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The Complete General Secretory Pathway in Gram-Negative Bacteria†

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† This review is dedicated to the memory of Phil Bassford, who was, quite simply, one of the best.

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INTRODUCTION TO THE GENERAL SECRETORY PATHWAY

Approximately 20% of the polypeptides synthesized by bacteria are located partially or completely outside of the cytoplasm. Most reach their final destination via the general secretory pathway (GSP), the first step of which is their insertion into and translocation across the cytoplasmic membrane. In gram-positive bacteria, fully translocated proteins are released into the external milieu (Fig. 1), whereas in gram-negative bacteria, they are released into the periplasm or are integrated into or transported across the outer membrane by one of several terminal branches of the GSP (Fig. 1 and 2).

In line with the most widely accepted terminology, proteins transported by the GSP are called secretory proteins (or presecretory proteins if they are made as precursors). Exported proteins are retained partially or wholly within the cell boundary (the outer membrane), whereas secretion leads to extracellular release (the term "excretion" is reserved for the transport of nonproteinaceous compounds, such as antibiotics, amino acids, and capsular polysaccharides). Secretory proteins are distinguished from other proteins by the presence of a secretory signal sequence (usually abbreviated to signal sequence) that characteristically includes an uninterrupted stretch of at least 10 mostly hydrophobic amino acids. This review describes what is known about the way signal sequence-bearing proteins reach their final destination.

TRANSLOCATION ACROSS THE CYTOPLASMIC MEMBRANE

The first section of this review deals with the early stages of the GSP, leading to transport across the cytoplasmic membrane. Since this topic was discussed in several excellent recent reviews (451, 473, 481, 603), most of this section will be restricted to recent developments and to a discussion of the mechanisms involved. Most of the work was carried out with *Escherichia coli*, but studies with other bacteria and with eukaryotes will be mentioned where pertinent.

Essential Elements

Some early studies indicated that presecretory proteins could be transported across the cytoplasmic membrane without involving any other membrane or cytoplasmic proteins. The model protein used in those studies (M13 procoat or pregpVIII) is now known to be a rare exception among presecretory proteins (see the section on Sec-independent translocation, below), and we recognize that presecretory protein export is usually catalyzed by several cytosolic and membrane proteins.

Methodology. In vivo studies aim to determine conditions

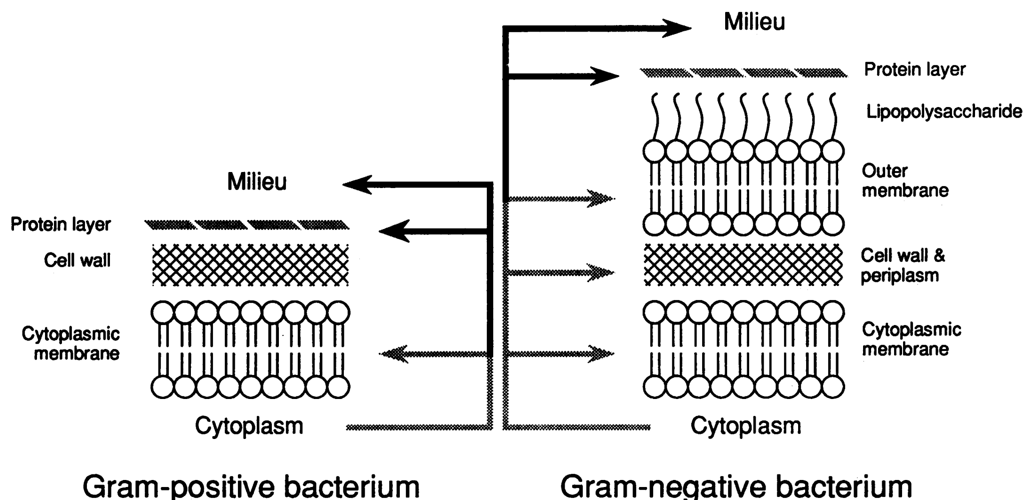

FIG. 1. Schematic representation of branches of the GSP in gram-positive and gram-negative bacteria. The basic pathway and its branches are indicated by arrows. Secreted (wholly extracellular) proteins follow the pathways indicated by both shaded and black arrows; exported (i.e., extracytoplasmic but not extracellular) proteins follow only the pathways shown as shaded arrows. The figure is not meant to represent sites through which the proteins must pass before reaching their target.

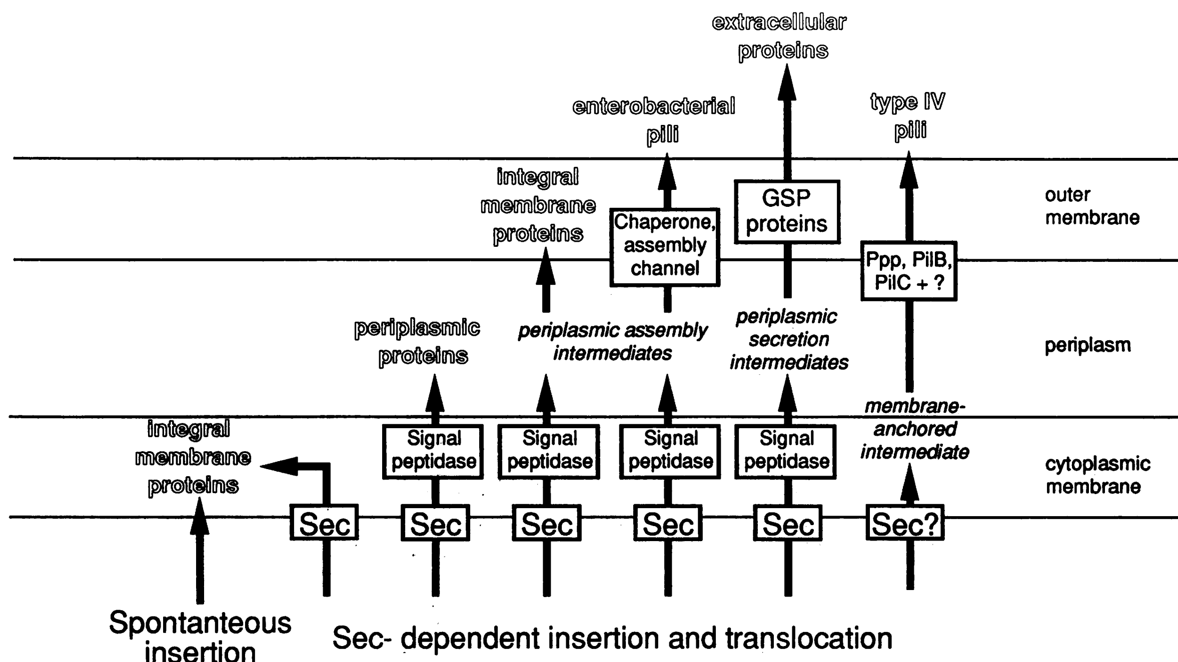

FIG. 2. Main terminal branches in the GSP of gram-negative bacteria. The figure illustrates most of the pathways for envelope protein export and extracellular protein secretion that are discussed in this review. The sites at which proteins accumulate at the end of these pathways are shown in relief characters. Intermediate stages are shown in italics, and proteins involved in their export and sorting are boxed. Sec indicates the proteins that are encoded by the *sec* genes and required for the early stages of the GSP. A specific chaperone and a translocator/assembly OMP are required for the assembly of the most common types of pili found in members of the *Enterobacteriaceae*. The second stage in extracellular secretion usually, although not always, requires several proteins (the GSP proteins) that form the main terminal branch of the GSP. Type IV pili are apparently assembled from cytoplasmic membrane-anchored precursors that are processed by prepilin peptidase (Ppp) and are assembled by a process that, in *Pseudomonas* species, involves at least the PilB and PilC proteins.

which reduce or increase protein export. Most protein export inhibitors have pleiotropic effects, making results difficult to interpret. Much more information can be gleaned from studying genetically altering signal sequences or proteins involved in export. Mutations that reduce protein export (usually indicated by the accumulation of unprocessed presecretory proteins) have been directly selected by a variety of techniques. One of the most widely used methods, developed and refined mainly in the laboratories of Jon Beckwith and Phil Bassford, relies on the fact that certain presecretory protein–β-galactosidase hybrids are toxic to cells (because of jamming of the export machinery by a blocked translocation intermediate) and confer a Lac^- phenotype (because of the inability of the β-galactosidase moiety to tetramerize) when they are produced at high levels (246, 317). Mutations that allow growth of these strains, confer a Lac^+ phenotype, or both have been found to affect either the sequence of the signal sequence of the hybrid protein, rendering it less efficient, or genes coding for components of the export machinery, rendering them partially or totally defective. More recently, a powerful alternative method for selecting mutations that reduce the activity of components of the export machinery was developed by Beckwith and his colleagues on the basis of the observation that the level of the mRNA coding for one component of the export apparatus, SecA protein is markedly increased when protein export efficiency is compromised (see, e.g., references 180 and 483) (see the section on SecA protein, below).

Most of the "export-down" mutations so far described are in the six *sec* genes and have severe effects on export only when the cells are grown at abnormally high or low temperatures or in rich media (conditional-lethal phenotype). This indicates that the *sec* genes are essential for viability under normal laboratory growth conditions (well-aerated, exponentially growing cultures in rich medium at 37°C). Conditional lethality may indicate that the *sec* gene product is inactive or unstable or fails to interact with other components of the export machinery or with presecretory proteins under nonpermissive conditions, that its synthesis is arrested under nonpermissive conditions, or that reduced levels or activity can be tolerated only under permissive conditions. The effects of export-debilitating *sec* mutations created in vitro cannot, of course, be easily tested because they cannot be maintained except in cells that also carry a wild-type copy of the gene. In future, it should be possible to construct strains in which the sole, wild-type copy of a *sec* gene is under the control of a tightly regulated promoter, allowing the effects of gradual depletion of its product to be tested as the gene is expressed at progressively reduced levels.

A novel class of mutations certain *sec* genes, the *prl* mutations, was obtained by selecting for restoration of the export of presecretory proteins with debilitated signal sequences. *prl* mutations alter the sequence of the Sec proteins without dramatically affecting the ability of the cells to export proteins with normal signal peptides; consequently, they do not affect cell viability. Cells which carry both a *prl* allele and a wild-type copy of the same gene are able to channel secretory proteins into either of two distinguishable export machineries (one containing the product of the *prl* allele and one containing the wild-type Sec protein), depending on the presence of absence of a mutation affecting the

activity of the signal peptide. Thus, if the merodiploid cells produce a presecretory protein–β-galactosidase hybrid with a signal peptide mutation, it will be channeled exclusively into the export machinery containing the Sec protein encoded by the *prl* allele and will therefore not block the export of other, natural presecretory proteins that can use either of the two machineries. Tom Silhavy and his colleagues introduced conditional-export-negative mutations into the wild-type *sec* genes in such *prl*/*sec*$^+$ merodiploids to demonstrate in a simple yet elegant assay that different *sec* gene products may be involved in distinct steps in protein export. A detailed description of this approach was presented by Bieker-Brady and Silhavy (38), and a broader review of different genetic approaches to studying protein export was presented by Schatz and Beckwith (481).

In vitro studies on protein export aim to reconstitute protein transport by using purified components under well-defined conditions. The basic idea of the in vitro translocation assay is to mix presecretory proteins with inverted cytoplasmic membrane-derived vesicles or with liposomes. Presecretory proteins that are translocated across the membranes of these vesicles or liposomes will become inaccessible to exogenously added proteases, whereas nontranslocated secretory proteins will remain protease accessible. A further indication of at least partial insertion or translocation is provided by the processing of the signal sequence by signal peptidase whose catalytic site is located on the periplasmic (luminal) side of the membrane of inverted vesicles. In the earliest versions of this assay, inverted cytoplasmic membrane vesicles from *E. coli* cells were added to in vitro transcription-translation systems producing radiolabeled presecretory proteins (371a, 461a). Subsequently, it became possible to purify presecretory proteins from *E. coli* cells and to study translocation in reactions in which the vesicles or cytosolic extracts were obtained from *sec* mutants grown at nonpermissive temperatures or from which one of the Sec proteins had been depleted. Most recently, essential components of the export machinery have been individually purified and functionally reconstituted in liposomes (see, e.g., references 5, 138, and 550; a detailed review is given in reference 603).

