translocation of proteins and bacteriophage across this membrane. Yops that are injected into eukaryotic cells must cross three membranes. It has been proposed that, for these Yops, the type III machine forms an injection device extending from the bacterial to the eukaryotic cytoplasm.

The main terminal branch of the general secretory pathway in Gram-negative bacteria, or the type II secretory pathway, is used by a wide variety of bacteria to transport exoproteins from the periplasm to the exterior, following their Sec-dependent translocation across the cytoplasmic membrane (Filloux, this volume). Some 14 or so products of linked genes, moderately to highly conserved in all the bacteria in which they have been found, form the export machinery. The clue to why the type II machinery should be so complex comes from the finding that the components include four polypeptides with N-termini resembling those of pilin subunits and a prepilin peptidase. The proteins they resemble are crucial components in the formation of type IV pili (long cell surface appendages at the poles of the producing bacteria). The prepilin peptidase is required for the processing of these 'pseudopilins', and, based on their strange fractionation (when overproduced they fractionate with the outer membrane), these subunits have been proposed to form a 'pseudopilus' – a rudimentary structure spanning the periplasm and connecting the inner and outer membranes. Other components of the type II machinery include a peripheral cytoplasmic membrane ATPase, which might be involved in driving the export of pseudopilins to the periplasm, and an outer membrane secretin, which, in its multimeric form, has a large central pore, some 95 nm wide. Further components are believed to energize gating of/transport through the pore, via a TonB-like energy transduction process. The pseudopilus, assuming it really exists, might either push exoproteins through the pore, or it might act like a cork to keep the pore blocked when not in use. Type II exoproteins do not share regions of amino acid sequence similarity, and molecular genetic analysis has revealed that their secretion signals are 'patch' signals, made up from different portions of the linear amino acid sequence. Conflicting data on the precise constitution of the secretion signal in specific exoproteins have led to the view that either the secretion signal is recognized as the exoprotein folds, or that it comprises a series of signals that are recognized sequentially