ABC Transporters: Bacterial Exporters

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INTRODUCTION

Proteins, peptides, polysaccharides, and many other molecules that are synthesized in the bacterial cytoplasm must often cross one or more membranes to reach their final destination. Many bacterial proteins are transported across the cytoplasmic membrane via the Sec pathway (139, 151, 191). Sec-dependent secretion requires that the secreted product be a protein with an N-terminal signal sequence, severely limiting the type of molecule that can be transported across membranes by using this system. Nonproteinaceous secreted products as well as extracellular proteins from gram-negative bacteria (which must cross both inner and outer membranes) cannot use the Sec pathway. In addition, there appear to be structural features of some proteins that are inherently incompatible with use of the Sec pathway (100, 116). Therefore many molecules must find another way to leave the cytoplasm. It has become apparent from results obtained in the last few years that this problem is often solved by the existence of dedicated export systems that facilitate membrane translocation with a large degree substrate specificity (80, 121, 140, 184).

Two major groups of dedicated export systems have been identified recently from gram-negative bacteria: the ABC transporters and the pullulanase-like family of transporters (80, 139, 150). This review will focus on the bacterial ABC transporters and will include information on transporters from both gram-negative and gram-positive systems.

Requirement for an ATP-Binding Domain

A feature common to dedicated exporter systems is that they, like the Sec system, require energy to facilitate translocation. Although the precise mechanism of secretion in these systems is still largely unknown, many of them appear to utilize ATP hydrolysis as a source of energy. Systems that use ATP require a component that binds ATP. Structural domains that can efficiently bind ATP and facilitate its hydrolysis have been highly conserved throughout evolution, and therefore it is not surprising that proteins making up the dedicated export systems display a highly conserved ATP-binding motif. This motif is known by several names including the Rossman fold (148), the Walker motif (182), and the Doolittle motif (28) and consists of two conserved sites (A and B) that form an ATP-binding pocket (148) (see Fig. 3). The ATP-binding site occurs at the end of an α-helix; the residues GXGKST form a turn, bringing the lysine residue in close proximity to the phosphates in the Mg^{2+}-ATP. The aspartic acid residue in the B site is in close proximity in space to the A site, and its negative charge may interact with the Mg^{2+} molecule (182). The consensus sequence was defined by comparing distantly related sequences in the alpha and beta subunits of ATP synthase,

myosin, and many kinases. This consensus in the amino acid sequence involves a very small number of residues and is likely to be present also in proteins that do not bind nucleotides (11, 28). Thus, proteins that display this consensus need not be functionally homologous.

The bacterial permeases, which are multiprotein complexes involved in nutrient uptake, have been extensively analyzed and shown to have homologous protein components with ATP-binding consensus sites (2). The permease systems all have a conserved component which includes a 200-amino-acid (aa) region that displays a high degree of sequence identity and that contains the ATP-binding consensus sites A and B. This extended region of similarity clearly distinguishes these permease components from other nucleotide-binding proteins such as ATP synthase, myosin, and kinases. This extended region is also present in many bacterial proteins that form part of dedicated export systems. This observation serves as the unifying theme presented in this review. Several excellent reviews describing other aspects of the family of proteins containing this conserved region have been written recently (3, 8, 80).

This highly conserved region has been called the ATP-binding cassette (ABC), and proteins which contain this region are called ABC transporters (82) or, alternatively, traffic ATPases (3). For the purposes of this review, we subdivide the superfamily of proteins that contain the ABC into three subfamilies based not on evolutionary differences but, rather, on differences in their source and generalized function as an importer or exporter. Each subfamily is described briefly, but the main emphasis here is placed on the description of the ABC exporters from bacteria.

OVERVIEW OF ABC TRANSPORTERS

Bacterial ABC Importers (Periplasmic Permeases)

The bacterial periplasmic permeases compose a large subfamily of ABC transporters and have been carefully studied (2, 83). These systems have several distinguishing features. They are all multisubunit import systems with a similar structural organization (Fig. 1). They all include a hydrophilic, membrane-associated protein containing an ABC type of ATP-binding domain. In several systems, this conserved component has been shown to associate tightly with two hydrophobic proteins (102). A unique feature of the bacterial ABC importers is that they all have a periplasmic binding protein that interacts with the incoming substrate, binds to it, and presents it to the import complex in the inner membrane. Bacterial ABC importers have the ATP-binding domain and the membrane-spanning domains (MSDs) present on separate polypeptides. In contrast, most but not all of the ATP-binding domains in the bacterial exporter systems are present on the same polypeptide as the MSDs.
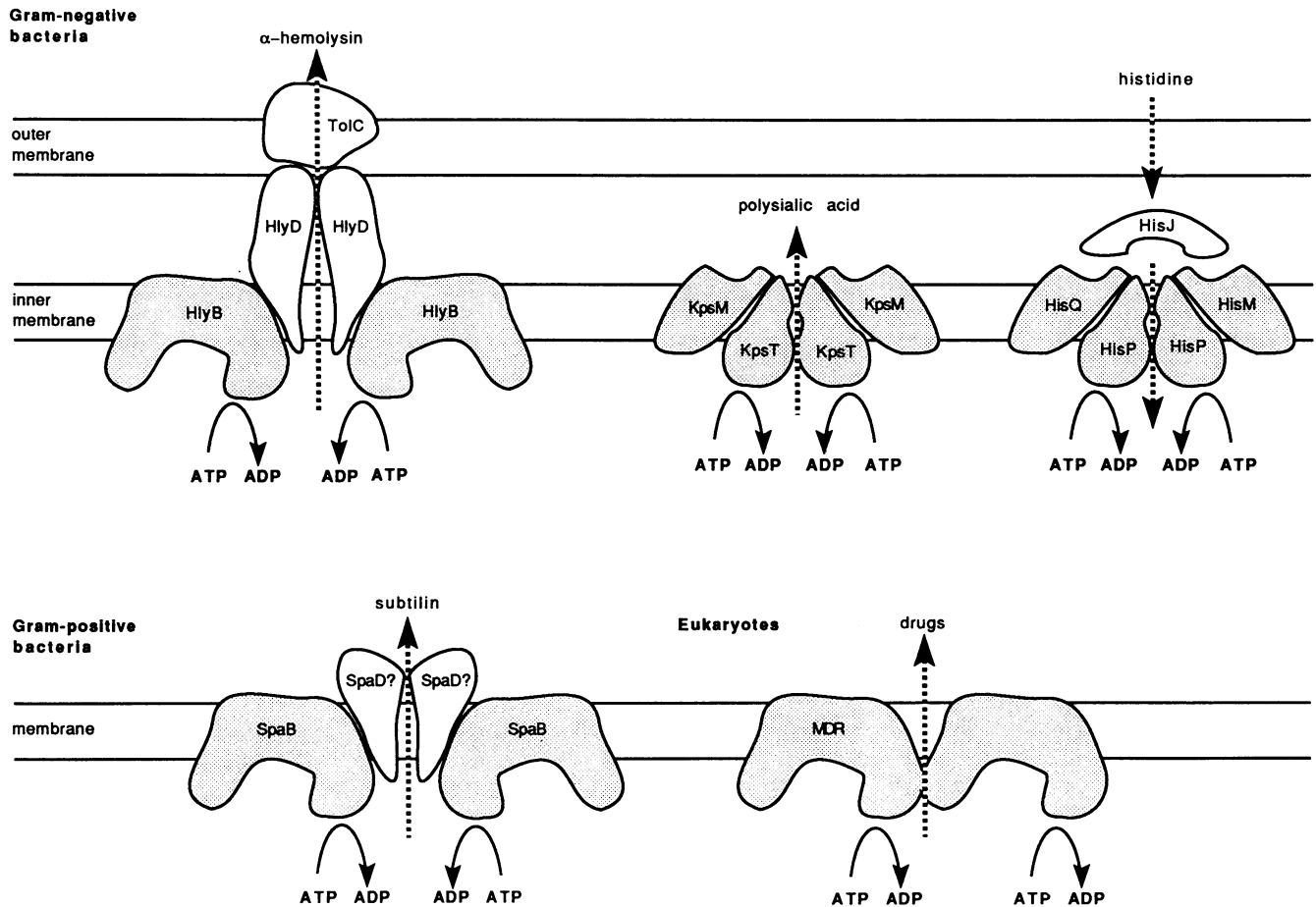
FIG. 1. Structural models of various ABC transporters. The prototype systems included are the *E. coli* alpha-hemolysin exporter, the *E. coli* polysialic acid exporter, the *S. typhimurium* histidine importer, the *B. subtilis* subtilin exporter, and the mammalian P-glycoprotein drug exporter. The bacterial exporters are drawn as dimers, consistent with the model of Higgins (80) and others, who propose a minimum of four required "core components." There is no experimental evidence that the bacterial export complexes form dimers. The core components in each complex are shaded.

Eukaryotic ABC Transporters

A second subfamily is composed of the ABC transporters found in eukaryotes. All the eukaryotic ABC transporters have their ATP-binding domain on a single polypeptide with the MSDs. In fact, most of the eukaryotic ABC transporters have a tandem duplication of the structure and do not appear to require other subunits for their function (Fig. 1). Several of these systems are of significant medical importance and have been under intense study since their discovery. These include the multidrug resistance protein, P-glycoprotein, which exports chemotherapeutic drugs from tumor cells when overexpressed (35) and the cystic fibrosis transmembrane regulator found to be defective in patients with cystic fibrosis (145). Other eukaryotic ABC exporters (reviewed in reference 80) include pfMDR, which exports antimalarial drugs from *Plasmodium falciparum* (44), STE6, which exports a-type mating factor from *Saccharomyces cerevisiae* (113, 127), and a group of transporters involved in antigen presentation (27, 128, 167, 178). Other, more recently identified eukaryotic ABC transporters include atpgp, an *Arabidopsis thaliana* P-glycoprotein homolog (30); pmd1, the leptomycin B resistance gene from *Schizosaccharomyces pombe* (133); hmt1, which encodes a cadmium-specific phy-

tochelatin in *S. pombe* (135); LEMDR06 and LEMDRF2, two multidrug resistance genes in *Leishmania donovani* (75); SNQ2, a quinoline resistance gene in *S. cerevisiae* (160); NG-TRA, which is a putative hormone transporter expressed in adrenal glands (6); and ALD, the putative X-linked adrenoleukodystrophy gene in humans (130).

Bacterial ABC Exporters

The third subfamily of ABC proteins is made up of the bacterial ABC exporters, the largest and fastest-growing group. There are over 40 identified systems (Table 1). A few ABC proteins (FtsE, HepA, MsbA, SurB, Orf1) are included in Table 1 because their structure is consistent with their having a translocation function, even though their role as an exporter has not been demonstrated. We describe the common features of these ABC exporters with particular emphasis on key prototype systems. At the end, we present a survey of all the known and putative bacterial ABC exporters with the hope that it can serve as an easy reference for those who wish to investigate a particular system in more detail.

All ABC transporters in the bacterial export subfamily have the conserved ATP-binding motif. The domain contain-