Signal peptides. All secretory proteins have a signal sequence, whose primary function is to help direct them to the cytoplasmic membrane. A signal sequence that is cleaved by a signal peptidase will be referred to here as a signal peptide. In eukaryotic cells, structurally similar signals direct proteins into the secretory pathway via the membrane of the endoplasmic reticulum. To avoid confusion, frequently used alternative terms will not be used here because they have other meanings (e.g., leader peptide and leader sequence are used in studies of gene transcription and translation) or because they describe eukaryotic, organelle-specific routing signals (e.g., transit peptide for chloroplasts, presequence for mitochondria) that are structurally quite distinct from signal sequences.

Studies that defined the essential features of signal peptides have already been reviewed (430). Figure 3 shows the essential features of three different types of signal peptide that are most readily distinguished by the sequence and position of the processing site. These differences are unimportant for the present discussion; it is sufficient to note that signal peptides are exclusively amino terminal; that they have a long, hydrophobic region (H domain) that is usually preceded by one or more positively charged residues in a short, generally hydrophilic region (the N domain); and that no two signal peptides from distinctly different presecretory proteins have exactly the same sequence. Shortening or introducing charged or strongly polar residues into the H domain or eliminating the basic amino acids from the N domain usually reduces export efficiency.

All bacterial signal peptides have essentially similar structural features, although those of presecretory proteins from gram-positive bacteria tend to be longer and to have more basic residues in their N domain (576). Signal peptides are processed during translocation across the cytoplasmic membrane, and are therefore unlikely to influence subsequent events in the GSP. It has been proposed, however, that signal peptides on presecretory proteins destined for secretion or for export to different envelope compartments in gram-negative bacteria are structurally distinct (517). This idea was based on the analysis of a limited number of proteins and is not supported by experimental evidence showing that signal peptides on presecretory proteins destined for different locations can be exchanged without affecting the sorting process (250, 416, 417, 553).

SecB protein. Six Sec proteins are known to be components of the GSP in *E. coli* (Table 1). Two of them, SecA and SecB, are found in the cytosol, although SecA is also found associated with the cytoplasmic membrane (see the next section). SecB is probably a tetramer and may be part of a protein complex that binds to presecretory proteins (368, 587, 588, 598; for a review, see reference 301). Other components of this complex have not been identified. SecB is apparently not essential for viability (but see below), and inspection of its predicted sequence does not reveal any features that might indicate its function. However, it binds to most presecretory proteins but to only very few cytosolic proteins (300, 301, 342), indicating that it is a specific GSP component.

Characterized *secB* mutations reduce the efficiency of export of many but not all presecretory proteins (302, 302a). This finding led to the concept of distinct SecB-dependent and SecB-independent classes of presecretory proteins (302a). In fact, this distinction is not clear-cut; most "SecB-dependent" proteins are exported, albeit slowly, in SecB$^-$ mutants, and the SecB-independent export of other proteins occurs only under certain conditions (see, e.g., references 272 and 457). This might explain why *secB* null mutations are lethal only under fast-growth conditions. Furthermore, most studies on SecB dependence used strain CK1953 (302a), which carries a Tn*5* insertion that converts the first AUG codon of *secB* into GUG. This may not completely eliminate SecB synthesis (153), and presecretory proteins with a high affinity for SecB could conceivably capture all residual SecB to prevent the "SecB-dependent" export of other proteins while themselves being exported normally. However, SecB has been shown to bind only weakly to one of the so-called SecB-independent proteins (209, 300), implying that at least some presecretory proteins can be exported without interacting with SecB.

Blobel and his colleagues interpreted their observation that SecB bound to pre-maltose-binding protein but not to a derivative lacking most of its signal peptide to indicate that SecB binds directly to signal peptides (588). Although SecB may bind to at least one other signal peptide (11), a more widely accepted explanation for this observation is that signal peptides retard protein folding sufficiently (see the section on translocation competence, below) to allow SecB protein to bind to multiple sites on the polypeptide backbone (10, 179, 331, 452, 449a, 555, 594) and especially to β-sheet structures (60a, 113, 342). Thus, SecB is basically similar to general molecular chaperones, which bind to exposed

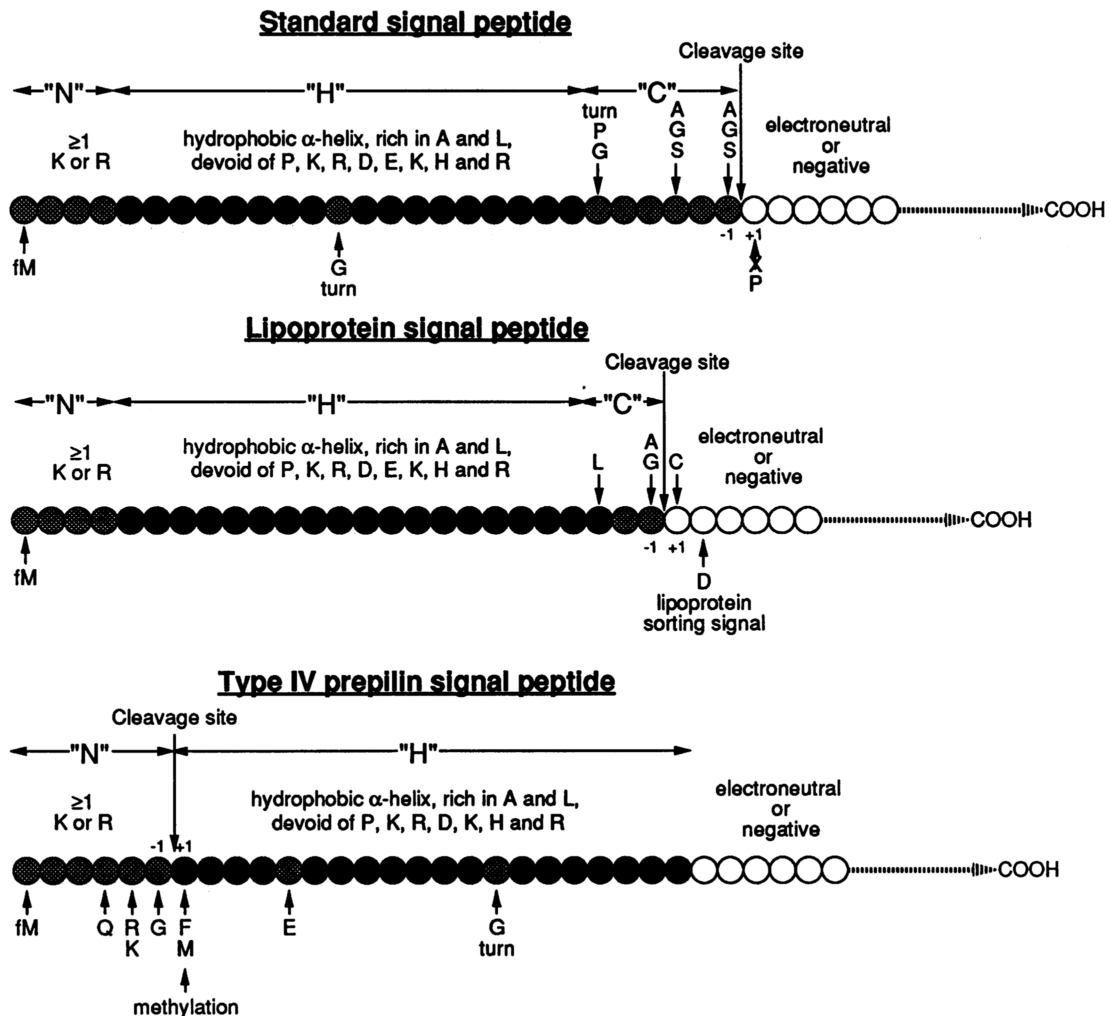

FIG. 3. Schematic representation of the principal features of the three types of signal peptide found in precursors of bacterial secretory proteins. Each amino acid within the functional elements of the signal peptides is indicated by a solid or shaded circle. Solid circles represent mainly hydrophobic or apolar residues, and shaded circles represent polar or charged residues. Open circles represent residues elsewhere in the polypeptide. Signal peptides have up to three domains ("N," "H," and "C"). The N domain (2 to >15 residues) is polar and carries a net positive charge. The H domain (>8 residues) is composed of predominantly hydrophobic residues and alanine and lacks strongly polar or charged residues. The high leucine and alanine content causes signal peptides to adopt an α-helical configuration in apolar environments. The C domain is usually less hydrophobic and contains the signals that are recognized by LepB or LspA signal peptidases. For convenience, the type IV prepilin signal peptides are considered not to have the C domain since they are cleaved between the N and H domains (see the text for details), although residues typically found in the C region of other signal peptides, including the turn-inducing amino acids and the "A-A" box, are often present at the end of the H domain or just downstream from it. Amino acids are indicated in the single-letter code. Lipoprotein signal peptides are usually shorter and have more hydrophobic amino acids in the H region. The turn residue upstream from the cleavage site is also less readily identifiable than in standard signal peptides. The amino-terminal cysteine residue of these polypeptides must be modified to glycerylcysteine for cleavage to occur and is usually fatty acylated (see text). The significance of these and other features of these signal peptides and of residues in the rest of the precursor polypeptides is discussed in the text.

polypeptide segments (see the section on translocation competence, below), except that SecB recognizes only proteins, including some cytoplasmic proteins (342), that fold slowly.

SecA protein. SecA, which is essential for viability, is probably a dimer or higher oligomer when in solution (3, 252, 350; reviewed in reference 388). It binds and hydrolyzes ATP (325, 326, 504), and its sequence (491) includes a segment located in the amino-terminal half of the polypeptide that is identical to a sequence (Walker box A) normally found only in nucleotide-binding proteins such as ATPases or kinases (581). Segments of SecA containing only this region of the polypeptide bind ATP (350), but the entire SecA molecule has been reported to bind at least three ATP molecules (325, 388), suggesting the existence of additional, nonconsensus nucleotide-binding sites. The role of ATP hydrolysis in protein translocation is discussed below in the section on energy requirements.

Although the absence of any segment of significant hydrophobicity suggests that SecA is a cytosolic protein, it is also found associated with membranes as well as with ribosomes (74, 324). Thus, SecA could interact with secretory proteins as they emerge from ribosomes, while they are free in the cytosol, or when they dock at the cytoplasmic membrane. In vitro studies show that translocation can be catalyzed solely

TABLE 1. Components of the *E. coli* cytoplasmic membrane secretory protein translocation system and their presence in other bacteria

Group and characteristics	Name	Size (kDa)	Location	Presence in other bacteria	Reference(s)
Secretory chaperonin (pilot protein[a])	SecB	18	Cytoplasm	Widespread in members of *Enterobacteriaceae*	115, 301
General chaperonins	DnaK	69	Cytoplasm	Universal	469
	GroEL	62	Cytoplasm	Universal	
	GroES	11	Cytoplasm	Universal	
Secretory ATPase (pilot protein)	SecA	102	Cytoplasm, ribosome, peripheral cytoplasmic membrane	*B. subtilis*, widespread in members of *Enterobacteriaceae*	115, 388, 392, 472, 541
Translocase	SecD	67	Cytoplasmic membrane	NI[b]	180
	SecE	14	Cytoplasmic membrane	*B. subtilis*	244, 483
	SecF	39	Cytoplasmic membrane	NI	180
	SecY	48	Cytoplasmic membrane	*B. subtilis*[c]	244, 373, 534a
	Band 1[d]	15	Cytoplasmic membrane	NI	67, 68
	Ydr[d]	19	Cytoplasmic membrane	NI	244
Signal peptidases	LepB[e]	36	Cytoplasmic membrane	*S. typhimurium*, *P. fluorescens*, *B. subtilis* (probably widespread)	41, 104, 561
	LspA[f]	18	Cytoplasmic membrane	*P. fluorescens*, *Enterobacter aerogenes* (probably widespread)	126, 243, 624
	Ppp[g]	ca.25	Cytoplasmic membrane	*P. aeruginosa* (PilD/XcpA), *V. cholerae* (TcpJ), *K. oxytoca* (PulO), *B. subtilis* (ComC)	23, 24, 264, 364, 384–386, 437
Others[h]	4.5S RNA (ffs)		Cytoplasm	*B. subtilis* (6S), *Thermus thermophilus*, *P. aeruginosa*, *Halobacterium halobium* (7S), *Mycoplasma pneumoniae*	418, 419, 462, 515, 533
	Ffh	48	Cytoplasm	NI	72, 466
	FtsY	?	Cytoplasmic membrane	*Sulfolobus solfataricus*	189, 419, 448, 466, 533

[a] See text.
[b] NI, not identified.
[c] Genes coding for proteins with >20% overall sequence identity to SecY have been cloned and sequenced from a variety of different sources, ranging from a plastid genome (136) to *S. cerevisiae* (526, 528). Only in *S. cerevisiae* and *B. subtilis* has any direct participation in protein traffic been demonstrated.
[d] Participation in translocation has not been fully established (see text).
[e] Also called leader peptidase or signal peptidase I.
[f] Also called prolipoprotein signal peptidase, lipoprotein signal peptidase, or signal peptidase II.
[g] Prepilin peptidase; the gene-based designation varies according to the organism from which it is derived and its function.
[h] The role of these macromolecules in protein translocation has not been firmly established. They are included here because their homologs in eukaryotes are involved in secretory protein translocation.

by membrane-associated SecA under certain circumstances, although this is unlikely to occur in vivo (73, 101). SecA associates more tightly with membranes containing acidic phospholipids (phosphatidylglycerol and cardiolipin) and the SecE/Y integral membrane components of the GSP (68, 326) and may even penetrate into the lipid bilayer (60, 388, 556a).

SecA synthesis increases when export is blocked or saturated. It is the only component of the GSP that responds in this way, suggesting that overproduction of SecA is sufficient to compensate for any temporary saturation or jamming of the GSP that might normally occur. This novel regulatory response is governed by SecA itself, which binds to a site upstream from its translation start codon in its polycistronic mRNA, presumably to prevent translation. Translation is increased when translocation is blocked, possibly because the size of the cytoplasmic pool of SecA diminishes as it is increasingly bound to blocked translocation intermediates (131, 490).

SecA can be cross-linked to presecretory proteins whose signal peptides include a basic N domain (4), and *prlD* mutations in the *secA/prlD* gene improve the export of presecretory proteins with defective signal peptides (159). These observations suggest but do not prove that SecA binds to signal peptides. Indeed, as discussed below, SecA probably binds at multiple sites along the length of presecretory proteins during translocation (see the section on energy requirements).

Integral membrane Sec proteins. Four Sec proteins have several long stretches of hydrophobic amino acids that probably span the cytoplasmic membrane. The first of these to be characterized was SecY, which seems to span the membrane 10 times with its amino and carboxy termini in the cytosol (7, 244). This organization is similar to that of cytoplasmic membrane proteins involved in solute transport (e.g., LacY lactose permease [75]). *secY* mutations that either block protein translocation or improve the translocation of secretory proteins with debilitated signal sequences (*prlA*) affect residues throughout the polypeptide (244).

SecE protein is thought to have three transmembrane segments (483), but a truncated variant that retains only the carboxy-terminal one is still active (379, 482), and SecE from *Bacillus subtilis* has only a single transmembrane segment (244). All characterized export-blocking *secE* mutations reduce the level of *secE* expression rather than altering the structure of the protein. SecD and SecF each appear to span the cytoplasmic membrane six times. Parts of their primary sequences are quite similar (180), suggesting that they perform related functions. None of the mutations that allow the export of proteins with debilitated signal peptides map to either of these loci, indicating that they may act at a late stage in export.

The two other proteins that might form part of the translocase are less well characterized. The synthesis of large amounts of one of these, Ydr protein, suppresses the export-negative effects of a *trans*-dominant mutation in *secY* in a diploid strain that carries a wild-type *secY* allele (244, 503). This suppression phenotype is different from the suppression of conditional *sec* mutations which was observed previously by others and which is often due to reduced translation (316, 430). Instead, suppression of the *trans*-dominant *secY* mutation by high levels of Ydr is presumed to indicate that it and SecY normally form a complex and that the product of the mutant *secY* allele titrates Ydr to prevent its interaction with wild-type SecY. Large amounts of Ydr saturate the defective SecY, allowing some functional SecY-Ydr complexes to form. Overexpression of *secE* or *secDF* also suppresses the *trans*-dominant effect of this *secY* allele (244), implying that the five proteins form a complex.

Purified SecE-Y often contains appreciable amounts of another protein, band 1 (68), which may increase the efficiency of translocation (67) in the in vitro assay based on purified components (5, 68, 103, 138). Band 1 cannot be separated from SecY without releasing SecE, making it difficult to determine whether it is an essential component of the translocase. Its structural gene has recently been cloned (602): its further analysis will no doubt soon reveal whether it is unique and whether it affects protein export in vivo, as well as providing a means of producing large quantities for purification and use in reconstitution studies.

Most of the results obtained by genetic and biochemical approaches are in broad agreement, but some differences remain to be clarified. For example, the reason why SecD and SecF are essential for protein export in vivo but have no effect in vitro (349) is not immediately apparent. Currently used in vitro assays may be inadequate to reveal a requirement for SecD and SecF (see the section on release from the translocation channel, below). However, the fact that precursors of all secretory proteins accumulate in all conditional *sec* mutants (except *secB* mutants) under nonpermissive conditions does not necessarily mean that they are involved in the translocation of all proteins. For example, if a late stage in the export of only a few secretory proteins requires SecD, SecF, or both, their absence would eventually cause all channels to become blocked by translocation intermediates under nonpermissive conditions, and therefore the translocation of all presecretory proteins (including those not needing SecD or SecF) would be adversely affected.

Another puzzling observation was reported by Blobel's group, who found that reconstituted proteoliposomes devoid of SecY were active in translocation and proposed that SecY is not an essential translocase component (590). This seems unlikely in view of the extensive evidence indicating direct SecY involvement in translocation (37, 244, 326, 378, 589), but a plausible explanation has not yet been found.

Antibodies against SecY coimmunoprecipitate SecE and band 1 (68), and overproduction of SecE or SecY is possible only when the other (or a segment thereof) is similarly overproduced (348, 379). These observations support that idea that SecE, SecY, and band 1 form a preexisting complex, avoiding the need to recruit them separately for each translocation event. However, SecE and SecY can be separated under relatively mild purification conditions and then recombined to form an active translocase (348), indicating that they are not necessarily tightly associated.

The stoichiometry of translocase components has not yet been determined, but results of one study indicate that SecE must be five times more abundant than SecY for maximally efficient translocation in vitro (5). SecE was originally estimated to be present at only 80 copies per cell (348), but this figure has now been revised upward to 300 to 600 copies (349, 482), close to the estimated number of copies of SecY, i.e., 200 to 400, and SecD, i.e., 450 to 900, and larger than the estimated number of copies of SecF, i.e., 30 to 60 (349). Thus, the upper limit for the number of translocases in *E. coli* is around 500. *E. coli* exports over 10^6 periplasmic and outer membrane polypeptides per generation (25 min under optimal conditions), or approximately 80 polypeptides ($>10^4$ amino acid residues) per min per translocase, which seems extraordinarily efficient. By using alternative methods based on the jamming of protein export, it has been calculated that there are approximately 20,000 export sites in *E. coli* (246) and 25,000 in *B. subtilis* (404), allowing the export of two

secretory proteins per min, but these values may be overestimates because of incomplete jamming under the conditions used.

Secretory protein translocase in eukaryotes. Only one of the tentatively identified components of the translocase of the endoplasmic reticulum of higher eukaryotes, the so-called signal sequence receptor, has been characterized at the molecular level (427). It does not have any similarity to the *E. coli* Sec proteins, and its involvement in protein translocation has recently been challenged on the basis of in vitro studies (361). More information is available from studies with the yeast *Saccharomyces cerevisiae*. For example, Sec61 protein, one of three membrane proteins that form a complex required for secretory protein translocation (124), has 20% amino acid identity to *E. coli* SecY protein (526, 528), suggesting that they have a common evolutionary origin. Translocation intermediates can be cross-linked to Sec61 protein (371, 476), implying that it is in close apposition, if not direct contact, with secretory proteins in the translocation channel.

Recognition and Insertion

Proteins destined for import into the various organelles of eukaryotic cells are distinguished solely by their targeting signals. Each organelle has one or more receptors that specifically recognize either the cognate targeting signal or a pilot protein (or protein complex) bound to it (reviewed in reference 430). Prokaryotes do not have this diversity of potential target membranes, and receptors or pilot proteins would be unnecessary if signal peptides spontaneously insert into the cytoplasmic membrane at translocation sites.

Membrane phospholipids. Purified signal peptides have been shown to interact with the surface of, and then penetrate into, artificial phospholipid monolayers and bilayers (271), leading to the adoption of an α-helical (28, 29, 61) or β-sheet (456) structure and to direct contact between the H region and the fatty acyl chains of the lipids (271). Synthetic signal peptides with a debilitated H segment are less able to interact with lipids (62, 66, 230, 354, 456), indicating that the observed interactions may be functionally relevant. Signal peptides interact most strongly with membranes rich in acidic phospholipids (270), probably because of electrostatic interactions involving the N domain (304). This may lower the energy barrier to full insertion into the bilayer by distorting the surface of the membrane (62). These electrostatic interactions may be involved in the first of the two experimentally observed stages in the interaction between signal peptide-bearing precursors and the *E. coli* cytoplasmic membrane (546). Depletion of acidic phospholipids in *E. coli* mutants or in vesicles leads to in vivo and in vitro defects in protein export, respectively (129, 216, 304, 326), although this may be because SecA interacts less well with these membranes rather than because signal peptide insertion is less efficient (see the section on SecA protein, above).

Electrostatic interactions with acidic phospholipid head groups could explain why the N domain remains on the cytoplasmic side of the membrane while the carboxy-terminal end of the signal peptide is "flipped" through the membrane (tail-first orientation [Fig. 4]) (29, 152, 304). Removal of basic amino acids from the N domain or insertion of these amino acids in or around the C domain should therefore have the same effect as acidic phospholipid depletion and might lead to the insertion of signal peptides in the reverse orientation. Experimental observations tend to support this prediction (15, 47, 239, 318, 340, 535, 620). Negatively charged residues in the N domain also prevent head-first insertion, although they are somewhat less effective (four glutamates, for example, are as effective as one lysine [15]). Thus, basic and acidic residues do not necessarily cancel each other out. Instead, the polarity of signal peptide insertion is determined by which extremity carries the strongest insertion polarity signal, which may be composed of both acidic and basic residues. The absence of basic residues from the N domain is not always sufficient to dramatically reduce protein export, however (48, 242, 444, 569). Other features of the signal peptide such as high overall hydrophobicity (220, 340, 444) may compensate for their absence, but there does not appear to be an inverse correlation between the length or hydrophobicity of the H domain and the number of basic amino acids in the N domain of natural signal peptides (220). Instead, signal peptides that lack basic amino acids from their N domain are more likely to have a glycine or proline residue in the H domain (340), but the significance of this correlation is unknown. Limited numbers of basic amino acids are sometimes found in or just after the C domain, although excessive numbers will block export (15, 321).