TABLE 1. List of bacterial ABC exporters

Transported substrate	ABC homolog	Translocator structural organization				Organism	Reference
		ABC	MSD	AF[a]	OMF[a]		
Proteins							
HlyA (alpha-hemolysin)	HlyB	HlyB—C	HlyB—N	HlyD	TolC	*Escherichia coli*	42a
HlyA (alpha-hemolysin)	HlyB	HlyB—C	HlyB—N	HlyD	TolC	*Proteus vulgaris*	107
HlyA (alpha-hemolysin)	HlyB	HlyB—C	HlyB—N	HlyD		*Morganella morganii*	105
AppA (hemolysin)	AppB	AppB—C	AppB—N	AppD		*Actinobacillus pleuropneumo-niae*	17
AshA (hemolysin)	AshB	NA[b]	NA	AshD		*Actinobacillus suis*	12
LktA (leukotoxin)	LktB	LktB—C	LktB—N			*Actinobacillus actinomycetem-comitans*	68
LktA (leukotoxin)	LktB	LktB—C	LktB—N	LktD		*Pasteurella haemolytica*	171
CyaA (cyclolysin)	CyaB	CyaB—C	CyaB—N	CyaD	CyaE	*Bordetella pertussis*	63
PrtA,B,C (proteases A, B, C)	PrtD	PrtD—C	PrtD—N	PrtE	PrtF	*Erwinia chrysanthemi*	117
AprA (alkaline protease)	AprD	AprD—C	AprD—N	AprE	AprF	*Pseudomonas aeruginosa*	31, 69
Peptides							
CvaC (colicin V)	CvaB	CvaB—C	CvaB—N	CvaA	TolC	*Escherichia coli*	62
CylL (hemolysin/bacteriocin)	CylB	CylB—C	CylB—N			*Enterococcus faecalis*	60
SpaS (subtilin)	SpaB	SpaB—C	SpaB—N	SpaD		*Bacillus subtilis*	18, 103
NisA (nisin)	NisT	NisT—C	NisT—N			*Lactococcus lactis* 6F3	36
EpiA (epidermin)	EpiT	EpiT'	EpiT			*Staphylococcus epidermidis*	153
CF (competence factor)	ComA	ComA—C	ComA—N	ComB		*Streptococcus pneumoniae*	92
PedA (pediocin PA-1)	PedD	PedD—C	PedD—N			*Pediococcus acidilactici*	124
LcnA (lactococcin A)	LcnC	LcnC—C	LcnC—N	LcnD		*Lactococcus lactis* subsp. *lactis*	170
McbA (microcin B17)	McbEF	McbF	McbE			*Escherichia coli*	51
Nonprotein substrates							
Capsular polysaccharide	KpsMT	KpsT	KpsM			*Escherichia coli* K5	164
Capsular polysaccharide	KpsMT	KpsT	KpsM			*Escherichia coli* K1	138
β-1,2-glucan	NdvA	NdvA—C	NdvA—N			*Rhizobium meliloti*	168
β-1,2-glucan	ChvA	ChvA—C	ChvA—N			*Agrobacterium tumefaciens*	13
Daunorubicin/doxorubicin	DrrAB	DrrA	DrrB			*Streptomyces peucetius*	67
Capsular polysaccharide	BexABC	BexA	BexB, BexC?			*Haemophilus influenzae*	110
Capsular polysaccharide	CtrBCD	CtrD	CtrC, CtrD?			*Neisseria meningitidis*	490
Heme for cytochrome *c*	CycVWX	CycV	CycW, ORF263			*Bradyrhizobium japonicum*	143
Heme for cytochrome *c*	HelABC	HelA	HelB, HelC			*Rhodobacter capsulatus*	7
Erythromycin resistance	MsrA	MsrA—N,C				*Staphylococcus epidermidis*	147
Tylosin resistance	TlrC	TlrC—N,C				*Streptomyces fradiae*	149
Macrolide resistance	SrmB	SrmB—N,C				*Streptomyces ambofaciens*	156
Macrolide resistance	CarA	CarA—N,C				*Streptomyces thermotolerans*	156
Incomplete systems (substrate or transporter not identified)							
? (envelope polysaccharide)	HepA	HepA—C	HepA—N			*Anabaena* sp. strain PCC7120	86
? (required to restart growth)	SurB	SurB—C	SurB—N			*Escherichia coli*	162
? (suppresses *htrB* phenotype)	MsbA	MsbA—C	MsbA—N			*Escherichia coli*	98
? (aerial mycelium formation)	AmfA	AmfA—C	AmfA—N			*Streptomyces griseus*	178a
? (aerial mycelium formation)	AmfB	AmfB—C	AmfB—N			*Streptomyces griseus*	178a
? (essential, cell division)	FtsE	FtsE				*Escherichia coli*	56
? (lipooligosaccharides?)	NodIJ	NodI	NodJ			*Rhizobium leguminosarum*	37
? (lipooligosaccharides?)	NodIJ	NodI	NodJ			*Bradyrhizobium japonicum*	181
? (downstream of NtrA)	ORF1	ORF1				*Rhizobium meliloti*	1
NodO (Ca-binding protein)	N.A.	NA	NA			*Rhizobium leguminosarum*	34
PrtSM (zinc metalloprotease)	N.A.	NA	NA			*Serratia marcescens*	118, 172
FrpA (Fe-binding protein)	N.A.	NA	NA			*Neisseria meningitidis*	174
GalA (gallidermin)	GalT	NA	NA			*Staphylococcus gallidermidis*	154
LcnG (lactococcin G)	Lcn?	NA	NA			*Lactococcus lactis*	74

[a] AF, accessory factor; OMF, outer membrane factor.
[b] NA, not applicable.

ing this motif can be on the same polypeptide as the MSDs (as in the eukaryotic ABC exporters) or on a polypeptide separate from the hydrophobic domains (as in the bacterial ABC importers). Every bacterial ABC exporter has the components necessary to form the conserved structural organization described in Fig. 1. Notable exceptions are the antibiotic resistance proteins CarA, MsrA, SrmB, and TlrC, which have two ABC cassettes and no identified MSDs.

Accessory factors. Many of the bacterial ABC exporters require additional proteins, besides the protein(s) which

include the ABC and the MSD, to form a functional complex. These additional factors have been identified in several gram-negative systems and shown to be needed when the secreted product is destined for immediate release into the extracellular medium. These proteins are referred to here as accessory factors. In several of the prototypes, the accessory factor fractionates mostly to the inner membrane, with minor amounts fractionating to the outer membrane (24, 123), consistent with the hypothesis that the accessory factor is anchored in the inner membrane and spans the periplasm. The accessory factor probably acts to connect the inner and outer membranes and functions to facilitate the export of products through both membranes of the gram-negative cell. The gene encoding the accessory factor is always found linked to the gene encoding the ABC protein.

Many gram-negative bacterial ABC exporters do not have accessory factors, notably those involved in the transport of nonprotein substrates. In these systems, the final destination of the exported product is the periplasm or outer membrane. In a few cases, additional proteins are required to complete translocation of the product into the outer membrane. However, in these systems, outer membrane transport is a distinct process and the proteins involved are different from the accessory factor proteins described above. Several of the gram-positive secretion systems have linked sequences that have some similarity to the accessory factors characterized in gram-negative bacteria (18, 103, 129, 170). However, in the export process of gram-positive bacteria, the secreted product has only one membrane to traverse, so the role of the accessory factor in gram-positive systems is not clear.

A third export-related gene is also observed in several of the gram-negative export systems (63, 117, 185). This gene encodes an outer membrane protein required for secretion in several characterized systems. In Table 1, the third component is referred to as the outer membrane factor. The gene encoding the outer membrane factor can be found either closely linked to the other export genes (63, 117) or physically quite distant (185). In the latter case, the outer membrane factor has been shown to have cellular functions apart from its export function (185). Lastly, genes involved in synthesis of lipopolysaccharide also seem to be required for protein export in several gram-negative systems, suggesting that lipopolysaccharide is also involved in the export process (187).

List of bacterial export systems. Table 1 lists all known bacterial ABC exporters, including several putative exporters. The list is divided into three major groups based on the characteristics of the transported product (and not on phylogeny of the ABC transporter, which is discussed later). These substrate groups are proteins, peptides, and nonprotein substrates. Some other systems have been included even though they have not been completely described.

Table 1 further describes these systems by including the components identified in each case which make up the transporter apparatus. Since there are different structural combinations among the bacterial ABC exporters, the structural organization is broken down into distinct units, the ABC, MSD, accessory factor, and outer membrane factor.

PROTOTYPE BACTERIAL ABC EXPORTERS

Protein Transport Systems

The best-characterized class of bacterial exporters is involved in the extracellular secretion of a family of proteins called the RTX (repeats in toxin) toxins (19) and the related

extracellular proteases (183). At present, 10 protein export systems have been identified and characterized from gram-negative bacteria. These RTX protein transport systems share many common features: they all have a large ABC transporter (600 to 750 aa) that contains N-terminal MSDs and a C-terminal ABC, and they all require an accessory factor and an outer membrane factor. The transported product in each of these systems is a large protein ranging in size from 50 kDa (protease A of *Erwinia chrysanthemi*) to 216 kDa (the *Bordetella pertussis* cyclolysin), and each product appears to be transported into the extracellular medium without any detectable periplasmic intermediate. The translocation complex recognizes an export signal located in the C-terminal 60 to 150 residues of the transported protein.

E. coli **alpha-hemolysin. (i) Sequence information.** The *Escherichia coli* alpha-hemolysin determinant was the first ABC export system identified in prokaryotes (42a). It has been sequenced and characterized now from several sources including the chromosome of uropathogenic strain J96 (42a), strain LE2001 (96), and plasmid pHly152 (78). It is highly conserved in these strains. Each hemolysin determinant contains an operon encoding four proteins, HlyCABD. The organization of the Hly operon is shown in Fig. 2. *hlyA* encodes the 1,023-aa (107-kDa) alpha-hemolysin (HlyA), *hlyB* encodes the 707-aa ABC exporter, *hlyD* encodes the 477-aa accessory factor, and *hlyC* encodes a 170-aa protein that has no secretion function but facilitates the activation of HlyA by addition of a fatty acid group (94). The outer membrane factor for alpha-hemolysin is the chromosomally encoded, 495-aa TolC protein (132, 185).

A large amount of genetic and biochemical data has been accumulated for the *E. coli* hemolysin, and these results are the subject of several detailed reviews (8, 9, 19, 65, 87, 88, 91, 190). Here, we will summarize several key points and discuss recent results.

(ii) HlyA secretion signal. The secretion of HlyA has been shown to require neither an N-terminal signal sequence nor the *secA* gene (53, 65). By using deletions, gene fusions, and point mutants, the secretion signal in HlyA has been localized to the C-terminal 48 to 60 amino acids (77, 100, 101, 108, 169). This C-terminal secretion signal does not resemble a typical N-terminal signal sequence, nor is it removed during HlyA secretion (42). There is surprisingly little primary sequence conservation in the C-terminal secretion signals of HlyA and the other RTX toxins (87, 91, 101, 169). Instead, several secondary structure features—the α-helical region, the aspartate box, and the amphipathic helix—have been proposed to be important for signal recognition (87, 91, 101, 169). Experiments with protein fusions have shown that the C-terminal signal in HlyA can be used to secrete heterologous proteins out of the cell in a HlyBD-dependent fashion (88, 100, 122). These include proteins that normally cannot be secreted by the Sec-dependent pathway. Over 100 mutations have been generated in the C-terminal region by several research groups. Several conclusions can be drawn from the analysis of these mutants. (i) The HlyA signal region is very tolerant of mutations. In fact, no missense mutations were found that completely abolished HlyA secretion. (ii) Surprisingly, mutations which should alter the proposed secondary structures conserved among the RTX toxins do not greatly affect HlyA secretion. (iii) Several of the putative structural domains in the C-terminal signal which were thought to be critical (the α-helical region, the aspartate box, and the amphipathic helix) are actually not essential for HlyA secretion. (iv) There appear instead to be
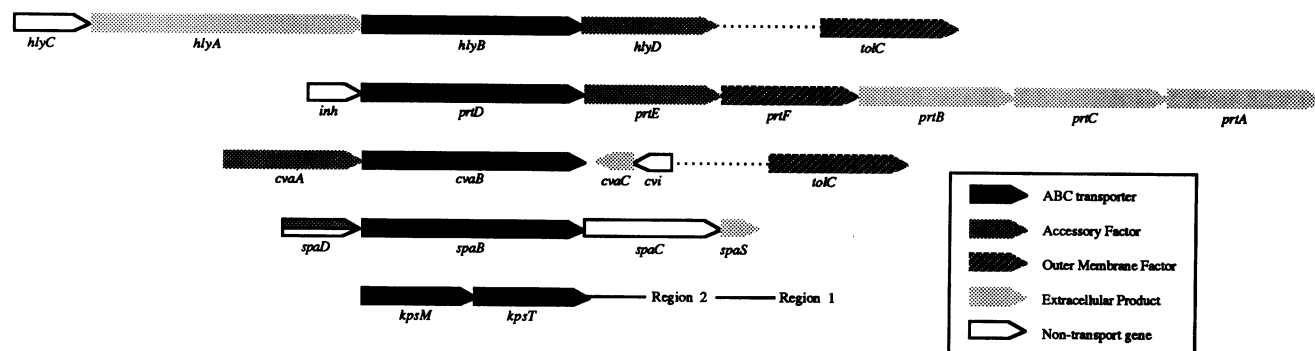
FIG. 2. Organization of selected bacterial ABC exporter operons. The systems included are *E. coli* alpha-hemolysin (*hlyCABD*) (42a), *Erwinia chrysanthemi* protease (*inh, prtDEFBCA*) (117), *E. coli* colicin V (*cvaABC, cvi*) (62), *B. subtilis* subtilin (*spaDBCS*) (18) (called *spaBTCS* by Klein et al. [103]), and the *E. coli* capsular polysaccharide transporter (*kpsMT*) (138, 164). The *tolC* gene is unlinked to the alpha-hemolysin and the colicin V operons. The symbol for the *spaD* gene means that *spaD* has some sequence similarity to other accessory factors but may not be part of the functional exporter. The gene sizes are roughly to scale. See the text for more details of these systems.

important contact residues scattered throughout the C-terminal secretion signal (101, 169).

(iii) **Structures of HlyB and HlyD.** The HlyB and HlyD exporters have been studied in some detail. Both proteins fractionate primarily to the inner membrane (96, 123, 157, 173, 188). Topological analysis of HlyD shows that it has one transmembrane domain and that most of the protein is on the periplasmic side of the membrane (157, 188). These results are consistent with the sequence hydrophobicity data and with the model that predicts that HlyD spans the periplasm as part of the secretion complex. The HlyB protein sequence can be divided into three large domains, an N-terminal hydrophilic domain of about 150 aa, a 275-aa central hydrophobic domain, and a 275-aa C-terminal domain that contains the ABC. Hydropathy analysis of HlyB predicts that it would have six transmembrane domains, all localized within the central hydrophobic domain. This would be consistent with the predicted topology of all the other ABC transporters. However, experiments from two research groups provide evidence that, instead, HlyB contains eight transmembrane domains: two in the N-terminal domain and six in the central hydrophobic domain (52, 188). The topological models predicted in these papers differ from models based on hydropathy analysis and from each other. Therefore, at this point it is difficult to conclusively identify the topological domains of HlyB.

HlyB has been difficult to characterize biochemically because of its membrane localization and low yield. Recently, HlyB has been tagged with epitopes from P-glycoprotein to allow further biochemical analysis (96). In addition, antibodies to HlyB have been successfully generated (173). Both of these studies confirmed the inner membrane localization of HlyB in vivo. Mutagenesis of HlyB has been carried out to characterize its function and to identify regions which may interact with HlyA. Linker insertion mutations and point mutations in the MSD of HlyB have been shown to affect HlyA secretion levels (8, 9, 96). Surprisingly, a deletion that removes the MSDs in the N-terminal 467 aa of HlyB and replaces them with part of TetC appears to be able to cause secretion of low levels of HlyA in an HlyBD-dependent fashion (173). This suggests that some secretion signals are in the MSD but that residual secretion activity is retained within the C-terminal ABC region.