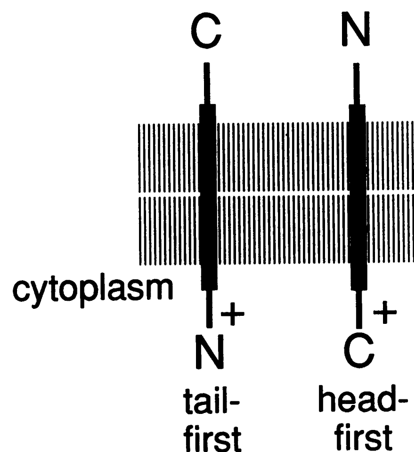

FIG. 4. Charge-dependent orientation of signal peptide insertion. Signal peptides normally have a positively charged amino-terminal (N) extremity (+) and insert in a tail-first orientation, possibly because of electrostatic interactions with negatively charged phospholipid head groups. Reversal of the charge distribution at the ends of the peptide converts it into an insertion signal, causing head-first insertion.

The interaction between signal peptides and membrane lipids could initiate protein export, but how do the generally hydrophilic sequences beyond the C region in the mature part of the polypeptide get pulled into and across the membrane? A few presecretory proteins are exported without the involvement of Sec proteins (see the section on Sec-independent translocation, below). In these cases, the mature part of the presecretory protein must be translocated through the lipid part of the membrane, which raises the possibility that at least partial insertion of the segment downstream from the C domain (the export initiation domain [16]) occurs with other, Sec-requiring presecretory proteins (see the section on the translocation channel, below). The signal peptide may initially form a miniloop, with residues beyond the export initiation domain being gradually drawn into the lipid bilayer as the signal peptide straightens to span the membrane (Fig. 5). Hydrophobic residues in the signal peptide might somehow shield hydrophilic residues in the

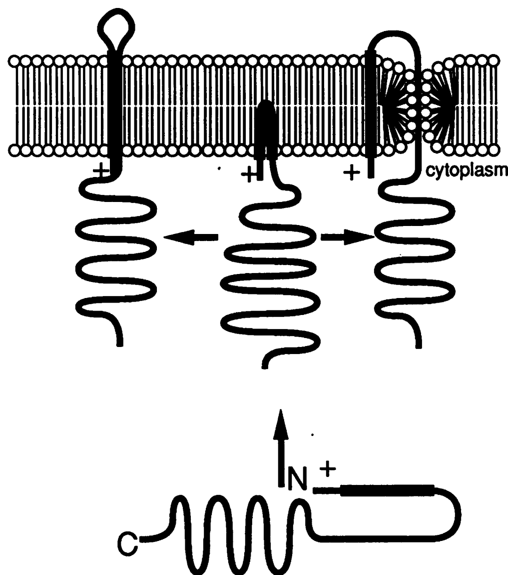

FIG. 5. Models for receptor-independent signal peptide insertion into the cytoplasmic membrane. Partial insertion of the signal peptide is proposed either to be followed by formation of a membrane-spanning loop in which the signal peptide pairs with and protects the export initiation domain (left) or to induce the phospholipids to adopt a nonbilayer conformation that traps the export initiation domain in a channel lined by polar lipid head groups (right). Insertion usually occurs before translation is completed. +, presence of basic amino acids in the signal peptide N domain; N and C, amino and carboxy termini of the protein, respectively.

export initiation domain from contact with the fatty acids. Alternatively, signal peptide-induced phase changes in the membrane (29, 128, 270) may allow the export initiation domain to be enveloped by lipids in nonbilayer conformation so that it is protected from the fatty acyl chains by the polar lipid head groups (Fig. 5) (128).

Pilots and receptors. Signal peptides are clearly able to interact directly with membrane lipids and may indeed do so to initiate translocation. However, translocation seems to occur at a very limited number of sites (see the section on integral membrane Sec proteins above), and productive interaction between signal peptides and the cytoplasmic membrane might therefore be guided by specific receptor proteins.

Either of the two soluble Sec proteins (SecA and SecB) could pilot nascent presecretory proteins to the translocase. SecB probably binds to multiple sites on presecretory proteins rather than specifically to signal peptides (see the section on SecB protein, above), in line with the widely accepted view that SecB is a GSP-specific molecular chaperone (see the section on translocation competence, below). However, de Cock and Tommassen (116) proposed that its primary function is to pilot secretory proteins to the cytoplasmic membrane by interacting with membrane-associated SecA protein (12, 210, 315) (see below). Thus, SecB and SecA could be pilot and translocase-associated docking proteins, respectively. Alternatively, cytoplasmic SecA protein bound to presecretory proteins may interact with integral membrane components of the translocase or with acidic phospholipids (60, 556a) (see the section on SecA protein, above), thereby guiding presecretory proteins to the cytoplasmic membrane or to the translocase. Clearly, such a pilot-docking protein system might obviate the need for a specific signal peptide receptor in the translocase complex, thereby relegating the signal peptide to the purely nonspecific role of anchoring the presecretory protein to the cytoplasmic membrane.

One of the major, circumstantial arguments against specific signal peptide recognition by any component of the GSP is that the sequences of these peptides are highly variable. If such a signal peptide receptor does exist, it could be any one of the four cytoplasmic membrane Sec proteins or membrane-bound SecA. Experimental approaches used to identify similar receptors in other systems (anti-idiotype antibodies [393, 394; but see also references 163 and 357], selective proteolysis of surface proteins [406, 407], cross-linking [1, 33, 190, 219, 604], affinity purification [322], binding inhibition by specific antibodies [520, 521] or competitive ligands [71, 89, 193, 406, 493], and extraction and reconstitution [406, 529]) have not been widely used to search for signal peptide receptors in bacteria. Antibodies against the N terminus of SecY are reported to prevent precursor binding to inverted membrane vesicles (589), as does trypsin treatment, which may inactivate SecY (537, 538). Synthetic bacterial signal peptides prevent in vitro translocation into cytoplasmic membrane vesicles (85), but the insertion of large amounts of synthetic signal peptide into membranes might perturb them sufficiently (128, 270) to affect several processes, including translocation. Simon and Blobel (513) recently reported that signal peptides can open the putative translocation channel in artificial cytoplasmic membrane bilayers (see the section on the translocation channel, below) but only when added to the cytoplasmic side. This was interpreted to show that signal peptides bind to a specific receptor on the cytoplasmic face of the translocase. However, signal peptides normally insert in a tail-first orientation (see the section on signal peptides, above) and would be in reverse orientation to the translocase, and thus possibly unable to open the channel, if inserted from the periplasmic side of the membrane.

The fact that *prl* mutations in *secE* or *secY* facilitate the export of proteins with debilitated signal peptides (149, 330, 445, 474, 524) or export initiation domains (330, 445) has been interpreted to indicate that weakened signals can be recognized by receptors rendered less stringent by the *prl* mutation (244). Genetic evidence suggests that SecE is required before SecY (37) and hence is more likely than SecY to be the signal peptide receptor. Alternatively, SecE may be the docking protein that binds SecA while SecY may be the signal peptide receptor. Surprisingly, *prl* mutations are generally well tolerated and do not appear to cause the export of normally cytoplasmic proteins. This implies that even presecretory proteins with severely debilitated signal peptides are nevertheless specifically selected for export in the *prl* mutants. The residual signal peptide may therefore retain some receptor-binding activity, or the signal peptide may not be the only feature of presecretory proteins which determines that they will be exported. Thus, an alternative explanation for the export of presecretory proteins with debilitated signal peptides in *prl* mutants is that they retain their ability to bind SecB and SecA, which could then guide them to the translocase complex in the absence of a signal peptide-receptor interaction.

It is clearly still too early to be categorical about how presecretory proteins find the translocase. The compelling evidence that signal peptides have a strong affinity for membrane lipids cannot be overlooked, but it is equally possible that the hydrophobic H domain of the signal peptide

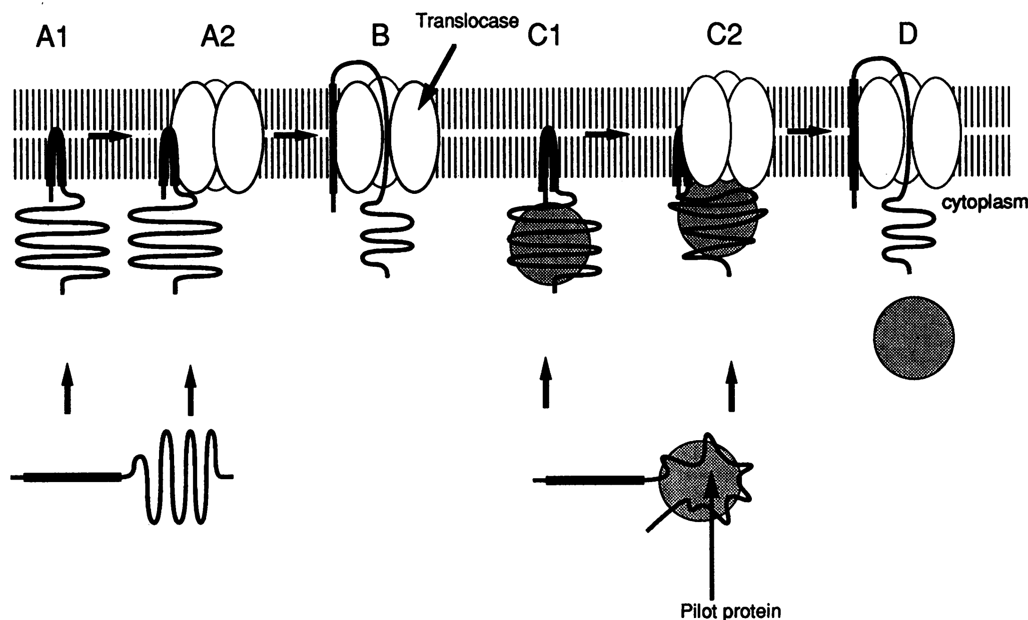

FIG. 6. Models for receptor-dependent docking and insertion of signal peptide-bearing precursor proteins into the cytoplasmic membrane at specific translocase sites. The model on the left (A to B) shows spontaneous insertion (A1) of a looped signal peptide (thick line), which diffuses in the plane of the membrane until it encounters its receptor in the translocase (A1 and A2) or which binds directly to a receptor on the translocase (A2), which then opens laterally to engulf the export initiation domain (B). In the alternative model on the right (C and D), a pilot protein bound to the mature part of the secretory protein binds to a specific receptor in the translocase (C1 or C2) and can thus facilitate direct contact between the signal peptide and its receptor either before or after insertion into the lipid phase of the membrane (D).

is important for recognition by its cognate receptor. One attractive idea is that signal peptides interact with translocase components (SecE or SecY) at their interface with the membrane lipids. Pilot proteins bound elsewhere to the presecretory protein may ensure contact between it and the translocase either before or after signal peptide insertion into the lipid bilayer, as illustrated in Fig. 6. It should be possible to devise tests based on vesicle fusion techniques or on the sequential expression of *sec* genes to determine whether presecretory proteins whose signal peptides are inserted into the cytoplasmic membrane devoid of translocase components can then be translocated when the missing components are supplied.

Translocation

Translocation channel. As we learn more about the general aspects of protein traffic, it becomes increasingly unlikely that no more than a few exceptional proteins can be completely translocated through the lipid part of a biological membrane. It is widely believed that translocated segments of presecretory proteins, which are largely to totally devoid of segments of significant hydrophobicity, cross the bacterial cytoplasmic membrane via a specific channel formed, in *E. coli*, by one or more of the integral membrane Sec proteins. They are therefore completely protected from contact with the hydrophobic environment of the lipid bilayer. This considerably reduces the energy costs that would otherwise be incurred in translocation of large, polar polypeptide segments through the lipid bilayer. Although still far from proven, this view was recently considerably strengthened by the observation that trapped in vitro translocation intermediates can be cross-linked to SecY protein and do not appear to be in close apposition to membrane lipids (602).

A parallel has already been drawn between SecY and solute permeases in the bacterial cytoplasmic membrane, all of which have several membrane-spanning segments that line a channel through which specific solutes are transported. It may be instructive to consider secretory polypeptides as nothing more that a series of amino acids which, instead of being transported individually through a permease, are transported as a single long chain rather like a string of beads. However, unlike small solutes, which enter the permease at one side of the membrane and exit at the other, secretory proteins are believed to form loop structures in the cytoplasmic membrane, and may be anchored to this membrane by their lipid-embedded signal peptide, before they enter the translocase channel. To explain how the secretory protein gains access to the inside of the translocase, Simon and Blobel (513) propose that the channel opens laterally to engulf the export initiation domain as or after it is pulled into the membrane. Such an event is represented schematically in Fig. 7, panel I, in which the resting translocase (for reasons of simplicity, only the SecY component is shown) is depicted as a collapsed bundle of transmembrane segments without a central channel. This would prevent the leakage of cytosolic solutes across the membrane when the translocase channel is not being used. The signal that triggers channel opening could be the signal peptide itself, as proposed by Simon and Blobel (513), or the presence of the export initiation domain at least partially embedded in the bilayer (Fig. 5). In Fig. 7, panel II, the presence of one or both initiates the unbundling of the translocase channel, whose signal peptide temporarily forms the 11th transmembrane segment, forcing the export initiation domain into the lumen of the channel as it forms. The channel becomes open at each end only when the export initiation domain is fully within the lumen of the translocase and further translocation can occur. Extramembranous

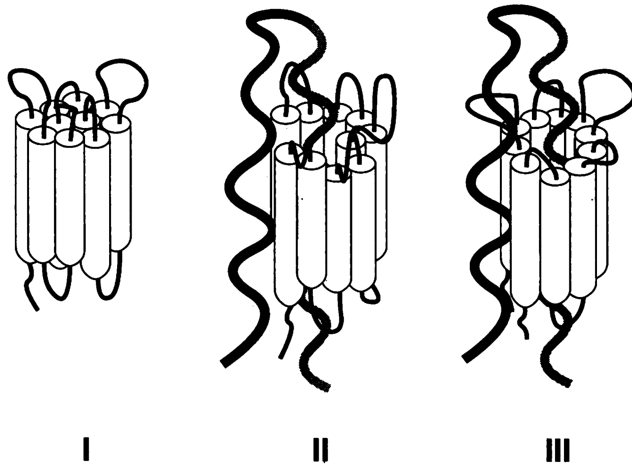

FIG. 7. Schematic representation of a model depicting the signal peptide-induced lateral opening of a translocase channel as viewed from the top (periplasmic) side of the cytoplasmic membrane. The collapsed channel is represented as composed of a single polypeptide that spans the membrane via 10 antiparallel hydrophobic segments (barrels) joined by hydrophilic loops (lines), such as might be the topology of SecY. In the closed state, the channel that runs perpendicular to the membrane is occluded because the transmembrane segments are bundled together (I). Contact with a signal peptide (thick line) or with an export initiation domain (shaded line) induces lateral opening of the channel to permit the entry of the latter within its lumen while blocking the entry of phospholipids (II). The lateral opening then closes in the form of a channel with the export initiation domain in its lumen (III). Additional components of the translocase may perform such functions as channel occlusion or gating and signal peptide–docking-protein receptor (see text).

loops of SecY or other components of the translocase (e.g., SecE) may help seal the channel during this transition stage, and other components of the translocase (e.g., SecA) may facilitate the unbundling and lateral opening (see the section on energy requirements, below). The walls of the channel presumably fit snugly around the secretory polypeptide to prevent ion leakage, although halide ions apparently diffuse through the cytoplasmic membrane (possibly through the translocation channel) during protein translocation, and especially during translocation pausing (488). The way in which the channel is gated provides a challenging subject for future research.

A translocation channel has been tentatively identified in the endoplasmic reticulum membrane by electrophysiological techniques (512). The method used is based on the fact that the channels remain open when translation is artificially terminated by puromycin. The ribosome remains bound to its receptor(s) in the membrane (387, 480) and covers the now-unoccupied channel, allowing ions to diffuse across the membrane (and presumably through the ribosome as well). This approach cannot be used with bacterial cytoplasmic membranes, which do not have a ribosome receptor (519), but Simon and Blobel (513) recently showed that small amounts of signal peptide induce the formation of similar channels in bilayers formed from the *E. coli* cytoplasmic membrane, presumably by tricking the translocase channel to open even though an export initiation domain cannot enter its lumen. The authors appear to rule out the possibility that the channels result from signal peptide-induced deformation of the lipid bilayer (128, 270), but similar channels have been observed on the insertion of other hydrophobic peptides into artificial bilayers (see, e.g., reference 391). In the experiments described by Simon and Blobel, the channels formed only when the signal peptides were inserted on the cytoplasmic side of the membrane, suggesting specific signal peptide recognition by a receptor component of the translocase (see the section on pilots and receptors, above). It should be relatively simple to assay channel-forming activity in membranes depleted of, for example, SecY protein, which would provide proof that the putative translocase was required for channel formation.

Channel induction by isolated signal peptides would probably be lethal to *E. coli* cells, because the electrochemical potential across the membrane would be rapidly dissipated. It is surprising that the effects observed by Simon and Blobel (513) were long lasting since processed signal peptides are normally rapidly degraded by membrane peptidases, presumably to avoid their lethal accumulation (430). Likewise, signal sequences that are not processed or degraded (see the section on bitopic membrane proteins, below) would presumably remain bound to their receptor and keep the channel open even when translocation is completed, which they do not. The signal peptide is unlikely to be within the lumen of the channel since it does not impede ion flow in the experiments reported by Simon and Blobel (513). Thus, although the observations made by Simon and Blobel are worthy of serious consideration, several aspects need further investigation before definitive conclusions can be drawn.

An export-specific domain in the cytoplasmic membrane? As discussed above, protein export is an extremely efficient process operating at a very limited number of sites in the cytoplasmic membrane which, it is argued, contain preassembled translocase. Subcellular fractionation and preliminary electron-microscopic data (546) suggest that translocation occurs at unique patches on the cytoplasmic membrane that may correspond to regions of contact with the outer membrane. The existence and significance of these sites is controversial (268), and the idea that they may form vesicles containing unique membrane components involved in membrane biogenesis is attractive but largely untested (616). Membrane-associated ribosomes (see the section on cotranslational translocation, below) (347) or translocation intermediates that accumulate following energy depletion (181), Sec protein depletion (556), or premature folding of nontranslocated segments (see the next section) might provide useful markers for translocation sites and could be cross-linked to translocase components (192a, 211, 267, 292, 371, 476, 522, 548).

Translocation competence. Polypeptides are generally believed to be threaded across membranes in a locally unfolded state (a simple analogy would be a thread being pulled through fabric). Since bacterial protein export can occur posttranslationally (258, 449), a considerable length of presecretory proteins might be exposed in the cytosol and fold into more highly ordered structures prior to translocation (see the next section). We therefore need to know how much tertiary structure can be tolerated by the translocation machinery and whether folding is prevented or retarded prior to translocation.

Purified presecretory proteins must be denatured or otherwise prevented from adopting more highly ordered structures to remain competent for import into vesicles (98, 99, 303). This, together with other evidence (86, 87, 146, 496, 564–566), suggests that extensive tertiary and especially quaternary structure makes precursor proteins translocation incompetent because they cannot be unfolded and thus

become jammed during translocation. Nevertheless, even branched or highly folded polypeptides can be translocated across certain eukaryotic organelle membranes (566, 567, 582), and the question of how much highly ordered structure can be tolerated by different protein translocation systems is still unresolved.

Signal peptide–β-galactosidase hybrids are the best-characterized examples of highly folded presecretory proteins that cannot be efficiently exported by *E. coli* (172, 246, 317). Although several other examples of similar phenomena have been reported, there is nothing intrinsic to cytoplasmic proteins that prevents their export when fused to signal peptides (430; see references 43 and 535 for recent examples). A crucial factor determining whether signal peptide–cytoplasmic-protein hybrids can be exported efficiently may be the rate at which they fold and oligomerize prior to translocation (430). Translocation-competent presecretory proteins are not necessarily devoid of secondary or tertiary structure, however (314). For example, a small loop formed by a disulfide bond does not prevent in vitro translocation of a natural presecretory protein of *E. coli* (542, 544), and an artificially biotinated presecretory hybrid protein is exported in vivo (457). Thus, limited tertiary structures (kinks-in-the-thread analogy) do not cause loss of translocation competence, but more highly ordered structures (knots-in-the-thread analogy, corresponding to prefolded β-galactosidase [317], trimethoprim-complexed dihydrofolate reductase [172], or aggregated states [314]) may do so.

Considerable effort has been devoted to determining the conditions that allow presecretory proteins to retain translocation competence. Signal peptides retard the refolding of denatured presecretory proteins (307, 331, 396). Their ability to similarly retard the folding of nascent chains in vivo would increase the time span during which molecular chaperones could bind to presecretory proteins to further retard their premature folding. Several excellent reviews present different views of the function of molecular chaperones in the folding of nascent polypeptides, in the refolding of denatured polypeptides, and in the maintenance of polypeptides in a form suitable for homo- or hetero-oligomerization (188, 309, 312, 469). From these, it seems that molecular chaperones prevent illicit interactions between different parts of the same polypeptide by keeping them apart while allowing other, permissible interactions to occur. Bacteria have many cytoplasmic molecular chaperones. Most of them presumably bind to presecretory proteins since, although each of them probably recognizes different structural features, they do not have strict binding-site specificities (164, 310, 502). Several of them may perform the same or overlapping functions in protein export. At least one major general chaperone, GroEL (a member of the 60-kDa heat shock protein family, often referred to as HSP60), can maintain purified, denatured presecretory proteins in a translocation-competent state when the denaturant is diluted out (44, 315), as can another cytosolic protein (trigger factor) (98, 99), which does not appear to be involved in export in vivo (199). Other general molecular chaperones probably have similar activities, but the only documented effects of manipulating the levels of major general chaperones on protein export in *E. coli* are the slightly reduced rate of pre-TEM β-lactamase processing in the absence of GroEL or GroES (HSP10) (305) and the improved export of signal peptide–β-galactosidase hybrids when GroEL or DnaK (HSP70) is present at such high levels (408) that it presumably blocks the premature folding that normally prevents β-galactosidase export.

SecB, like general molecular chaperones, has also been reported to prevent denatured presecretory proteins from losing translocation competence (91, 209, 303, 315, 594, 595) (but see the section on pilots and receptors, above, for an alternative explanation). Although SecB has been shown to retard tertiary-structure formation in presecretory proteins (91, 179, 331), it does not abolish it altogether (60a). Furthermore, since tertiary-structure formation does not necessarily lead to the loss of translocation competence (see above), the ability of SecB to prevent aggregation (60a, 314) may be of greater importance in protein export than its ability to retard early folding steps.

General molecular chaperones require ATP as a cofactor to bind polypeptide segments and hydrolyze ATP during protein release (177, 308, 323, 346, 355, 518). SecB protein does not have a consensus nucleotide-binding site, and presecretory proteins bind SecB in the absence of ATP (586) and can be displaced by other presecretory proteins without ATP hydrolysis (209). ATP hydrolysis by SecA protein might provoke SecB release as translocation starts, however (see the section on energy requirements, below).