(iv) **Functional complementation.** Since the HlyA substrate is able to tolerate many mutations and still remain functional

for secretion, the HlyBD exporter must have a relaxed specificity for export of HlyA-like molecules. This hypothesis is confirmed by looking at the ability of HlyBD to functionally complement mutations in exporters from related ABC export systems. HlyBD have been shown to efficiently secrete many related proteins including *Morganella morganii* and *Proteus vulgaris* HlyA (105), AppA (72), LktA (15, 84, 171), CyaA (125, 159), and NodO (152). At a lower level, HlyBD can also secrete ColV (39), AprA (69), PrtSM (118, 172), and PrtB (23). Thus, many proteins that are secreted through a dedicated ABC exporter can be transported by HlyBD, even though some of them have very little sequence similarity with HlyA.

(v) **HlyA secretion pathway.** The mechanism of HlyA secretion by HlyBD has been carefully studied. In cells carrying the wild-type HlyBD exporters, HlyA can be found in the cytoplasm, the outer membrane, and the culture supernatant. No periplasmic HlyA has ever been detected during secretion (43, 66, 108, 134). In cells that lack either HlyBD or HlyB alone, HlyA localizes to the cytoplasm and the inner membrane; this suggests that some HlyA can target to the membrane independently of HlyBD. HlyA from cells lacking HlyD are also cytoplasmic (65). However, when HlyB is expressed along with two-thirds of HlyD, HlyA targets to the outer membrane, suggesting that this HlyD derivative still retains partial secretion functions (134). The secretion process can be divided into early and late stages on the basis of energy requirements (106). Early steps, possibly HlyA binding to the membrane secretion complex, require the proton motive force. In contrast, late translocation to the outer membrane is independent of the proton motive force, perhaps relying on ATP hydrolysis by HlyB (106). A three-step model for HlyA secretion has been proposed (91, 169): (i) the C terminus of HlyA associates with the inner membrane; (ii) once in the membrane, HlyA interacts with HlyB; and (iii) a secretion complex that includes HlyB, HlyD, and TolC then facilitates secretion directly through both membranes.

Erwinia chrysanthemi **proteases.** The protease secretion system of *E. chrysanthemi* shares most of the functional features with the hemolysin system described above, such as an ABC export system and C-terminal secretion signal. In addition to these features, the extracellular protease systems from *E. chrysanthemi*, *Pseudomonas aeruginosa*, and *Serratia marcescens* share additional commonalities and compose a distinct subgroup of ABC exporters (183).

E. chrysanthemi is a phytopathogenic enterobacterium that secretes three related proteases, PrtA, PrtB, and PrtC. The genes required for this function were cloned and expressed in *E. coli* (Fig. 2) (186). The proteases are distinct but closely related in sequence and size; PrtA is 50 kDa, PrtB is 53 kDa, and PrtC is 55 kDa. Most detailed studies have been carried out on PrtB, which displays slight sequence similarity with HlyA in the RTX repeat region. The deduced protein sequences also reveal that these proteases are processed to remove the N-terminal 16 to 18 residues (25, 55). These processed leaders are not Sec-dependent export signal sequences. Instead, secretion of the proteases requires the three linked genes *prtD*, *prtE*, and *prtF* (117). PrtD is the ABC exporter, PrtE is similar to HlyD, and PrtF has sequence similarity with TolC.

By using protein fusions and deletions of the PrtB protein, a minimum secretion signal region has been localized to the C-terminal 39 residues of PrtB (23). Thus, the proteases have a C-terminal signal similar to the HlyA secretion signal. PrtDEF and HlyBD were shown to have only slight functional conservation. Complementation experiments showed that HlyBD secretes PrtB at only less than 2% and that PrtDEF does not secrete detectable HlyA (23). More recently, CyaA-PrtB and HlyA-PrtB fusions were used to further characterize the C-terminal PrtB secretion signal (119). A large C-terminal region, which contained the RTX repeats, was required for the secretion of larger HlyA-PrtB and CyaA-PrtB fusions. The authors hypothesize that larger regions of PrtB might be necessary to facilitate the unfolding of the fusion polypeptide when it interacts with the PrtDEF secretion proteins (119).

The secretion proteins PrtD, PrtE, and PrtF have also been characterized when expressed in *E. coli* (24). PrtD is localized in the inner membrane, PrtE is localized to both the inner and outer membranes, and PrtF is localized in the outer membrane. This is consistent with the localization of HlyBD and TolC and provides independent evidence about the structure of the ABC-type secretion complexes. Protease accessibility studies also confirm that the Prt proteins have a membrane organization similar to that found in the Hly system, although they do not address the question of six or eight MSDs (24). The protease exporters are expressed at relatively high levels in *E. coli* and appear to be more amenable to biochemical analysis than the exporters from hemolysin and colicin V.

Peptide Transporters

The second major group of bacterial transport systems facilitates the secretion of ribosomally encoded peptide antibiotics (for a review, see reference 104) and related small proteins. This group consists of at least seven systems from gram-positive bacteria and two from gram-negative bacteria. Several features distinguish these transport systems from the systems described above that secrete large proteins. The extracellular product in each of these systems is a small protein/peptide (<11 kDa) which lacks any RTX repeat motifs or any HlyA-like C-terminal export domain. In the one peptide secretion system where a secretion signal has been characterized, colicin V, this signal has been localized to the N-terminal region (62). The colicin V secretion signal shares some primary sequence conservation with the N terminus of lactococcin A, but this conservation does not extend to the other peptides in this subfamily. Most of the secreted peptides undergo significant processing and unusual posttranslational modifications that give rise to residues such

as dehydroalanine and lanthionine (45, 95). Peptides containing lanthionine have been termed the lantibiotics (95).

The ABC exporters responsible for peptide secretion are typically on one large polypeptide that contains both the ABC and an MSD, much like HlyB and PrtD. In only one example, the microcin B17 secretion system, the ABC and the MSD are on separate polypeptides (51). Several other features of the microcin B17 secretion system distinguish it from the other systems in this group. No accessory factor has been found, and during microcin B17 secretion, a periplasmic intermediate has been observed (22, 51).

The prototypical colicin V exporter requires both an accessory factor and outer membrane factor (62). In contrast, the systems from gram-positive bacteria, having no outer membrane barrier to cross, may be able to bypass the requirement for the additional components. Although obviously no outer membrane factor component can exist in the gram-positive systems, several of them do have linked genes that encode potential HlyD-like accessory factors (18, 103, 129, 170), and in the lactococcin A system a mutation in the accessory factor is found to abolish lactococcin A activity (170).

E. coli colicin V. Colicin V is an 88-residue peptide antibiotic whose production is encoded in large, low-copy-number virulence plasmids found in *E. coli* and other members of the *Enterobacteriaceae* (38, 189). Colicin V is active against a number of gram-negative bacteria and kills sensitive cells by disrupting their membrane potential (193). The genes encoding colicin V production, activity, and immunity were cloned from plasmid pColV-K30 and sequenced (61, 62). Four linked genes were identified: *cvaC* encodes the 103-aa pro-ColV, *cvi* encodes a 78-aa immunity protein; and *cvaA* and *cvaB* encode 413- and 698-aa secretion proteins, respectively (see Fig. 2). CvaC, the colicin V primary translation product, does not contain a Sec-dependent N-terminal signal sequence. However, the N-terminal 15 residues are removed concomitant with export (40). In addition, mass spectroscopy has shown that, unlike many other peptide antibiotics, colicin V does not undergo posttranslational covalent modifications (40). Secretion of colicin V from *E. coli* requires the ABC exporter CvaB, the accessory factor CvaA, and the unlinked outer membrane factor TolC (61, 62).

The secretion signal in CvaC was localized to the 39 N-terminal residues by using CvaC-PhoA fusions and point mutations (62). CvaC mutations G14D, G14N, and G38R all decrease secretion levels without affecting intracellular colicin activity. Active colicin V can also be secreted via the HlyBD and the PrtDEF secretion systems at lower efficiencies, despite the lack of similarity between the HlyA/PrtB secretion signals and the CvaC secretion signal (39). The HlyBD system was shown to recognize an N-terminal signal in CvaC, but the CvaC export mutants differentially affect export through HlyBD and CvaAB, suggesting differences in signal specificity between the two systems (39).

The colicin V secretion pathway has been characterized by genetic and biochemical methods. In one experiment, the N-terminal signal sequence from the OmpA protein was fused to the 88-aa mature colicin V peptide and shown to facilitate export of colicin V into the periplasm, showing that colicin V is not incompatible with the Sec-dependent pathway but that it could not pass through the outer membrane unaided (40). During CvaAB-mediated secretion, free colicin V was shown not to accumulate in the periplasm, and mutations in *cvaA*, *cvaB*, or *tolC* also do not result in free periplasmic colicin V. These results suggest that the three

transport proteins form a single export complex that directly secretes colicin V into the extracellular medium, consistent with what is seen for the hemolysin secretion system (40). The colicin V system has been reviewed recently (38).

Nonprotein Substrates

Many ABC export systems are known to secrete nonprotein molecules such as lipophilic drugs, antibiotics, and polysaccharides. Among the bacterial ABC exporters there is a surprisingly wide range of substrate structural diversity. In some systems, a single HlyB-like ABC exporter is sufficient to move polysaccharide to the outer membrane of *Rhizobium* and *Agrobacterium* cells (13, 168). In other systems involved in export of capsular polysaccharide, transporters that look very much like inverse periplasmic permeases are found (49, 110, 138, 164). Perhaps most surprisingly, antibiotic efflux systems have been described that lack any identifiable MSD (147, 149, 156). It is yet to be seen whether any unlinked MSDs will be identified for these efflux systems.

None of the secretion systems for nonprotein substrates have accessory factors like HlyD or outer membrane factors like TolC. Consistent with this observation, none of the systems secrete their product directly into the extracellular medium. In the capsular polysaccharide systems, additional proteins are required to complete translocation of the product into the outer membrane. However, in these examples outer membrane transport is a distinct process and the proteins involved are different from the accessory factor proteins described above.

Several members of this exporter subfamily have been reviewed recently and given the name of ABC-2-type transporters (144). It is interesting that these multicomponent systems have so much in common with the periplasmic permeases and yet clearly facilitate export. This observation reinforces the idea that all the ABC systems share a common structural organization and functional history and that there is not a clear structural distinction between exporters and importers (3).

Agrobacterium tumefaciens ChvA and *Rhizobium meliloti* NdvA. The plant pathogen *Agrobacterium tumefaciens* causes crown gall tumors when present on wounded dicotyledonous plants. Tn*5* mutagenesis identified two chromosomal genes which were required for virulence, *chvA* and *chvB* (29). These mutants fail to produce extracellular β-1,2-glucans. The β-1,2-glucans are oligomers 18 to 24 aa long with a molecular mass of 4 to 6 kDa (194, 195), and are involved in the attachment of the bacteria to plant cells. *chvB* mutants are avirulent and affect glucan production but not glucan secretion (141).

β-1,2-Glucans have also been implicated in the attachment of *Rhizobium meliloti* to legumes (54), a process essential for the formation of symbiotic nitrogen-fixing nodules. The *ndvA* and *ndvB* genes were isolated by hybridization to *chvA* and *chvB* and found to be required for nodule development (32). The *ndv* genes are functionally equivalent to their *chv* counterparts; they also function to produce and secrete β-1,2-glucans. *ndvA* mutants do not produce extracellular β-1,2-glucans, although the protein-sugar intermediate is observed in cytoplasmic extracts (168). Sequence analysis shows that *ndvA* encodes a 616-aa ABC export protein with a predicted molecular mass of 67.1 kDa. NdvA probably functions to export the 4- to 6-kDa β-1,2-glucan oligomers to the periplasm, from where they are then localized to the surface of the outer membrane.

The *chvA* gene was sequenced soon afterward and found to encode another ABC exporter (13). ChvA is a 588-aa protein with a predicted molecular mass of 64.7 kDa. *chvA* mutations were also shown to accumulate glucan in the cytoplasm and to lack detectable glucan in the periplasm (13). ChvA and NdvA are highly homologous in function and sequence (76% amino acid identity). The discovery of NdvA and ChvA widened the family of ABC exporters to include systems that secrete nonproteinaceous molecules and to include proteins that function to facilitate secretion to the periplasm of gram-negative bacteria.

Capsular polysaccharide exporter—*E. coli* KpsMT. Many clinical strains of *E. coli*, *Haemophilus influenzae*, and *Neisseria meningitidis* produce capsular (K) polysaccharides that are major virulence determinants (10). To identify genes from *E. coli* serotypes K5 and K1 that encode the capsular polysaccharide determinant, cosmids from K1 and K5 were cloned into the noncapsular *E. coli* K12 and shown to be sufficient to allow K12 to produce capsule (10). The capsular polysaccharide determinant was shown to contain three phenotypically distinct genetic regions associated with polysaccharide expression. Region 1 is necessary for the transport of mature, lipid-linked polysaccharide across the outer membrane and its assembly into a capsule. Region 2 is serotype specific and encodes the enzymes for synthesis and polymerization of specific K antigen. Region 3 is involved in the energy-dependent translocation of the polysaccharide across the inner membrane (10).