SecB might bind to nascent polypeptides as they emerge from the ribosome or only as the last element in a cascade of chaperone-binding reactions (311), and it need not necessarily displace general molecular chaperones. Indeed, defects in protein export as a result of the absence of SecB protein can be overcome by high-level expression of the heat shock regulon, which includes the genes for several general molecular chaperones (12). Thus, the normally SecB-dependent export may occur in the absence of SecB if presecretory proteins are swamped by general molecular chaperones. The major heat shock chaperones GroELS and DnaK do not seem to be necessary for this suppression effect, but, as discussed above, this may be because several general molecular chaperones can fulfill the same role.

Cotranslational translocation. Signal peptides could insert into the cytoplasmic membrane and initiate translocation the moment they emerge from the ribosome, i.e., after about 70 amino acids have been polymerized. Indeed, polysomes synthesizing presecretory proteins are enriched in the membrane fraction of cells (450) because of signal peptide-directed membrane interactions (455), showing that translocation and translation can occur simultaneously, possibly with little or none of the nascent chain exposed in the cytosol (Fig. 8, ribosome A). mRNAs coding for exported proteins are usually long lived, so that once a polysome has been anchored to the membrane by a signal peptide, it may stay there until the mRNA is degraded and translation ceases. Some of the many nascent presecretory proteins being produced by a single polysome may not immediately dock at the translocation channel. The signal peptides of these nascent chains may insert into the lipid bilayer (as in Fig. 8, ribosome B), or the completed nascent chain may be released into the cytosol (as in Fig. 8, ribosome F) (posttranslational export). In both cases, a considerable length of the nascent chain may be temporarily exposed to the cytosol (258, 449). However, since the calculated rate of protein translocation (>10^4 residues/min; see the section on integral membrane Sec proteins, above) is at least 10 times higher than the rate of translation, these exposed segments of presecretory proteins should be rapidly sucked into the translocation channel once translocation is initiated, causing the rapid transition from the situation represented in Fig. 8, ribosome E, through that in Fig. 8, ribosome D, in which very little of the nascent polypeptide is exposed in the cytosol.

The degree of coupling between export and translation

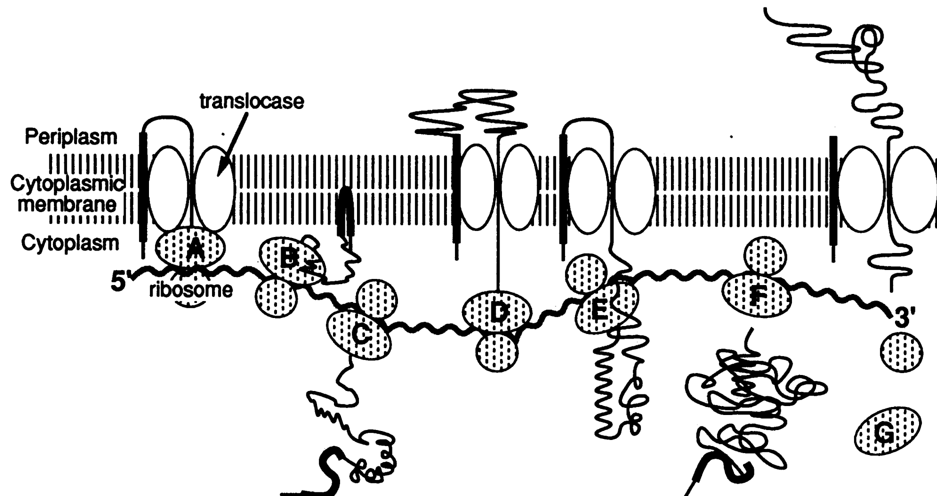

FIG. 8. Schematic representation of cotranslational, translation-linked, and posttranslational export of presecretory polypeptides synthesized from the same mRNA. Ribosomes are shown associated throughout the length of the mRNA. Ribosome A is also closely associated with translocase, giving rise to cotranslational translocation. Polypeptides synthesized by ribosomes B, C, and F have not yet docked at the translocase. Ribosomes D and E represent examples of translation-linked translocation in which substantial lengths of the nascent chain are exposed in the cytoplasm. Translocation of the polypeptide synthesized by ribosome G continues after translation termination, and the polypeptide synthesized by ribosome F is released into the cytosol before translocation is initiated (posttranslational translocation).

should influence the requirement for SecB and general molecular chaperones (Fig. 9). For example, tight coupling between translation and translocation should obviate the need for molecular chaperones (Fig. 9A). SecB dependence has been shown to decrease as signal peptide hydrophobicity is increased (92), possibly because this improves the chances of early docking and initiation of translocation (170) (but see the section on translocation competence, above). However, it is difficult to determine the respective chaperone requirements of co- and posttranslational export because truly cotranslational translocation (Fig. 8, ribosome A, and 9A) and translation-linked translocation (Fig. 9B) are not easily distinguished.

Energy requirements. Like solute transport, protein export is an energy-driven process; however, unusually, it requires both ATP hydrolysis (82, 84, 184) and the ΔpH or the $\Delta\psi$ components (21, 506) of the proton motive force (PMF) (83, 139, 619; for a review, see reference 182).

In vitro studies show that secretory protein translocation requires ATP hydrolysis by SecA protein (82, 184, 325), but this requirement has never been directly demonstrated in vivo, presumably because the affinity of SecA for ATP is so

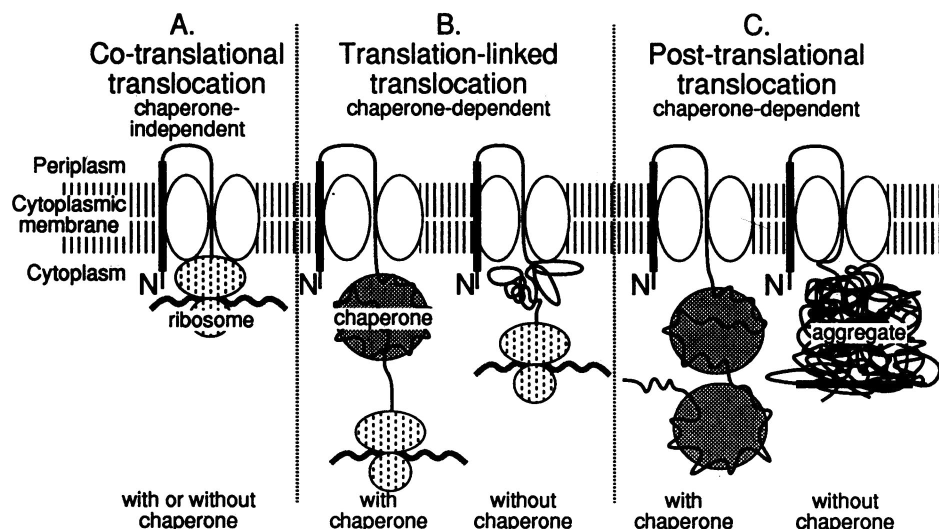

FIG. 9. Model for the chaperone dependency of the three different modes of translation-translocation coupling. (A) Cotranslational translocation is chaperone independent. (B) Translation-linked translocation is chaperone dependent because segments of the presecretory protein between the ribosome and the translocase may fold into a conformation that cannot be translocated. The ribosome may also exert some constraints on protein folding prior to translocation. (C) Posttranslational translocation requires chaperones to prevent premature folding that might lead to the formation of aggregates or other structures that cannot be translocated. N, amino terminus.

high that ATP levels cannot be reduced sufficiently to prevent export without stalling protein synthesis. However, the toxic effects of sodium azide were recently shown to be largely due to inhibition of SecA ATPase and consequent inhibition of protein export (166, 389). The low constitutive ATPase activity of SecA is markedly stimulated by acidic phospholipids, SecB protein, SecE/Y protein, and signal peptide and mature secretory protein sequences (102, 210, 325, 326).

Initially, it was thought that in vitro protein translocation was driven by ATP hydrolysis alone. Indeed, in vitro translocation can be driven to completion by using ATP alone, although higher levels of SecA and ATP are required than when a PMF is imposed (83, 183, 191, 506, 543, 615). However, recent studies suggest that the PMF is the driving force for translocation and that it may operate alone during periods when SecA is not bound or at late stages in translocation (139, 183, 486, 543). Furthermore, translocase-bound SecA exhibits high ATPase activity even when translocation is not occurring (486) or when it is driven backward by reversing the polarity of the PMF (137), which again implies that ATP hydrolysis does not drive translocation. Even when coupled to translocation, ATP hydrolysis occurs at rates that would be prohibitively high in vivo, implying that the in vitro system lacks an important control element (102).

Both acidic and basic residues close to the export initiation domain slow protein translocation in vitro, but their effects can be overcome by increasing either ΔpH or Δψ (262). Translocation of a presecretory protein completely devoid of charged residues is also improved by the imposition of a PMF. These data show that electrophoresis of positively charged residues or protonation (139) cannot alone (if at all) account for the requirement for the PMF, but there is evidence that proton antiport may occur during translocation (68, 139).

Thus, ATP hydrolysis may promote early stages of insertion and translocation, with the PMF completing the process once translocation is initiated. The PMF has also been proposed to affect the diameter of the translocation channel (262). For example, the presence of a small disulfide-bonded loop in a presecretory protein that can normally be translocated in vitro without imposing a PMF blocks translocation unless a PMF is established (544) (see the section on translocation competence, above). Since the disulfide bond is not reduced during translocation (542), the size of the channel may increase according to the potential applied to accommodate abnormally bulky structures that cannot be resolved by the putative unfoldase activity of SecA (see below). Intriguingly, in vivo studies show that different exported proteins require different PMF levels for efficient export (106). It should be possible to determine whether proteins which are exported in vivo despite having a considerable degree of tertiary structure (see, e.g., reference 457) (see the section on translocation competence, above) require a higher PMF than other, less tightly folded proteins. The PMF is also proposed to catalyze SecA-ADP displacement from secretory proteins (506) or ADP removal from SecA to permit it to recycle (504), and it is also proposed to induce conformational changes in secretory proteins (335), but none of these claims have been fully substantiated.

The exact role of ATP hydrolysis in translocation remains unclear. It may induce channel opening or the release of SecB protein bound to the precursor. SecB protein apparently prevents presecretory proteins from aggregating but not from adopting extensive tertiary structure (see the section on translocation competence, above). ATP hydrolysis by SecA may therefore catalyze the dissolution of residual tertiary structure that might otherwise impede translocation in reactions analogous to those performed by ATP-dependent general molecular chaperones (see the section on translocation competence, above). Alternatively, ATP hydrolysis may allow SecA to be released from the secretory polypeptide after distinct translocation steps. Replacement of ADP by ATP might then allow SecA to perform repeated unfolding reactions through continued dissociation from and reassociation with translocation intermediates, as though it were moving backward on an ascending escalator (Fig. 10) (137, 486).

The requirement for both the PMF and ATP hydrolysis for efficient translocation does not rule out the possibility (152) that energy derived from folding of secretory polypeptides actually pulls the trailing part of the protein through the translocation channel or prevents reverse movement of partially translocated protein (514). An example of how such a system might function is provided by studies on protein import into mitochondria, showing that the interaction of imported proteins with a molecular chaperone in the matrix of the organelle drives import (260, 343). This idea gains support from the recent observation by Wickner and his colleagues that the in vitro translocation of a short segment of a blocked translocation intermediate can occur in the absence of both SecA and an imposed PMF when the translocation block is released by allowing the untranslocated segment to unfold (602). Indeed, the ability of high levels SecA to promote in vitro translocation in the absence of an imposed PMF could be explained by the SecA-mediated dissolution of tertiary structure in nontranslocated segments of translocation intermediates, allowing the protein to be drawn through the translocation channel by further folding of the translocated segment. The possible role of molecular chaperones in posttranslocation folding in bacteria is discussed below (see the section on protein folding in the periplasm).