Two genes have been identified and sequenced from region 3 of *E. coli* serotypes K1 and K5 and found to encode components of an ABC transporter. In K5, the *kpsT* gene encodes a 224-aa, 25.5-kDa ABC protein and *kpsM* encodes a 258-aa, 29.5-kDa hydrophobic protein (164). In K1, *kpsT* encodes a 219-aa, 24.9-kDa ABC protein and *kpsM* encodes a 258-aa, 29.6-kDa hydrophobic protein (Fig. 2) (138). Insertion mutations in *kpsM* or *kpsT* result in polysaccharide that is cytoplasmic and shorter than surface polymers (112). A mutation in the ATP-binding domain of K1 KpsT (K44E) results in a nonfunctional protein (138). Additional mutants with amino acid substitutions in the ATP-binding domains are also nonfunctional (163), and KpsT has also been shown to bind to 8-azido-ATP, providing biochemical evidence for ATP binding (163). PhoA fusions to KpsM suggest that it is membrane associated and that parts of it are exposed to the periplasm (138), and more extensive analysis of KpsM with fusions to β-lactamase support the model predicted from the hydropathy profile, i.e., that KpsM contains six MSD (163).

Genes in region 3 of serotypes K5, K7, K12, and K92 are able to functionally complement each other in *trans*, suggesting that *kpsM* and *kpsT* are functionally equivalent between serotypes (146). Apparently, the KpsMT exporter is capable of transporting a variety of acidic polysaccharides. These *E. coli kps* systems are highly homologous to the capsular polysaccharide genes of *H. influenzae* and *N. meningitidis* described below. The authors suggest that KpsMT acts to transport the growing polysaccharide chain to the periplasm, where it is then secreted to the surface by specific periplasmic proteins, such as KpsD, which are encoded by region 1 (163, 164).

Incomplete Systems

Several systems have only been partially described. These include systems where the ABC transport protein has been cloned and sequenced but a substrate for the transporter has not been clearly identified. They also include a few systems

that contain a secreted protein that is homologous to a known ABC substrate such as hemolysin or epidermin. In these systems, characterization of the operon is not yet complete and putative ABC transporters that facilitate secretion are likely to be identified. Several such systems are described in the survey below.

SEQUENCE AND PHYLOGENETIC ANALYSIS OF BACTERIAL ABC EXPORTERS

To better understand the evolutionary relationship between the bacterial ABC exporters, we carried out a detailed sequence comparison of the ABC regions from 29 systems. For this analysis, only the ABC domains were compared. Although the MSDs all appear to have similar hydrophobicity profiles, there is much less primary sequence conservation between the MSDs, making detailed sequence comparison more difficult.

The ABC sequence comparison results are shown in Fig. 3, 4, and 5. Figure 3 shows a multiple sequence alignment of the bacterial ABC exporters, including a consensus sequence. The degree of sequence identity varies markedly along the 190 aa in the ABC, suggesting the presence of distinct subdomains within the ABC. The degree of sequence similarity across the ABC domain is shown graphically in Fig. 4. The most highly conserved sequences cover the extended regions around the A and B sites, specifically residues 10 to 34 and 115 to 150. The central subdomain of the ABC has much lower primary sequence conservation, possibly resulting from differences in substrate specificity. Overall, the primary and secondary structures of the ABC domain are conserved between the various bacterial ABC exporters and throughout the entire superfamily of ABC-transport systems.

Analysis of the phylogenetic tree in Fig. 5 shows that the bacterial ABC exporters can be divided into two primary branches. One branch contains all the transport systems where the ABC is present on the same polypeptide as the MSD (group A). The other branch contains all the systems where the ABC and MSD are found on separate polypeptides (group B). We have previously shown that the ABC domains from mouse and human P-glycoprotein belong with the sequences in group A and that the ABC domains from several periplasmic permease systems (MalK, HisP, OppD, OppF, and PstB) as well as from Orf1, NodI, and FtsE belong with the sequences in group B (38). Therefore, the evolutionary relatedness of these domains seems to be more a function of their disposition with the MSD than with their substrate recognition. On the basis of these observations, two hypotheses can be made: the superfamily of ABC transporters originated from a shared common ancestor and the earliest branching of the superfamily resulted in two ABC protein families, those that became fused with an MSD and those that did not.

The transporters in group A appear to function only in export. Proteins in group A include the 17 sequences in Fig. 5 known to have a function in export, as well as the eukaryotic exporter P-glycoprotein, described previously (38). Additional pairwise alignments of the eukaryotic exporters STE6 and the cystic fibrosis transmembrane regulator suggest that they also fall in group A (data not shown). The proteins MsbA and SurB, whose functions are not yet known, also fall within group A, although very near to the base of that branch. From this, it is difficult to predict an export function yet for MsbA or SurB. Within group A are ABC exporters which do and do not contain a tandem

duplication of its functional domains. None of the prokaryotic proteins in group A contain this tandem duplication. In contrast, eukaryotic ABC exporters have been found both with and without the tandem duplication (80). This suggests that the tandem duplication did not arise until after the split between prokaryotes and eukaryotes.

The bacterial ABC exporters in group A can be further subdivided. One node within group A contains proteins identified from gram-positive systems (ComA, PedD, CylB, NisT, and SpaB). They appear to more related to each other than to any of the other ABC exporters. The RTX toxin exporters also make up a distinct subfamily in group A, but, interestingly, the protease exporters PrtD and AprD do not fall within this grouping, even though they are quite similar to the RTX exporters.

ABC transporters from group B can facilitate import or export. Examples include the polysaccharide exporters, the putative heme exporters, the microcin B17 exporter, and the daunorubicin exporter, all shown in Fig. 5, as well as the other periplasmic permease importers (38, 83). Interestingly, no eukaryotic ABC transporters have yet been identified that would fall into group B. Several proteins described in this review (Orf1, NodI, and FtsE) fall in group B and do not have any known transport function.

INSIGHTS FROM BACTERIAL ABC EXPORTERS

Several excellent reviews of the ABC transporters include detailed discussion of the bacterial permeases and eukaryotic transporters (3, 8, 80). These reviews also give careful consideration to fundamental aspects of the ABC transporters, including transporter structure, energy coupling during translocation, transporter regulation, and the similarities between channels and transporters. The reader is referred to these for further information. Our goal has not been to repeat these earlier works but to extend the coverage to the third subfamily of ABC transporters, the bacterial exporters. Perhaps we can gain some new insight into the ABC transporters by considering here some of the fundamental issues from the point of view of the bacterial exporters.

How Do ABC Transporters Recognize Such a Wide Range of Substrates?

An intriguing fact about the ABC transporters is that they can transport such a wide range of structurally unrelated products. The periplasmic permeases import oligopeptides, amino acids, sugars, phosphate, metal ions, and vitamins. The eukaryotic exporters facilitate the secretion of lipophilic drugs, peptides, and pigments (80). The bacterial exporters have an equally wide range of specificity. Transported products for bacterial ABC exporters include protein toxins as large as 216 kDa, small peptide antibiotics, polysaccharides, antibiotics, and possibly even heme molecules.

Results from the bacterial systems suggest that there is specificity involved in these transport systems but that the specificity is rather relaxed, allowing for the recognition of related ABC-protein substrates by various ABC transporters. Mutagenesis of several ABC-protein substrate secretion signals shows that these signals are able to tolerate many mutations and still be secreted. Mutagenesis of the ABC transporters has identified residues in the MSD that affect transport, but, surprisingly, a mutant with deletion of the MSD still retains some function. We suggest that efficient secretion is mediated through the MSD, which retains little sequence conservation among the ABC exporters, but that a

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             1             15 16            30 31            45 46            60 61            75 76            90 91           105
 1 HlyB      INLSIKQGEVIGIVG RSGSGKSTLTKLIQR FYIP--ENGQVLIDG HDLALADPNWLRRQV GVVLQDNVLLNRSII DNISL-----ANPGM SVEKVIYAAKLAGAH
 2 HlyB-Pv   INLNIKQGEIIGIVG RSGSGKSTLTKLIQR FYIP--ENGQVLIDG HDLALADPNWLRRQV GVVLQDNVLLNRSII DNIAL-----ADPGM PVEKVIHAAKLAGAH
 3 LktB-Aa   INLDISQGEVIGIVG RSGSGKSTLTKLIQR FYIP--EQGQVLIDG HDLALADPNWLRRQV GVVLQDNVLLNRSIR ENIAL-----TNPGM PMEKVIAAAKLAGAH
 4 LktB-Ph   VNLEIRQGEVIGIVG RSGSGKSTLTKLLQR FYIP--ENGQVLIDG HDLALADPNWLRRQI GVVLQDNVLNRSIR  ENIAL-----SDPGM PMERVIYAAKLAGAH

 5 AppB      VNLSIQQGEVIGIVG RSGSGKSTLTKLIQV FYIP--ENGQVLIDG HDLALADPNWLRRQV GVVLQDNVLLGRSIR DNIAL-----ADPGM PMEKIVHAAKLAGAH
 6 CyaB      VSLRIAPGEVVGVVG RSGSGKSTLTRLIQR MFVA--DRGRVLIDG HDIGIVDSASLRRQL GVVLQESTLFNRSVR DNIAL-----TRPGA SMHEVVAAARLAGAH
 7 ChvA      VSFTAKAGETVAIVG PTGAGKTTLINLLQR VYDP--DSGQILIDG TDISTVTKNSLRNSI ATVFQDAGLLNRSIR ENIRL-----GRETA TDAEVVEAAAAAAAT
 8 NdvA      VSFKAKAGQTIAIVG PTGAGKTTLVNLLQR VHEP--KHGQILIDG VDIATVTRKSLRRSI ATVFQDAGIMNRSIG ENIRL-----GREDA SLDEVMAAAEAAAAS

 9 HetA      ITLTIERGKTTALVG ASGAGKTTLADLIPR FYDP--TEGQILVDG LDVQYFEINSLRRKM AVVSQDTFIFNTSIR DNIAY-----GTSGA SEAEIREVARLANAL
10 MsbA      INLKIPAGKTVALVG RSGSGKSTIASLITR FYDI--DEGEIIMDG HDLREYTIASLRNQV ALVSQNVHLFNDTVA NNIAY----ARTEQY SREQIEEAARMAYAM
11 CvaB      LSLSVAPGESVAITG ASGAGKTTLMKVLCG LFEP--DSGRVLING IDIRQIGINNYHRMI ACVMQDDRLFSGSIR ENICG-----FAEEM DEEWMVECARASHIH
12 AprD      LTLAIPAGSVVGVIG PSGSGKSSLARVVLG IWPT--LHGSVRLDG AEIRQYERETLGPRI GYLPQDIELFAGTVA ENIAR-------FGEV QADKVVEAARLAGVH

13 PrtD      IHFSLQAGETLVILG ASGSGKSSLARLLVG AQSP--TQGKVRLDG ADLNQVDKNTFGPTI GYLPQDVQLFKGSLA ENIAR-------FGDA DPEKVVAAAKLAGVH
14 ComA      INLTVPQGSKVAFVG ISGSGKTTLAKMMVN FYDP--SQGEISLGG VNLNQIDKKALRQYI NYLPQQPYVFNGTIL ENLLL----GAKEGT TQEDILRAVELAEIR
15 LcnC      IELSIKENEKLTIVG MSGSGKSTLVKLLVN FFQP--TSGTITLGG IDLQQFDKHQLRRLI NYLPQQPYIFTGSIL DNLLL----GANENA SQEEILKAVELAEIR
16 PedD      VSLTIPHHQKITIVG MSGSGKTTLAKLLVG FFEPQEQHGEIQINH HNISDISRTILRQYI NYVPQEPFIFSGSVL ENLLL----GSRPGV TQQMIDQACSFAEIK

17 CylB      ISFDIRKGDKVAIVG RSGSGKSTLLKLLAG LLQP--SNGEILYEG YPLSNNSNNRRNIFY VNQNAHIFNETIEKN ISLEFK---PNSSIN EKKRLKGSMSKSKMD
18 SurB      ISLQVNAGEHIAILG RTGCGKSTLLQQLTR AWDP--QQGEILLND SPIASLNEAALRQTI SVVPQRVHLFSATLR DNLLL-------ASP GSSDEALSEILRRVG
19 NisT      INLSFEKGELTAIVG KNGSGKSTLVKIISG LYQP--TMGIIQYDK MRSSLMPEEFYQKNI SVLFQDFVKYELTIR ENIGLSDLSSQWEDE KIIKVLDNLGLDFLK
20 SpaB      INVSLHKGERVAIVG PNGSGKSTFIKLLTG LYEV--QQGDIILNG INIKELDMDSYMNQI AALFQDFMKYEMTLK ENIGFGQIDKLHQTN KMHEVLDIVRADFLK

21 BexA      INFELQKGEKIGILG RNGAGKSTLIRLMSG VEPP--TSGTIERSM SISWPLAFSGAFQGS ------------LTGM DNLRFICRLYDVDPD YVT--RFTKEF---S
22 CtrD      INFSLQKGEKVGILG RNGAGKSTLVRLISG VEPP--TSGEIKRTM SISWPLAFSGAFQGS ------------LTGM DNLRFICRIYNVDID YVK--AFTEEF---S
23 KpsT-K1   LNIIFPKGYNIALIG QNGAGKSTLLRIIGG IDRP--DSGNIITEH KISWPVGLAGGFQGS ------------LTGR ENVKFVARLYAKRDE LNERVDFVEEF---S
24 KpsT-K5   LNIEIPSGKSVAFIG RNGAGKSTLLRMIGG IDRP--DSGKIITNK TISWPVGLAGGFQGS ------------LTGR ENVKFVARLYAKQEE LKEKIEFVEEF---A