Release from the translocation channel. The final step in translocation is release from the translocation channel. The channel could simply close or collapse behind the last residue of the secretory protein. Alternatively, specific release factors might eject fully translocated secretory polypeptides from the channel and close it. Such factors ought to be part of the translocation channel complex, and mutations affecting their activity would be expected to block translocation. Genetic studies indicate that SecD and SecF are involved in a late step in translocation (38), in line with the fact that they differ from SecY and SecE in having relatively large periplasmic domains. Indeed, anti-SecD antibodies inhibit secretory protein release from the cytoplasmic membrane when added to spheroplasts (osmotically stabilized cells with permeabilized outer membranes that have been treated with lysozyme to degrade the peptidoglycan) (362a). Thus SecD and SecF might be "translocation release factors" or catalysts of translocation-coupled polypeptide folding on the periplasmic face of the membrane (see the section on general molecular chaperones, below). Their absence would thus reduce or block translocation in the same way as observed for protein import into mitochondria (260) or into the endoplasmic reticulum (374, 476) in the absence of the molecular chaperones that interact with newly translocated proteins.

Signal peptide cleavage is not required for translocation channel clearance, since naturally unprocessed secretory polypeptides (see the section on bitopic membrane proteins, below), presecretory proteins with altered signal peptidase

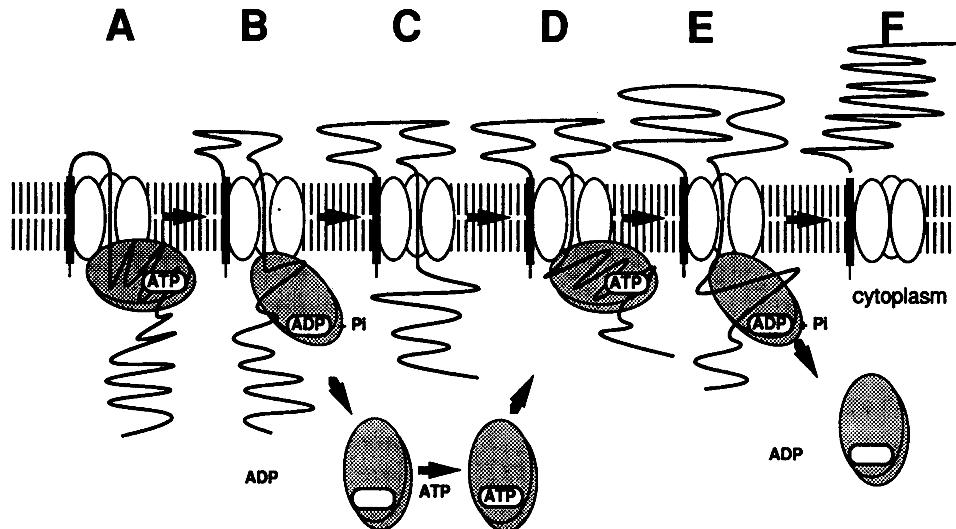

FIG. 10. Model for the recycling of SecA protein dimers during protein export. The cycle starts with signal peptide insertion and opening of the translocation channel. ATP-charged SecA (shaded) is shown bound to a prescretory protein and anchored to the translocase and probably partially embedded in the membrane lipids (A). ATP hydrolysis releases SecA from the presecretory protein, allowing translocation of the released segment and leaving ADP in the SecA nucleotide-binding site (B). SecA is then released into the cytosol (C), wherupon ADP is replaced by ATP, allowing SecA to reassociate with the presecretory protein further toward the C terminus (D). The cycle is then repeated until the protein is fully translocated (D to F). The signal peptide (black bar) is shown as being cleaved only after translocation is complete, but presumably it can be cleaved at any stage between A and F.

cleavage sites (158), or presecretory proteins made in the absence of signal peptidase (105) all have their extreme carboxy termini exposed on the periplasmic side of the cytoplasmic membrane despite remaining membrane anchored.

Anything Else?

The in vitro assay described above identifies essential requirements (a molecular chaperone, SecA, SecY, and SecE) for the translocation of a very small number of presecretory proteins but is less useful for characterizing nonessential components. Genetic techniques identified two other translocase components (SecD and SecF), but the selection procedures used are not necessarily exhaustive and other approaches have led to the identification of other components (see, e.g., reference 244). It is by no means evident that conditional-lethal mutations can be obtained in all genes coding for components of the translocation machinery, and existing *sec* mutations have been tested with only a limited number of presecretory proteins. This raises the possibility that other components of the protein export machinery have been overlooked. Furthermore, almost all of the studies carried out so far were restricted to *E. coli*. Although recent results indicate that homologs of some of the GSP components of *E. coli* are present in other bacteria (Table 1), it is still far too early to conclude that all features of protein export are the same in all bacteria.

Recent studies on the products of *SEC* and other genes involved in protein import into the endoplasmic reticulum of the yeast *S. cerevisiae* seem particularly pertinent to this point. Initial selections based on the conditional-lethal phenotype and altered cell density of nonsecreting mutants failed to identify mutations affecting this step in secretion. Such mutations were obtained only by selecting for mislocalization of secretory protein-cytoplasmic protein hybrids. Furthermore, different types of mutants were obtained when different hybrid proteins were used, supporting the idea that reduction of the level of a given SEC protein may have more drastic effects on some secretory proteins than on others (125, 195, 468, 527, 528). These observations point to potential drawbacks of using a limited battery of selection and screening procedures to study export-defective mutants.

A bacterial SRP? One component of the GSP that might have been overlooked in bacteria is the equivalent of the eukaryotic signal recognition particle (SRP). SRP is a multifunctional protein-RNA complex that binds to signal sequences as they emerge from the ribosome and retards translation of secretory protein mRNA until the complex has docked with its receptor on the surface of the endoplasmic reticulum. The signal sequence is then released from the SRP and can interact with the endoplasmic reticulum membrane, restoring translation and initiating translocation (430). GTP hydrolysis is required for signal sequence release (96, 453).

Two components of eukaryotic SRP, the 7S RNA backbone and the 54-kDa (actually 55.7-kDa) signal peptide-binding protein (430), have structurally related counterparts in bacteria (Table 1). In vitro reconstitution assays show that the 54-kDa protein can assemble on *E. coli* 4.5S RNA, the product of the *ffs* gene, which is predicted to fold in a conformation similar to SRP 7S RNA (419). Furthermore, this incomplete hybrid SRP is functional in in vitro SRP-dependent eukaryotic secretory protein translocation assays (418). Conversely, expression of the gene for mammalian 7S RNA in *E. coli* suppresses the lethal effects of Ffs RNA depletion (462), indicating that the two RNAs are at least partially interchangeable.

The sequence similarity between the 48-kDa *ffh* gene product (72, 466) and the 54-kDa protein of SRP includes the so-called M domain of the 54-kDa protein that is involved in signal sequence recognition (218, 338) and the carboxy-terminal region of the 54-kDa protein that includes a characteristic GTP-binding site (466). Ffs RNA and Ffh protein

can be coimmunoprecipitated with anti-Ffh antibodies, suggesting that they form a stable, functional complex (418). Intriguingly, the product of the *E. coli ftsY* gene (189) shows substantial sequence similarity to the alpha subunit of the SRP receptor in the endoplasmic reticulum membrane (430), leading to the suggestion that it might function as the membrane-bound receptor for a rudimentary *E. coli* SRP complex formed from Ffh and Ffs (466).

Are these observations purely coincidental, or does *E. coli* have a rudimentary SRP and an SRP receptor in the cytoplasmic membrane. If the latter is true, what are their roles in protein export? The only evidence that Ffh and 7S-like RNA might be involved in protein export is that pre-β-lactamase accumulates when SRP-like RNA is depleted (372, 418, 462) or when Ffh is produced at abnormally high levels (462). Processing of several other presecretory proteins is unaffected, although synthesis of some presecretory proteins is reduced (372, 462). It should be possible to demonstrate whether Ffh protein interacts with signal peptides on bacterial presecretory proteins by cross-linking experiments similar to those demonstrating the interaction between a eukaryotic signal peptide and SRP 54-kDa protein (293, 604). It will also be important to test the effects of reducing the levels of *ffh* gene expression on protein export.

Clearly, the only way to resolve this question is to determine the primary functions of the *E. coli ffs*, *ffh*, and *ftsY* gene products. GTP-binding proteins such as Ffh and FtsY are relatively rare in bacteria. Only elongation factor EfTu has been studied in detail, and most other GTP-binding proteins have no known function (344). *ftsY* is located in an operon of genes involved in cell division (189), which may provide a clue to its function. Ffs RNA is enriched in ribosome fractions of lysed cells (implying that Ffh should also be ribosome associated), and its depletion disrupts translation (54), but its possible involvement in general translation control can be discounted on both experimental (418, 462) and theoretical (65) grounds. The fact that *Halobacterium halobium* 7S RNA is enriched in membrane-bound polysomes synthesizing prebacterioopsin (196) suggests that it might have a specific role in the translation of secretory protein mRNA, although bacteriospin differs from the extensively characterized presecretory proteins of *E. coli* in being a polytopic membrane protein with a very restricted distribution and an atypical propeptide (579).

In conclusion, the available evidence does not support the idea (454) that SRP provides the main pathway for routing to the *E. coli* cytoplasmic membrane translocase and that SecA and SecB are components of a salvage pathway. We must concede, however, that Ffs, Ffh, and FtsY might function in parallel to SecAB to direct presecretory proteins to the translocase, that they might be involved in a hitherto untested aspect of secretory protein synthesis or traffic (such as polytopic membrane protein export, SecB-independent presecretory protein export, or GSP-independent secretion [see, e.g., reference 77a]), or that they are vestiges of a secretory pathway that is still functional in bacteria other than *E. coli*. In this respect, it is noteworthy that depletion of SRP components in *S. cerevisiae* reduces the secretion of only some proteins (most notably of an integral membrane protein) without affecting other presecretory proteins at all (208). Thus, if secretory protein traffic in eukaryotes had been studied first in yeasts rather than in pancreatic cells (430), the pivotal role of SRP might still not have been discovered.

Sec-Independent Translocation

E. coli can export a small number of presecretory proteins with typical signal peptides even when SecA and SecY proteins have been severely depleted (the requirement for other Sec proteins has not yet been tested). The classical example is the precursor of gpVIII (procoat) of filamentous phages such as M13 (601). gpVIII is an integral membrane protein which, in addition to a typical signal peptide, has an internal, membrane-spanning stretch of hydrophobic amino acids that anchors it in the cytoplasmic membrane and which is essential for export (178, 298, 299). pregpVIII can be imported into liposomes devoid of cytoplasmic membrane-derived proteins and is correctly processed by liposomes into which signal peptidase has been incorporated (185, 511, 591). Other proteins that are exported by Sec$^-$ mutants at nonpermissive temperatures include a derivative of LepB signal peptidase which has an inverted topology similar to that of pregpVIII, except that the signal sequence is not cleaved (575) (insertion of part of wild-type LepB into the cytoplasmic membrane is Sec dependent), and honeybee prepromelittin (90).

Sec-independent translocation presumably occurs though the lipid portion of the cytoplasmic membrane rather than through a protein channel (see the section on recognition and insertion membrane phospholipids, above). Indeed, the fact that export of certain presecretory proteins such as pregpVIII and prepromelittin can be completed in the absence of SecA and SecY suggests that at least the initial insertion and translocation of other, normally GSP-dependent presecretory proteins might occur in the absence of pilot proteins, signal peptide receptor, and translocase. Although the export of pregpVIII and of prepromelittin, like that of GSP-dependent presecretory proteins, requires a PMF (90, 107), it may be used for a completely different purpose in Sec-independent and Sec-dependent export. It should be noted, however, that the PMF dependence of pregpVIII and prepromelitin was tested only in strains with functional SecA and SecY proteins (90, 107).