25 DrrA      LDLNVPAGLVYGILG PNGAGKSTTIRMLAT LLRP--DGGTARVFG HDVTSEPDTVRRRIS VTGQYASVDEGLTGT ENLVMMGRLQGYSWA RAR--ERAAELIDGF
26 NodI      LSFTIAAGECFGLLG PNGAGKSTITRMILG MTSP--SVGKITVLG AQEPGQVRLARAKIG IVSQFDNLDLEFTVR ENLLVYGLYFRMSTR EIE--TVIPSLLEFA
27 CycV      LDFEAVSGEAVAVVG RNGSGKTSLLRLIAG LLIP--AGG---TIA LDGGDAELTLPEQCH YLGHRDALKPALSVA ENLSFWADFLGGERL DAH--ESLATV----
28 HelA      VSFSLAAGHALVLRG PNGIGKTTLLRTLAG LQPP--LAG---RVS M--------PPEGIA YAAHADGLKATLSVR ENLQFWAAIHATDTV ET----ALARM----

29 McbF      LSLKIEQGELIGLLG ENPAGKTTLFNLIRG GVSN--YEGTLKRNF SGGELVSLPQVINLS GTLRNEEVLDLICCF NKL------TKKQAW TDV--NHKWNDNFFI
30 CONSENSUS INLSIPAGEV?AIVG RSGSGKSTLTKLIQG FYIP---DSGQILIDG HDLALVD?NSLRRQI GVV?QDNVLFN?SIR ENIAL-----A?EGA SMEKVV?AAELAGAH
                          A site
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             106          120 121           135 136           150 151           165 166           180 181           195
 1 HlyB      DFISELREGYNTIVG EQGAGLSGGQRQRIA IARALVNNPK--ILI FDEATSALDYESEHV IMRNMHKIC-KGRTV IIIAHRLSTV (488-667) 180
 2 HlyB-Pv   DFISELREGYNTIVG EQGAGLSGGQRQRIA IARALVNNPK--ILI FDEATSALDYESEHV IMRNMHKIC-QGRTV IIIAHRLSTV (488-667) 180
 3 LktB-Aa   DFISELREGYNTVVG EQGAGLSGGQRQRIA IARALVNNPR--ILI FDEATSALDYESENI IMHNMHKIC-QNRTV LIIAHRLSTV (488-667) 180
 4 LktB-Ph   DFISELREGYTTIVG EQGAGLSGGQRQRIA IARALVNNPK--ILI FDEATSALDYESEHI IMQNMQKIC-QGRTV ILIAHRLSTV (489-668) 180

 5 AppB      EFISELREGYNTIVG EQGAGLSGGQPNRIA IARALVNNPK--ILI FDEATSALDYESEHI IMRNMHQIC-KGRTV IIIAHRLSTV (488-667) 180
 6 CyaB      EFICQLPEGYDTMLG ENGVGLSGGQRQRIG IARALIHRPR--VLI LDEATSALDYESEHI IQRNMRDIC-DGRTV IIIAHRLSAV (493-672) 180
 7 ChvA      DFIDSRINGYLTQVG ERGNRLSGGERQRIA IARAILKNAP--ILV LDEATSALDVETEAR VKAAVDALR-KNRTT FIIAHRLSTV (354-533) 180
 8 NdvA      DFIEDRLNGYDTVVG ERGNRLSGGERQRVA IARAILKNAP--ILV LDEATSALDVETEAR VKDAIDALR-KDRTT FIIAHRLSTV (385-564) 180

 9 HetA      QFIEEMPEGFDTKLG DRGVRLSGGQRQRIA IARALLRDPE--ILI LDEATSALDSVSERL IQESIEKLS-VGRTV IAIAHRLSTI (383-562) 180
10 MsbA      DFINKMDNGLDTVIG ENGVLLSGGQRQRIA IARALLRDSP--ILI LDEATSRLDTESERA IQAATDELQ-KNRTS LVIAHRLSTI (362-542) 181
11 CvaB      DVIMNMPMGYETLIG ELGEGLSGGQKQRIF IARALYRKPG--ILF MDEATSALDSESEHF VNVAIKNM---NITR VIIAHRETTL (512-689) 178
12 AprD      ELVLRLPQGYDTVLG VGGAGLSGGQRQRIA LARALYGAPT--LVV LDEPNSNLDDSGEQA LLAAIQALKARGCTV LLITHRAGVL (352-531) 180

13 PrtD      ELIILSLPNGYDTELG DGGGGLSGGQRQRIG LARAMYGDPC--LLI LDEPNASLDSEGDQA LMQAIVALQKRGATV VLITHRPALT (350-529) 180
14 ComA      EDIERMPLNYQTELT SDGAGISGGQRQRIA LARALLTDAP--VLI LDEATSSLDILTEKR IVDNLIAL---DKTL IFIAHRLTIA (503-681) 179
15 LcnC      ADIEQMQLGYQTELS SDASSLSGGQRQRIA LARALLSPAK--ILI LDEATSNLDMITEKK ILKNLLPL---DKTI IFIAHRLSVA (501-679) 179
16 PedD      TDIENLPQGYHTRLS ESGFNLSGGQKQRLS IARALLSPAQ--CFI FDESTSNLDTITEHK IVSKLLFMK--DKTI IFVAHRLNIA (505-686) 182

17 CylB      EVLLGIPQYEKTIVS ENGSNFSGGQRQKIA LARAFYSNVN--TLL LDEPTSAMDNISEFE VFSNLLDE---KRTV ITVAHRISTV (491-670) 180
18 SurB      LEKLLEDAGLNSWLG EGGRQLSGGEPGRLA IARALLHDAP--LVL LDEPTEGLDATTESQ ILELLAEM-MREKTV LMVTHRLRGL (359-536) 178
19 NisT      TNNQYVLDTQLGNWF QEGHQLSGGQWQKIA LARTFFKKAS--IYI LDEPSAALDPVAEKE IFDYFVAL-SENNIS IFISHSLNAA (371-555) 185
20 SpaB      SHSSYQFDTQLGLWF DEGRQLSGGQWQKIA LARAYFREAS--LYI LDEPSSALDPIAEKE TFDTFFSL-SKDKIG IFISHRLVAA (358-542) 185

21 BexA      ELGDYLYEPVKK--- -----YSSGMKARLA FALSLSVEFD--CYL IDEVIAVGDSRFAEK CKYELFEKR-KDRSI ILVSHSPSAM (24-184) 161
22 CtrD      ELGQYLYEPVKR--- -----YSSGMKARLA FALSLAVEFD--CYL IDEVIAVGDSRFADK CKYELFEKR-KDRSI ILVSHSHSAM (24-184) 161
23 KpsT-K1   ELGKYFDMPIKT--- -----YSSGMRSRLA FGLSMAFKFD--YYL IDEITAVGDAKFKKK CSDIFDKIR-EKSHL IMVSHSERAL (24-186) 163
24 KpsT-K5   ELGKYFDMPIKT--- -----YSSGMRSRLG FGLSMAFKFD--YYI VDEVTAVGDARFKEK CAQLFKERH-KESSF LMVSHSINSL (24-186) 163

25 DrrA      GLGDARDRLLKT--- -----YSGGMRRRLD IAASIVVTPD--LLF LDEPTTGLDPRSRNQ VWDIVRALVDAGTT- VLLTTQYLDE (27-201) 175
26 NodI      RLESKANTRVAD--- -----LSGGMKRRLT LAGALINDPQ--LLI LDEPTTGLDPHARHL IWERLRSLLARGKT- ILLTTHIMEE (31-205) 175
27 CycV      GLDHATHLPAAF--- -----LSAGQRRRLS LARLLTVRRP--IWL LDEPTTALDVAGQDM FGGLMRDHLARGGLI IAATHMALGI (20-189) 169
28 HelA      NLNALEHRAAAS--- -----LSAGKRRLG LARLLVTGRP--VWV LDEPTVSLDAASVAL FAEAVRAHLAAGGAA LMATHIDLGL (22-180) 159

29 McbF      RYDKIRRKRTYT--- -----VSYGEKRWLI ISLMVTLCKNARLFL LDEPTVGIDIQYRMM LWELINKITADGKT- VFFSTHIFDE (26-196) 171
30 CONSENSUS E?IS?LPEGY?T--G E-G-GLSGGQRQRIA IARALLNNPP--ILI LDEATSALDYESEHK IMDNLRAL--KGRTV IIIAHRLSTV
                          B site
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FIG. 3. Sequence alignment and consensus sequence of the 190-aa domain containing the ABC. The proteins listed are described in detail in Table 1 and in the text. The first 20 sequences fall into evolutionary group A, the group of ABC exporters that contain the ABC and MSDs on one polypeptide. The last nine sequences fall into evolutionary group B, which have the ABC and MSDs on separate polypeptides (see Fig. 5). The alignment was generated by using the pattern construction algorithm PIMA (pattern-induced multiple alignment) written by Smith and Smith at the Molecular Biology Computer Research Resource, Dana-Farber Cancer Institute (165, 166). All pairwise comparisons between sequences in the set were performed, and the resulting scores were clustered into families by using maximal linkage rules (165). Secondary-structure analysis was carried out by using PRSTRC (142). The alignments were refined manually by using MASE (41) to move gaps from conserved secondary-structure domains to predicted turn regions. The numbers in parentheses next to the alignments show the amino acid boundaries for the particular protein sequence. The number to the right of the parentheses on each line is the total size of the domain being compared from each system. The consensus was generated from MASE and simply shows the amino acid that occurs in each column with the highest frequency.

low level of secretion can occur through interaction with the ABC domain. Functional complementation experiments are consistent with this hypothesis.

Why Are Dedicated Exporters Required?

Proteins can be transported through an ABC transporter even though they contain any number of posttranslational modifications including fatty acylation and unusual residues such as lanthionine. Some proteins which normally use the Sec-dependent pathway can also be secreted through ABC systems (100, 122). However, more interestingly, many molecules that cannot be transported through the Sec pathway can be transported via an ABC transporter. Given the limits of the Sec pathway, the ABC pathway is a useful alternative.

In the gram-negative bacterial systems, the presence of additional factors such as the accessory factor and the outer membrane factor allow for the secretion of proteins directly

FIG. 4. Sequence similarity between 29 of the bacterial ABC export systems calculated across the 190-aa ABC domain. The sequence similarity score was determined by comparing the amino acid in each position with the consensus amino acid sequence. The degree of sequence similarity was calculated by using the similarity score rules devised by Smith and Smith (165): identical matches were given a score of 5, conservative changes were scored 1 to 3 depending on the degree of conservation, and mismatches were scored as zero. The 1-aa window is shown by the dotted line, and a 7-aa window is shown by the thick solid line. Sequences corresponding to several of the highly conserved regions are also shown above the graph.

through both membranes. This allows proteins which could not normally pass through the outer membrane to move through it. The ABC export system is very efficient in this respect and is a very simple system when compared with the general secretion pathway for pullulanase, which requires over 15 proteins to facilitate protein transport through the outer membrane (139).

One possible reason why the dedicated exporters are required is that the proteins that utilize them cannot be transported via the Sec pathway. In at least one case, this hypothesis is not correct. We have shown that the OmpA signal sequence, when fused to the mature colicin V peptide, can permit colicin V to translocate across the inner membrane by using the Sec pathway (40). Interestingly, this colicin V remains trapped in the periplasm. Thus, the dedicated colicin V exporter has a marked advantage over the Sec pathway because it facilitates export through both membranes. It would be interesting to see whether other ABC protein/peptide substrates can be secreted by the Sec pathway, especially those from gram-positive systems such as the lantibiotics, which do not have an outer membrane to cross but which do have extensive posttranslational modification.

What Does the Complex Look Like?

A basic ABC transporter structure has been proposed which includes four "core components," basically consisting of a dimer of ABCs and MSDs (Fig. 1) (80). This configuration is likely to be conserved among the bacterial ABC exporters, although the macrolide efflux systems currently are an exception since they lack MSDs. Most of the bacterial ABC exporters have their MSD and ABC in the same polypeptide. In these cases, the four core components would consist of a homodimer of each bacterial ABC exporter. There are few experimental data on the multimeric state of the bacterial ABC exporters, but the generation of antibodies and epitope tags to HlyB should allow such analysis to begin. (See Addendum in Proof.)

The presence of additional accessory factors is unique to bacterial ABC exporters. Accessory factors are present when secretion occurs through both membranes. In the absence of an accessory factor, some ABC transporters are still able to facilitate export into the periplasm. This raises an interesting question: is the accessory factor an integral part of the export complex? If the accessory factor is a peripheral component, the four core domains of the ABC exporter should be sufficient to facilitate translocation across the inner membrane (as in the NdvA system) even in the absence of an accessory factor and outer membrane factor. Thus, deletions of the accessory factor from other systems should result in periplasmic accumulation. Instead, deletion of the accessory factor in several systems results in cyto-
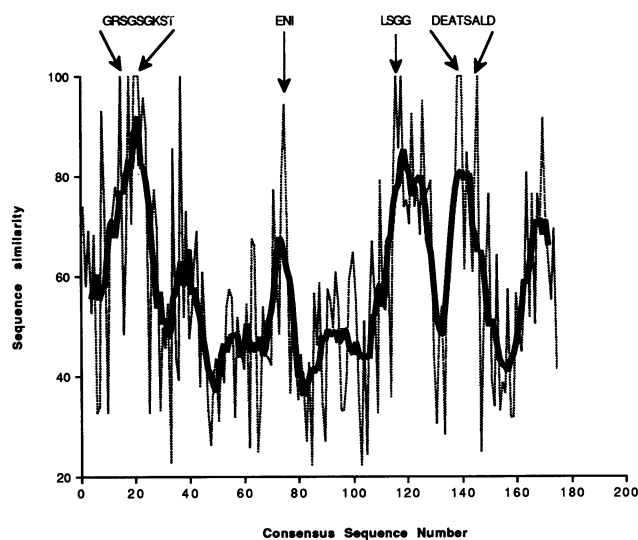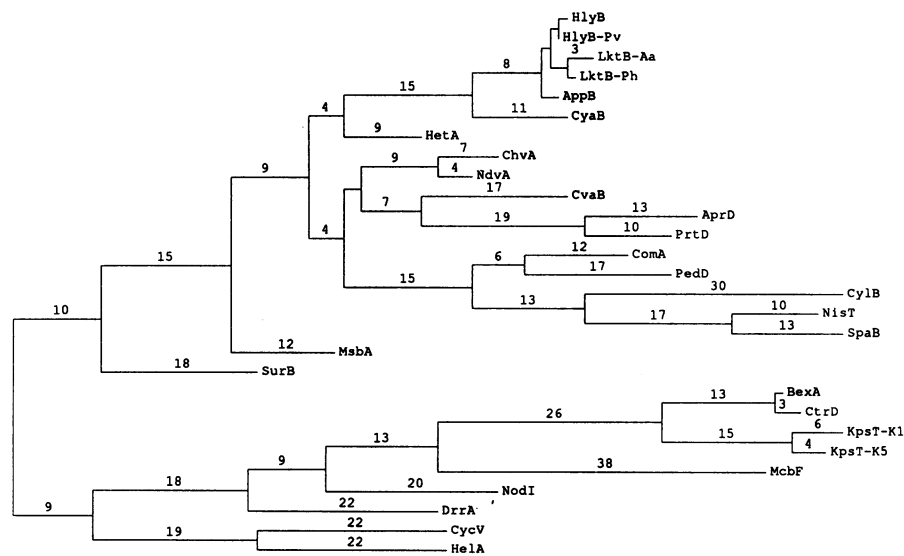
FIG. 5. Unrooted phylogenetic tree showing the evolutionary relatedness of the 190-aa domain containing the ABC. The computer program PAUP (Phylogenetic Analysis Using Parsimony) written by D. L. Swofford, Illinois Survey of Natural History, was used to generate the phylogenetic tree. The PAUP program uses DNA as well as protein sequence information when generating an evolutionary tree. For this analysis, DNA from the second position of each codon was used for the analysis, which is appropriate when comparing a diverse family of sequences. The total length of the tree is 607 units, and the consistency index for the tree is 0.427. The horizontal branch lengths indicate relative evolutionary distance.

plasmic accumulation of substrate (40, 65). These results suggest that there are several functionally distinct classes of bacterial ABC exporters. One class evolved in the presence of accessory factors and outer membrane factors and became dependent on all three components to form a functional export complex through both membranes of a gram-negative cell. The second class evolved to export molecules through a single membrane and can function independently of additional factors. To date, though, no physical interactions have been demonstrated between the ABC and the accessory factors, and the exact location of the accessory factors in the export complex has not been determined.

How Does the Export Complex Interact with Substrate?

The most detailed analyses of the interactions between substrate and exporter have been carried out on the bacterial permeases and eukaryotic exporters (3, 80). Specific interactions between bacterial ABC exporters and their substrates have not been well characterized, but a few ideas have been proposed. The secretion signal is located at the C terminus of several transported ABC protein substrates; therefore in these systems, secretion must be posttranslational. The large size of the proteins makes it likely that they pass through the ABC exporter in an unfolded state, but the chaperones GroEL, GroES, and SecB are not involved in HlyA or PrtB secretion (53, 184). There is, as of yet, no experimental evidence of unfolding, but if it does occur, it could be mediated either by host-encoded chaperones or by the secretion proteins themselves.

Newly synthesized HlyA can be found in the inner membrane even in the absence of the HlyBD exporters. This raises the possibility that interaction of HlyA with the exporters occurs within the plane of the membrane. Export of glucans and capsular polysaccharides also appears to occur after these oligomers are synthesized at inner membrane biosynthetic complexes. This type of membrane inter-

action is similar to the "flippase" model suggested for the P-glycoprotein, which recognizes and transports lipophilic drugs. It was suggested that P-glycoprotein interacts with drugs that are in the plane of the inner membrane and functions to flip them from the inner face of the lipid bilayer to the outer face (81). Localization of the transported substrate to the inner membrane could restrict the movement of these molecules and increase the likelihood of substrate-transporter interaction. Under these conditions, substrate specificity could be more relaxed, because only few types of molecules would be properly localized to interact with the export complex in the proper orientation.

CONCLUSIONS

The bacterial exporter subfamily of ABC transporters is a large and diverse family of proteins sharing a conserved ATP-binding domain and a common export function. In the past few years, the number of identified bacterial systems has greatly increased. Many proteins secreted by bacterial ABC exporters are important virulence factors or have important industrial applications. Other putative exporters have been found to be essential for bacterial growth and development and should provide insight into our understanding of basic cellular physiology. Each bacterial ABC exporter that is discovered demonstrates the diversity and the importance of this superfamily. Much has been learned about these exporters, and our knowledge about these systems should significantly contribute to our overall understanding of the superfamily of ABC transporters.

THE SURVEY

Proteus vulgaris and *Morganella morganii* Hemolysins

The hemolysins from *Proteus vulgaris* and *Morganella morganii* have the same genetic organization and functional

characteristics as the *E. coli* hemolysin system (105). Complementation experiments show that export proteins HlyB and HlyD are completely exchangeable between these two systems and the *E. coli* hemolysin system. The *M. morganii* hemolysin was also shown to be biochemically and immunologically related to the *E. coli* hemolysin (33).

The *hlyB* gene has been sequenced only from *Proteus vulgaris* (107). *P. vulgaris* HlyB is 707 aa and is highly homologous to the *E. coli* HlyB. In initial functional studies, several mutations were constructed in the region adjacent to the B site of *P. vulgaris* HlyB. Two conservative changes, G605A and K625R, had little effect on extracellular hemolysin levels, but the mutations G608R and K625I greatly reduced hemolysin activity. Biochemical analysis will be necessary to determine the effect of these mutations on *P. vulgaris* HlyB function.

Actinobacillus Hemolysins and Leukotoxins

At least three *Actinobacillus* species produce extracellular hemolysins or leukotoxins in the RTX family. *Actinobacillus pleuropneumoniae* and *A. suis* each secrete a 104-kDa RTX hemolysin (AppA and AshA, respectively) thought to be virulence factors in porcine pleuropneumonia, and *A. actinomycetemcomitans* secretes a closely related 125-kDa leukotoxin implicated in juvenile periodontitis. The *A. pleuropneumoniae* hemolysin was first studied immunologically, and antibodies raised against *A. pleuropneumoniae* hemolysin serotype 1 strain CM-5 were shown to cross-react with supernatants from other *A. pleuropneumoniae* serotypes, *Pasteurella haemolytica*, *A. suis*, and alpha-hemolysin-producing *E. coli* (26).

The *E. coli* HlyC, HlyB, and HlyD proteins were shown to be capable of secreting active *A. pleuropneumoniae* hemolysin from *E. coli* (72). More recently, the genes encoding one of the complete *A. pleuropneumoniae* hemolysin operons were cloned into *E. coli* and sequenced. One operon contains the *appCA* genes with a downstream *appB* pseudogene (16). An unlinked operon contains the *appBD* genes found with an *appA* pseudogene upstream. AppBD, HlyBD, and *P. haemolytica* LktBD all function in *E. coli* to secrete the AppA hemolysin (17). Analysis of operons from several different *A. pleuropneumoniae* serotypes suggest that there are, in fact, several distinct hemolysin determinants in each of the serotypes including AppA (17), HlyIIA (47, 72), and ApxIIIA (94a). The operons that contain these determinants appear to have undergone rearrangement by deletion to yield noticeable genetic variation (46, 94a). In *A. suis*, the hemolysin determinant has also been sequenced and found to be in a split configuration, where the *ashCA* and *ashBD* genes are unlinked and flanked by pseudogenes (12).

The *A. actinomycetemcomitans* leukotoxin production genes (*lktCA*) have sequence similarity to *hlyCA*. *A. actinomycetemcomitans* LktC is 168 aa. The 1,055-aa *A. actinomycetemcomitans* LktA protein toxin appears to remain associated with the outer membrane, in contrast to the extracellular secretion of hemolysin (109, 115). This membrane localization could be due to a C-terminal hydrophobic domain unique to the *A. actinomycetemcomitans* LktA. The gene encoding the *A. actinomycetemcomitans* leukotoxin ABC transporter is *lktB* (68). A 200-bp region of the gene encoding LktD has been sequenced and predicts a partial protein containing 72% identities with HlyD (109). LktB and LktD have not yet been shown to be required for secretion, and no other secretion functions have been identified.

Pasteurella haemolytica Leukotoxin

Pasteurella haemolytica serotype A1 is a virulence factor involved in a bovine respiratory pneumonia known as shipping fever. *P. haemolytica* secretes a ruminant-specific leukotoxin that migrates as a 102-kDa protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The genes encoding the leukotoxin system have been cloned and sequenced (84, 171). The operon is identical in form with the *E. coli* operon. LktC is 167 aa, LktA is 983 aa, LktB is 708 aa, and LktD is 478 aa. The Hly genes are highly homologous to the Lkt genes, with amino acid sequence similarities ranging from 50 to 90%. LktBD and HlyBD are also functionally conserved—HlyBD and LktBD are interchangeable and each set functions to secrete either toxin (15, 84, 171).

Bordetella pertussis Cyclolysin

Bordetella pertussis is one of the few prokaryotic organisms to secrete an adenylate cyclase, called cyclolysin. The *B. pertussis* cyclolysin is a key virulence factor and has multiple activities, including adenylate cyclase, invasin, and hemolysin. These properties are included on the single large polypeptide, CyaA (63). CyaA is produced as a 216-kDa protein which is secreted into the extracellular medium (79). Several groups have reported lower-molecular-mass species of cyclolysin (114, 126), but these appear to be proteolytic by-products. Genetic and functional analyses demonstrate that the N-terminal 450 aa have the adenylate cyclase and invasiveness activities while the C-terminal 1,250 aa have the hemolytic activity (63).

A total of five linked genes have been found to be required for cyclolysin production, activation, and secretion. *cyaA* encodes the 1,706-aa cyclolysin, and *cyaC* encodes the 185-aa protein required to activate the hemolytic moiety of CyaA (5). Three genes encode the secretion functions: *cyaB* encodes the 712-aa ABC transporter, *cyaD* encodes the 440-aa accessory factor, and *cyaE* encodes the 474-aa outer membrane factor (63). CyaE was the first outer membrane component shown to be associated with an ABC transport complex. All three genes have been shown to be required for secretion of the cyclolysin (63).

The HlyBD secretion system is capable of secreting CyaA when cloned into *E. coli* (125, 159). To facilitate analysis of cyclolysin, the complete secretion system has been reconstructed in *E. coli* (158). Surprisingly, the *cyaBDE* genes are synthesized in *E. coli* but are unable to promote CyaA secretion (158). In *E. coli*, CyaA secretion is facilitated by the presence of the HlyBD/TolC system, which recognizes a secretion signal at the C-terminal 217 aa of CyaA. When larger fragments of CyaA are fused to the C-terminal secretion signal, the efficiency of CyaA secretion decreases (159). Several groups studying heterologous secretion have observed that the size of the secreted product affects the efficiency of heterologous secretion (39, 119, 159).

Pseudomonas aeruginosa Alkaline Protease

P. aeruginosa is an opportunistic pathogen whose virulence is related to the secretion of several proteins, including an alkaline protease (90). The genes in the alkaline protease operon could function to secrete active protease from *E. coli*, but only when put under the control of the *tac* promoter (70). No processing of protease was observed, and protease secretion was found to be SecA independent (71).

The alkaline protease operon includes five genes. *aprA* encodes the 479-aa alkaline protease, and *aprI* encodes a 130-aa protease inhibitor. Secretion requires three other genes: *aprD* encodes the 593-aa ABC exporter, *aprE* encodes the 432-aa accessory factor, and *aprF* encodes the 481-aa outer membrane factor (31). Mutations in the AprDEF region prevent protease secretion in *E. coli*. The alkaline protease could be secreted from *E. coli* by PrtDEF with high efficiency and by HlyBD/TolC at low efficiency, consistent with their degree of sequence similarity (31, 69). This system has been recently reviewed (176).

Serratia marcescens Zinc Metalloprotease

S. marcescens produces an abundant extracellular metalloprotease. The gene encoding the zinc metalloprotease (*prtSM*) was cloned, sequenced, and found to encode a protein with 470 aa and a predicted molecular mass of 50.6 kDa. PrtSM lacks a signal peptide but possesses a 17-aa N-terminal propeptide which is cleaved off during processing. This protease was found to have significant sequence similarity to the *Erwinia chrysanthemi* and *P. aeruginosa* proteases (131). When expressed alone in *E. coli*, the protease is not secreted. However, significant amounts of extracellular protease were observed when the PrtSM protein was produced in the presence of either *Erwinia chrysanthemi* PrtDEF (118) or *E. coli* HlyBD/TolC (172). Additional experiments localized the secretion signal recognized by PrtDEF to the C terminus of PrtSM (118). To date, no PrtSM secretion genes have been identified from *S. marcescens*, but it is likely that an ABC-type exporter will be found associated with PrtSM secretion.

Rhizobium leguminosarum NodO

The nodulation region of the *R. leguminosarum* genome contains a gene called *nodO*, which encodes a 284-aa secreted calcium-binding protein (34). NodO does not have a signal sequence and is not processed at its N terminus (25a). The sequence is slightly similar to HlyA, CyaA, and LktA in the RTX glycine repeat region (25a, 34). NodO secretion from *E. coli* could be facilitated by either HlyBD/TolC or PrtDEF. All three secretion genes must be present for NodO secretion through either heterologous system, providing strong evidence that an ABC exporter is required for NodO secretion (152). There are two genes linked to *nodO* in the *R. leguminosarum* chromosome, *nodI* and *nodJ*, which encode a multicomponent ABC transport system (see below). However, surprisingly, *nodI* mutants still produce secreted NodO (34). The *ndvA* gene, also found in *R. leguminosarum*, is an unlinked ABC transporter involved in glucan export (see above). Mutants with mutations in *ndvA* were also able to secrete NodO (152). Thus, another, as yet unidentified, *R. leguminosarum* gene(s) must be involved in the secretion of NodO.

Neisseria meningitidis FrpA and FrpC

When starved for iron, *N. meningitidis* produces a variety of outer membrane proteins (174). One of these proteins, FrpA, has been cloned and sequenced and found be a 1,115-aa protein with sequence similarity to HlyA and other RTX toxins (174). A monoclonal antibody to FrpA cross-reacts with HlyA and CyaA. A second outer membrane protein, FrpC, has been identified by using cross-reacting antibody that is also related to the RTX toxins. No trans-porters have been identified for FrpA or FrpC, but their sequence and outer membrane localization suggest that an ABC transporter may play a role in their secretion (174).

Enterococcus faecalis Bacteriocin/Hemolysin

Sixty percent of clinical *Enterococcus* strains have a cytolytic toxin which lyses human, rabbit, and horse erythrocytes. This toxin is also bactericidal against a broad range of gram-positive bacteria (93). Toxin activity requires two components. The L component is an 11-kDa precursor of the lytic toxin which is activated extracellularly by the 27-kDa A (activator) component (64). The genes encoding these components are localized to the large transmissible plasmid, pAD1. Mutagenesis of pAD1 identified a 7.0-kb region required for bacteriocin/hemolysin production (93). A fragment from the L region was sequenced and found to encode a 714-aa, HlyB-like protein called CylB. Mutations in *cylB* prevent secretion of component L but, interestingly, do not affect secretion of component A. CylB is expressed in *E. coli* and can complement a *cylB* mutation in *trans*, but it fails to complement an *hlyB* mutation (60). The nucleotide sequences of components A and L, and other associated factors have not yet been determined. The sequence adjacent to *cylB* is likely to encode additional functions necessary for hemolysin/bacteriocin maturation or secretion. CylB was the first described ABC exporter identified in gram-positive bacteria.

Bacillus subtilis Subtilin

Subtilin is one of a number of lantibiotics produced and secreted by gram-positive bacteria. Mature subtilin is a 32-aa peptide antibiotic with many unusual amino acids including lanthionine and dehydroalanine (95). The subtilin operon was cloned from *B. subtilis* ATCC 6633 and sequenced by two groups, who report somewhat different results (18, 103). *spaS* encodes the 56-aa subtilin primary product, which is posttranslationally modified and processed to the mature 32-aa form. Adjacent to *spaS* is *spaC*, which encodes a 441-aa product that is probably involved in subtilin processing.

Two genes also linked to *spaS* probably have secretion-related functions: *spaB* (called *spaT* by Klein et al. [103]) and *spaD* (called *spaB* by Klein et al. [103]). SpaB is an ABC exporter predicted to have 599 or 614 aa depending on which ATG is used. Both groups have disrupted SpaB by cassette mutagenesis, with conflicting results. Chung et al. found that *spaB* insertions prevent external subtilin activity, whereas Klein et al. observed unusual clumping and slow-growth phenotypes but still observed external subtilin activity. Klein et al. suggest that their mutant is unable to secrete subtilin, leading to an internal accumulation which may be responsible for the observed effects. Neither experiment demonstrated a specific secretion function for SpaB.

The two published sequences for SpaD have significant inconsistencies. Both groups identify an identical 177-aa open reading frame (ORF), but the remaining details differ. The SpaD sequence of Chung et al. predicts a 177-aa protein with some sequence similarity to HlyD; the sequence of Klein et al. identifies the 177-aa ORF as part of a 390-aa protein that is homologous to the C terminus of EpiB, a 990-aa epidermin biosynthetic protein. Klein et al. show that mutants with mutations in SpaD (called SpaB in their paper) do not secrete subtilin, but no specific secretion function is

demonstrated. At this point, it is still unknown whether SpaD function is related to secretion or biosynthesis.

Lactococcus lactis Nisin

Nisin is a commercially important lantibiotic secreted by certain strains of *Lactococcus lactis* and active against a wide range of gram-positive bacteria. It is widely used as a food preservative. The nisin peptide (NisA) is ribosomally synthesized as a 57-aa propeptide (97) and processed and modified into a 32-aa mature form (95). The three-dimensional structure of nisin has been determined by nuclear magnetic resonance spectroscopy (180a). The nisin operon has been sequenced, and three adjacent genes have been found: *nisB*, *nisT*, and *nisC* (36). *nisT* encodes a 600-aa ABC export protein. Expression of *nisT* in *E. coli* is toxic, and no secretion function has yet been identified for *nisT*. *nisB* encodes a 993-aa membrane-bound protein, and *nisC* encodes a 418-aa protein. Both have homologues in the subtilin and epidermin systems and probably encode biosynthetic enzymes. No other export-related genes were found in the nisin operon.

Staphylococcus epidermidis Epidermin

Epidermin is another lantibiotic active against many gram-positive bacteria (95). Epidermin is synthesized as a 52-aa propeptide and processed to the tetracyclic 21-aa mature form (155). The epidermin genes are located on a 54-kb plasmid, and at least six genes are involved in epidermin synthesis (155). *epiA* encodes the 52-aa propeptide. Downstream of *epiA* are *epiB*, *epiC*, *epiD*, *epiP*, and *epiQ*, which all appear to have a role in epidermin biosynthesis (4, 153).

Two intriguing open reading frames are found in the opposite orientation upstream of *epiA*. One, called *epiT*, could encode a maximum of 148 aa and has a good Shine-Dalgarno sequence but no identifiable start codon. With a −1 frame shift, a further reading frame of 275 aa follows and continues past the end of the cloned fragment. The second ORF, called *epiT'*, has the consensus A site, but the sequence ends before the beginning of any identifiable B site. It is possible that these genes compose a two-part ABC exporter or that they form a single ABC exporter through a shift in reading frame. Surprisingly, the 13.5-kb region cloned from this plasmid is sufficient to yield extracellular epidermin when expressed in the nonproducing organism *Staphylococcus carnosus* (153), even though *epiT'* does not contain a complete ABC domain. Much work is necessary to elucidate the mechanism of epidermin secretion, but it appears that an ABC exporter may be involved.

Staphylococcus gallidermidis Gallidermin

Gallidermin is a lantibiotic produced by *S. gallidermidis* and is nearly identical in structure to epidermin (154). The *gdmA* gene encodes a 52-aa propeptide which is processed to a 21-aa mature form. Gallidermin differs from epidermin by only one amino acid: the N-terminal Ile in epidermin is a Leu in gallidermin (154). A gallidermin operon structure has been reported which matches that of epidermin (103). No DNA sequences have been published, but there is a clear possibility that gallidermin secretion is also mediated by an ABC exporter.

Streptococcus pneumoniae Competence Factor

Competence factor is a 10-kDa secreted protein required to facilitate genetic transformation in *Streptococcus pneumoniae* (175). The *com* locus was cloned (14), sequenced (92), and found to contain several genes required for transformation. One of these genes, *comA*, encodes a 717-aa ABC export protein with a predicted molecular mass of 80.3 kDa (92). Mutations in *comA* and the adjacent *comB* gene have a severe competence deficiency (14, 129). *comB* encodes a 49-kDa protein that has no significant similarities to known proteins (129). ComA and ComB are both hypothesized to be part of a dedicated export complex (129). If this is indeed the case, it would be the first example of a required accessory factor in a gram-positive secretion system. The gene(s) encoding the competence factor has not yet been identified and appears to be unlinked to the *comAB* locus.

Pediococcus acidilactici Pediocin PA-1

Pediocin PA-1 is a small, heat-stable bacteriocin produced from plasmid pSRQ11 in *Pediococcus acidilactici* PAC1.0 and is active against many gram-positive bacteria (76). The protein has been purified and sequenced and found to be a 44-aa, 4,629-Da peptide with sequence similarity to bacteriocins from other gram-positive bacteria (120). The pediocin production operon was cloned and sequenced (124). Four ORFs were found: *pedA*, *pedB*, *pedC*, and *pedD*. PedA is the 62-aa precursor of pediocin A; PedB and PedC are 112 and 174 aa, respectively, and have no known function. PedD is a 724-aa ABC exporter. The *pedD* gene was mutated and shown to be required for pediocin production.

Lactococcus lactis Lactococcins A and G

The lactococcin A system was cloned and characterized from several strains of *Lactococcus lactis* (89, 170, 179, 180). Lactococcin A is synthesized as a 75-aa precursor with a 21-aa N-terminal extension (89). The complete lactococcin A operon contains four genes: *lcnA* encodes the 75-aa precursor, and *lciA* encodes a 98-aa immunity protein (89), whereas *lcnC* and *lcnD* encode a putative export system (170). LcnC is a 716-aa ABC transport protein with a predicted molecular mass of 79.9 kDa. LcnD is a 474-aa protein (52.5 kDa) with slight sequence similarity to the HlyD and PrtE accessory factors. Both LcnC and LcnD have been inactivated by transposon mutagenesis and found to be essential for lactococcin A activity (170). Another newly discovered bacteriocin, lactococcin G from *L. lactis*, also requires additional genes for its synthesis. These genes have been cloned and sequenced. One encodes a putative immunity protein and the other encodes an ABC exporter (74).

Escherichia coli Microcin B17

Microcin B17 is a small, highly modified 43-aa peptide antibiotic that inhibits DNA replication (21). The proteins McbE and McbF act to transport microcin B17 into the periplasm, where it associates with the outer membrane and passes through into the extracellular medium. McbEF also play a role in conferring immunity to the producing cell by secreting microcin B17 out away from its cytoplasmic target, DNA gyrase (51). The *mcbEF* genes were cloned and sequenced from the microcin-producing plasmid pMccB17. McbE is a 241-aa hydrophobic protein with six potential transmembrane domains and a predicted molecular mass of

27.9 kDa. McbF is a 28.9-kDa, 247-aa protein that contains the consensus A and B sites common to ABC transporters. Insertion mutations in *mcbE* and *mcbF* result in a complete loss of extracellular microcin B17, but active microcin can be detected in cytoplasmic extracts (51). McbEF is the first multicomponent ABC transporter to be identified that is clearly involved in export (51).

Streptomyces peucetius DrrAB

The gram-positive bacterium *S. peucetius* produces the antitumor agents daunorubicin and doxorubicin. Several genes involved in the biosynthesis of daunorubicin and doxorubicin have been identified by being cloned into the nonproducing strain *S. lividans* (136). Genes that are involved in daunorubicin and doxorubicin resistance have been sequenced and found to include a multicomponent ABC transport system (67). The genes, *drrAB*, encode two proteins: DrrA, an ABC protein with 330 aa and a predicted molecular mass of 35.7 kDa; and DrrB, a very hydrophobic protein with 283 aa and a predicted molecular mass of 30.6 kDa. When the *drrAB* operon is cloned into *S. lividans*, the genes confer Dnr and Dxr resistance to the heterologous host. *drrAB* are cotranscribed and are expressed only during antibiotic production (67). The authors suggest that DrrA may energize transport of antibiotics by ATP hydrolysis through a membrane pore made by DrrB. The discovery of bacterial ABC exporters that facilitate export of Dnr and Dxr is particularly exciting because these are two of the antibiotics that are exported by the mammalian P-glycoprotein encoded by the *mdr* gene (35).

Haemophilus influenzae BexAB

A polymer of ribose and ribitol-5-phosphate makes up the capsule of type b strains of *H. influenzae* (20). The locus involved in capsule formation is organized as a directly repeated duplication separated by a 1.3-kb "bridge" region (85). Recombination that removes this bridge results in loss of capsule, suggesting that an essential promoter or gene is present in the region (110). The bridge region was sequenced and found to contain the gene *bexA*, which encodes a 24.7-kDa, 217-aa ABC transporter (110). A frameshift mutation in *bexA* results in accumulation of internal polysaccharide (110).

The region around *bexA* was then sequenced and three additional ORFs were identified: *bexB*, *bexC*, and *bexD* (111). BexB is 30.2-kDa, 265-aa hydrophobic protein homologous to KpsM. Although the organization of the *bex* locus is distinct from that of the *kps* locus, they each contain genes sufficient for production and export of capsular polysaccharide. The BexAB and KpsTM exporters seem to be structurally and functionally similar.

Neisseria meningitidis CtrCD

The capsular polysaccharides produced by *N. meningitidis* are also major virulence factors. The group B *N. meningitidis* capsule is composed of α-2,8-linked polysialic acid and is related to the *E. coli* and *H. influenzae* capsules described above. A 24-kb plasmid sufficient to produce capsule in *E. coli* K-12 was characterized, and five phenotypically distinct regions were identified (50). Mutations in region A abolish all polysaccharide synthesis; those in region B produce mutants that contain only cytoplasmic polysaccharide; those in region C produce mutants that contain

polysaccharide in the cytoplasm and periplasm; and those in regions D and F affect levels of polysaccharide (48).

Region C, which seems to be required for secretion of polysaccharide from the periplasm to the cell surface, was sequenced (49), and four genes, *ctrABCD*, were found. CtrC is a 264-aa, 30.1-kDa inner membrane protein homologous to BexB, and CtrD is a 215-aa, 24.6-kDa cytoplasmic ABC transporter homologous to BexA. Together, CtrCD could form an ABC transporter. These results are somewhat unexpected because the *ctrABCD* cluster is located in region C, which is implicated in outer membrane transport, while the homologous *bex* locus from *H. influenzae* is implicated in inner membrane transport. In a recent review, the authors propose that CtrBCD form an inner membrane ABC transport complex that associates with the outer membrane protein CtrA. This complex would recognize membrane-associated polysaccharide and facilitate its transfer to the outer membrane (48). In this scenario, CtrB and CtrC are heterodimeric subunits of the transport complex. However, the hydropathy analysis of CtrB is not consistent with that of CtrC, and CtrB may not have the transport role as proposed.

Bradyrhizobium japonicum CycVW

Bacterial *c*-type cytochromes consist of an apoprotein covalently attached to a 600-Da protoheme IX cofactor. Cytochrome *c* is found free in the periplasm. Pleiotropic Tn5 insertion mutations were identified in *Bradyrhizobium japonicum* that gave a negative reaction in the cytochrome *c* oxidase colony assay (143). These mutants were cloned and sequenced, and several ORFs were found. Three of these mutants no longer made *c*-type cytochromes and were designated *cycVWX*. CycV is a 200-aa, 21.1-kDa ABC transporter, and CycW is a 222-aa, 22.7-kDa protein predicted to be in the inner membrane. Mutations in the small (61-aa) protein CycX also abolish cytochrome activity. An additional ORF, ORF263, was predicted to encode a 263-aa inner membrane protein, but mutations in ORF263 did not abolish cytochrome activity (143). The authors speculate that CycVW may form a complex that transports out an essential component of cytochrome *c*. Other experiments suggest that the apoprotein is exported by a signal-sequence-dependent method and the heme group is attached to the apoprotein in the periplasm (137). That suggests that CycVW may export the cytochrome *c*-heme lyase or possibly the heme molecule itself.

Rhodobacter capsulatus HelABC

A region homologous to the *B. japonicum cyc* region was cloned and sequenced from the photosynthetic bacterium *Rhodobacter capsulatus* and subjected to insertion mutagenesis (7). Four genes were characterized and found to be required for cytochrome *c* biogenesis. HelA (the CycV homolog) is a hydrophilic 214-aa ABC transporter; HelB is a hydrophobic 218-aa protein homologous to CycW; HelC is a hydrophobic 242-aa protein homologous to ORF263; and ORF52 is a small (52-aa) protein homologous to CycX.

The observation that HelC is required for cytochrome biosynthesis in *R. capsulatus* contrasts with the *B. japonicum* result which suggests that ORF263 is not an essential gene. The small ORF52 and CycX proteins have no counterparts in other bacterial ABC systems. Their role in the translocation process and in cytochrome *c* production is not yet clear. Cytochrome *c*-PhoA fusions show that the apocytochrome is secreted to the periplasm in Hel⁻ mutants. The

authors suggest that the *hel* genes are not involved in import of iron or in export of holo- or apocytochrome *c*. Instead, they favor the model that the Hel complex exports the heme group to the periplasm where it is then attached to the apocytochrome.

Anabaena Sp. Strain PCC 7120 HepA

The cyanobacterium *Anabaena* sp. produces nitrogen-fixing heterocysts when starved for nitrogen under aerobic conditions (73). A specialized external polysaccharide layer is formed early in heterocyst development. A mutant that reduces the cohesiveness of the polysaccharide layer was identified and sequenced (86). The mutated gene was originally called *hetA* (86) but was recently renamed *hepA* to reflect the fact that it affects heterocyst envelope polysaccharide (36a). *hepA* encodes a protein with a maximum size of 607 aa, but some evidence suggests that a shorter (532-aa) protein might be produced instead. The HepA protein is similar to other members of the ABC exporter family and is hypothesized to be involved in export of the heterocyst envelope polysaccharide.

Escherichia coli SurB

The *surB* gene was identified by looking for genes required for *E. coli* survival during the stationary phase (161, 177). Mutations in one gene, *surB*, resulted in cells unable to exit from the stationary phase and resume aerobic growth at high temperature (162). The *surB* gene was cloned, mapped to 19.5 min on the *E. coli* chromosome, and sequenced. The sequence indicates that *surB* encodes a putative 573-aa ABC transporter with both an ABC and potential MSDs. The gene directly upstream of *surB* (called *orfU*) was also sequenced and found to contain a 243-aa ABC. The genetic organization of *orfU* and *surB* is particularly interesting. *orfU* ends with the sequence TAA, and the ATG of *surB* starts immediately afterward in the same frame. There is an intriguing possibility that OrfU and SurB form as a single polypeptide at some frequency as a result of readthrough. Insertion mutagenesis has demonstrated that *surB* is not essential for normal *E. coli* growth (162). *surB* or *orfU* may be allelic with *cydC*, which is involved in heme-*d* production or secretion. Consistent with this, a *surB* mutant does lack a functional cytochrome *d* oxidase (162). Therefore one *surB* function may be to aid in assembly of heme-*d* into the cytochrome *d* oxidase complex.

Escherichia coli MsbA

The *htrB* gene is required for growth of *E. coli* at temperatures above 33°C. Genes were identified that suppress a temperature-sensitive *htrB* mutant when present on multicopy plasmids (98, 99). One of the suppressor genes is *msbA*, which maps to 20.5 min on the *E. coli* chromosome and encodes a 582-aa protein with a predicted molecular mass of 64.5 kDa (98). The MsbA protein shares significant sequence similarities to the bacterial ABC exporters. Insertion mutations in *msbA* were found to be lethal, demonstrating that *msbA* is an essential gene in *E. coli*. This is the first example of an essential ABC exporter. The *msbA* gene is cotranscribed with another essential gene called *orfE*, which could encode a 328-aa, 35.6-kDa protein. The authors suggest that MsbA and OrfE could function together to increase the export of polyamines, which restore growth of *htrB* mutants at high temperatures, or that they could export a toxic

molecule that accumulates at nonpermissive temperatures in the absence of HtrB (98).

Escherichia coli FtsE

An operon involved in cell division was found at 76 min on the *E. coli* chromosome and contains three genes, including one encoding the putative ABC transporter, *ftsE* (57). *ftsE* is the second gene in a three-gene operon and encodes a 222-aa, 24.4-kDa protein. The genes in this operon are, in order, *ftsY-ftsE-ftsX* (57). FtsY is a 497-aa protein which runs as a 92-kDa protein on SDS-PAGE and has sequence similarity to the SRα protein of eukaryotes involved in protein secretion (59). FtsX is a hydrophobic 352-aa, 38.5-kDa protein. All three proteins are associated with the inner membrane (58). Conditional-lethal mutations were isolated in FtsE and found to cluster in the ABC region, between the A site and B site and near to important cystic fibrosis transmembrane regulator mutations (56). No specific transport function has been identified yet, but these studies suggest that FtsYEX may form a complex that transports septation-specific proteins to the periplasm (56).

Several genes that encode putative ABC transporters have now been identified on the chromosome of *E. coli*, including *msbA*, ORF, *surB*, and *ftsE*. It is important to remember that these genes do exist in the *E. coli* chromosome and that they may be able to complement other plasmid-borne ABC exporters at low levels. So far, though, none of these genes appear to complement mutations in ABC exporters such as HlyB, PrtD, or CvaB when these systems are expressed in *E. coli*, because mutations in these ABC transporters are not complemented when expressed in cells containing wild-type MsbA, SurB, OrfU, and FtsE.

Streptomyces griseus AmfA and AmfB

Genes involved in aerial mycelium formation in *Streptomyces griseus* were identified by looking for genes which can suppress an A-factor mutation when expressed at high copy number (178a). Among the genes found were two that encode putative ABC translocators, AmfA and AmfB. The authors hypothesize that AmfA and AmfB are involved in the transport of proteins and peptides that serve as intercellular signals during aerial mycelium formation.

Rhizobium leguminosarum and *Bradyrhizobium japonicum* NodIJ

NodIJ were identified on the symbiotic plasmid pRL1J1 of *R. leguminosarum* and shown to be involved in nodulation (37). *nodI* encodes a 311-aa ABC protein with a predicted molecular mass of 34.3 kDa. *nodJ* encodes a hydrophobic 259-aa protein with a predicted molecular mass of 27.7 kDa. There is no direct evidence that NodIJ has any transport role, and it is known that NodIJ does not function to secrete the hemolysin-like NodO protein (34). It was postulated that NodIJ may form a permease that imports a compound exuded from plant roots and taken up by *Rhizobium* cells during nodulation (37). Recently the NodIJ locus from *B. japonicum* was also cloned and sequenced and found to be highly homologous to *R. leguminosarum* NodIJ and the polysaccharide exporters (181). The authors of this report hypothesize that NodIJ may actually be involved in export of lipopolysaccharides which are produced by other *nod* genes (181), but the role of NodIJ as an importer or exporter has not yet been determined.

Rhizobium meliloti ORF1

A gene has been identified upstream of the transcriptional regulator *ntrA* in *R. meliloti* that is a member of the ABC transporter family (1). The gene, called ORF1, encodes a 230-aa protein and is part of a larger operon that contains additional unidentified upstream genes. Genes similar to ORF1 have been found upstream of *ntrA* in *Salmonella typhimurium* and *Klebsiella pneumoniae*. No function has been identified for these genes, although there is evidence that ORF1 is an essential gene in *R. meliloti* and is unrelated to *ntrA* function. No transport function has been identified for ORF1 yet, but the sequence suggests that it may encode an ABC transporter.

Staphylococcus epidermidis MsrA

A 1.9-kb DNA fragment containing novel erythromycin resistance genes from *S. epidermidis* was introduced into a sensitive *S. aureus* strain and shown to be functional (147). The fragment contained a gene, *msrA*, which encodes a 488-aa protein. MsrA contains two ATP-binding domains separated by a long Q-linker. Q-linkers make up a family of interdomain sequences found in many prokaryotic multidomain proteins but not yet seen in ABC transport proteins (192). MsrA alone, when subcloned into *S. aureus*, is sufficient to promote efflux of [^{14}C]erythromycin. This function is not unlike the immunity function observed by the microcin B17 exporters McbEF (51). If any hydrophobic proteins are required by MsrA to facilitate erythromycin resistance, they must also be present in *S. aureus*.

Streptomyces TlrC, SrmB, and CarA

The tylosin-producing strain *S. fradiae* contains at least three genes specifying tylosin resistance. One of these genes, *tlrC*, was cloned and sequenced and found to encode a 548-aa, 59.1-kDa protein that has the ABC-Q-ABC structure seen in MsrA (149). Additional macrolide resistance genes have been found in related *Streptomyces* strains: *srmB* from *S. ambofaciens* and *carA* from *S. thermotolerans* (156). The proteins TlrC, SrmB, and CarA have 66 to 76% sequence identity with each other. These macrolide resistance proteins share sequences common to other ABC transporters but have an unusual domain configuration and lack any identifiable hydrophobic components. Additional work is necessary to determine the precise functional mechanism in these systems and their precise place in the family of bacterial ABC exporters.

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ADDENDUM IN PROOF

Since the submission of this paper, at least five new bacterial ABC export systems have been identified or partially described. These include a third RTX toxin from *A.*
actinomycetemcomitans (Y.-F. Chang et al., DNA Cell Biol. 12:351–362, 1993), a leukotoxin system from a *P. haemolytica*-like organism (Y.-F. Chang et al., Infect. Immun. 61: 2089–2095, 1993), an ABC exporter in *Aeromonas salmonicida* associated with a secreted surface array protein (S. Chu and T. J. Trust, J. Bacteriol. 175:3105–3114, 1993), an oleandomycin resistance determinant from *Streptomyces antibioticus* (A. M. Rodriguez et al., Mol. Microbiol. 8:571–582, 1993), and FrpC, a second RTX toxin gene from *N. meningitidis* (S. A. Thompson et al., Mol. Microbiol. 9:85–96, 1993).

In addition, significant progress has been made in analyzing the ABC domain from HlyB. The C terminus of HlyB has been overproduced and shown to form a dimer possessing both ATP binding and ATPase activity, with activities similar to those of the importer MalK and the P glycoprotein (V. Koronakis et al., Mol. Microbiol. 8:1163–1175, 1993). These results provide direct evidence in support of several of the hypotheses discussed in this review.

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