In contrast to what one might expect, Sec-independent export of pregpVIII is not determined by special properties of its signal peptide, which can be replaced by signal peptides from other, GSP-dependent exported proteins without making pregpVIII export Sec dependent (297). The results of several studies showing that Sec-independent export of pregpVIII is determined by the mature part of the polypeptide, especially by the second hydrophobic segment and its flanking charged residues, led Kuhn and his colleagues to propose that electrostatic interactions between basic residues in the N region of the signal peptide (295) and downstream from the second hydrophobic segment and acidic phospholipids are followed by concerted insertion of both segments (Fig. 11). Interestingly, positively charged residues can be tolerated in the region just downstream from the signal peptide of gpVIII (299), whereas they are not tolerated in this position in proteins whose export is Sec dependent.

The nontranslocated carboxy terminus of gpVIII can be extended without affecting Sec independence (297), but extending the translocated region between the two hydrophobic segments abolishes Sec independence in the same way as the removal of the second hydrophobic segment does (296). The insertion of "inverted" LepB signal peptidase into the cytoplasmic membrane may be similar to that of pregpVIII, but export of prepromelittin (107) is somewhat different. Although promelittin is highly hydrophobic and

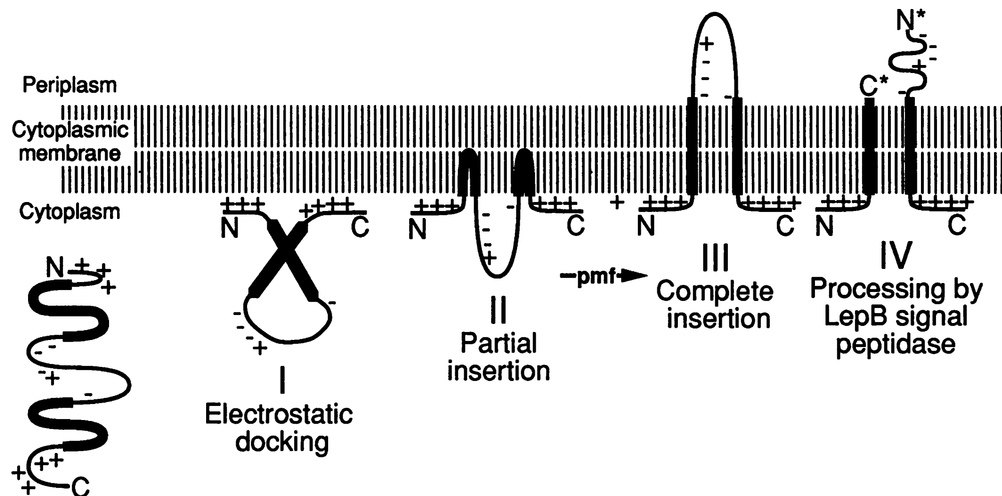

FIG. 11. Model for the posttranslational insertion and export of pregpVIII, the precursor of the major coat protein of filamentous phages such as M13 (procoat). The precursor contains two segments of relatively high overall hydrophobicity, the signal peptide and the membrane anchor. Electrostatic interactions between basic amino acids at each end of the precursor and negatively charged phospholipid head groups (I) are followed by partial, simultaneous insertion of both hydrophobic segments (II) and subsequently by PMF-dependent translocation of the central segment to the periplasmic side of the membrane (III) and cleavage of the signal peptide by LepB signal peptidase (IV). + and −, approximate positions of basic and acidic residues, respectively; N, amino terminus; N*, processed amino terminus; C, carboxy terminus; C*, processed carboxy terminus.

might therefore have a functional equivalent of the second hydrophobic segment of pregpVIII, the mature protein is recovered from the periplasm rather than the cytoplasmic membrane. However, extending the translocated segment by tagging it with dihydrofolate reductase abolishes Sec-independent export (107).

These observations on pregpVIII and prepromelittin suggest that only relatively short stretches of polypeptide can be translocated in the absence of the Sec export factors. As noted above, however, Sec independence may represent an artificial situation, since it is not clear from the published literature whether pregpVIII or prepromelittin export involves Sec proteins under normal conditions. If it does not, one might assume that the translocated segments are not long enough to provide binding sites for GSP components such as SecA. It is therefore important to determine whether there is an exact cutoff point to Sec-independent export and whether presecretory proteins can be engineered so that they cannot be secreted because they are both too long for Sec-independent export and too short to interact with SecA or other Sec proteins.

From being considered a model protein for export in *E. coli*, pregpVIII has declined to the level of an interesting oddity. However, pregpVIII could be an excellent, simple model for the export and assembly of other, more complex polytopic cytoplasmic membrane proteins that may be exported in a very similar way (see the section on integral cytoplasmic membrane proteins, below). In particular, the results of studies on pregpVIII remind us that the mere presence of a cleavable signal peptide does not automatically mean that a secretory polypeptide will interact with Sec proteins to be exported by the GSP.

Processing

Signal peptidases. Three types of bacterial signal peptidase have been identified. Most presecretory proteins are cleaved 5 to 7 residues downstream from the H domain by the major signal peptidase (LepB protein in *E. coli*; for a review, see reference 104), an integral cytoplasmic membrane protein that recognizes sequences with small amino acids at positions −1 and −3 (usually A or G) and a turn-inducing residue (usually G or P) at position −6 with respect to the cleavage site (141, 403, 571, 572) (Fig. 3). Peptides that include and extend beyond this region are cleaved by LepB, whereas mutations that alter the recognition sequence or amino acids flanking it in presecretory proteins can block processing (27, 127, 130, 158, 410, 471). Certain genetically altered presecretory protein variants bind avidly to LepB without being cleaved, leading to titration of the enzyme and to inhibition of cleavage of other presecretory proteins (27), whereas drastic alteration of the LepB recognition sequence may result in processing at alternate sites. Some of these resemble consensus LepB cleavage sites (157), and others do not (410).

Polytopic membrane proteins (see the section on integral cytoplasmic membrane proteins, below) are usually not cleaved by LepB signal peptidase. One apparent exception is cytochrome c_1 of the gram-negative bacterium *Bradyrhizobium japonicum*. This particular cytochrome c_1 is the carboxy-terminal segment of a tripartite precursor protein of which the polytopic cytoplasmic membrane protein cytochrome *b* is the amino-terminal segment. The hydrophobic endopeptide between the two, which is not found in other *b* cytochromes, resembles a typical signal peptide with a LepB cleavage site at its carboxy-terminal end. The two halves of the hybrid protein are normally separated by proteolytic cleavage at precisely this site, and, furthermore, destruction of the LepB consensus recognition sequence abolishes cleavage (547). A similar situation was observed with an artificially constructed hybrid protein comprising alkaline phosphatase (PhoA) fused to the third periplasmic domain of SecY protein in *E. coli*. This hybrid was cleaved by LepB at an artificially created consensus LepB site between the SecY and PhoA segments (6). These results suggest that LepB could process at least the last periplasmic segment of other

polytopic membrane proteins if they had appropriate cleavage sites. Eukaryotic signal peptidase may also have this property (32). A recently designed LepB cleavage cassette (377) might be used to test whether internal periplasmic loops of polytopic membrane proteins can be cleaved by LepB and hence provide a method for studying the kinetics of polytopic membrane protein insertion into the cytoplasmic membrane.

LepB does not belong to any of the four recognized major classes of proteases (serine protease, aspartate protease, cysteine protease, or metalloprotease), but it does have essential serine and aspartate residues (41, 536). Other residues essential to its activity have not yet been identified, and the precise location of its catalytic site and details of its mode of catalysis await further investigation. It should be possible to purify the periplasmic, carboxy-terminal segment of LepB that carries the catalytic site (39), to study its interaction with model peptides, and to determine its structure. LepB is estimated to be present at approximately 500 copies per cell (606), close to the most recent estimate of the number of translocase complexes (see the section on integral membrane Sec proteins, above).

Lipoprotein signal peptidase (LspA or signal peptidase II) is a polytopic cytoplasmic membrane protein (369) which is structurally unrelated to LepB, cleaves only presecretory proteins with a cysteine residue immediately after the cleavage site, and is characteristically inhibited by the antibiotic globomycin (126, 238). The cysteine residue must be converted to glycerylcysteine before cleavage occurs, and it is usually further modified by the addition of two fatty acids to the glyceryl residue and a fatty acid to the free amino group after signal peptide cleavage. Lipoproteins are readily identified by the metabolic incorporation of radioactive palmitate. Their signal peptides differ in several ways from those recognized by LepB; they are shorter and have a more hydrophobic H region, lack the G or P residue typically found at or around position -6, and have a leucine residue at position -3 (574) (Fig. 3). The importance of these differences is indicated by the fact that replacement of the cysteine residue, even when it leads to the creation of a potential LepB cleavage site, does not result in efficient cleavage by LepB (213, 287, 412, 413).

The third, most recently identified signal peptidase, type IV prepilin peptidase (385) (Table 1), is a polytopic cytoplasmic membrane protein that, unlike LepB and LspA, cleaves signal sequences on the cytoplasmic side of the membrane. Prepilin peptidase has been found in both gram-negative and gram-positive bacteria. A gene coding for a protein with significant sequence similarity to characterized prepilin peptidases may exist in the *E. coli* K-12 chromosome, but neither the gene product nor its activity has been identified (600). The cleavage site is highly conserved (Fig. 3) (143, 283, 437, 531). Prepilin peptidases cleave a very small number of precursor proteins, including some which have been found in certain *E. coli* strains (191), which either are the subunits of type IV pili (264, 283, 531) or are required for secretory protein translocation across the outer membrane (24, 386, 437) or transformation (probably DNA uptake) in *B. subtilis* (8) (see the section on the main terminal branch of the GSP, below) (Table 1).

Does cleavage indicate translocation? Two questions that have never been satisfactorily answered are whether signal peptidases are part of the translocase and whether cleavage indicates that complete translocation has occurred. Cleavage by all three peptidases occurs rapidly, often within the time span of translation. Insertion of the signal peptide and export initiation domain into the membrane may be sufficient for cleavage since the catalyic site of LepB is on the periplasmic face of the membrane and since processing of blocked translocation intermediates has been reported in vivo (455) (see the section on Sec-independent translocation, above). Furthermore, LepB can be purified in its active form in the absence of all other proteins, suggesting that it is not tightly associated with the translocase (606). On the other hand, translocase-depleted cytoplasmic membrane vesicles do not process presecretory proteins (see, e.g., reference 155). Indeed, the absence of signal peptide processing in in vivo and in vitro experiments is routinely considered to indicate that translocation has not occurred, even though translocation of the catalytic carboxy-terminal region of LepB through the membrane is itself Sec dependent (318). The situation with prelipoproteins is even more complex since they are processed by LspA only after being modified by glyceryl transferase, implying that the two enzymes may be associated. Prepilin peptidase is somewhat different because cleavage occurs on the cytoplasmic face of the membrane and may be Sec independent (264).

Eukaryotic signal peptidases. Endoplasmic reticulum signal peptidase has the same cleavage specificity as LepB signal peptidase of *E. coli* (592) but is composed of several polypeptides, some of which are very similar to each other but all of which are quite distinct from LepB (45, 614). Chloroplasts and mitochondria also have an enzyme with similar cleavage site specificity to LepB (14, 205, 492), and the sequence of the yeast mitochondrial enzyme is similar to that of LepB (31). The mitochondrial and chloroplast enzymes process proteins that are translocated from the mitochondrial matrix across the inner membrane or from the chloroplast stroma into or across the thylakoid membranes, respectively. Since these proteins have amino-terminal signal peptide-like sequences, it would be interesting to determine whether other proteins involved in thylakoid protein import or in membrane protein assembly in mitochondria or chloroplasts are related to *E. coli* Sec proteins.

INTEGRAL CYTOPLASMIC MEMBRANE PROTEINS

Integral membrane proteins exhibit a wide variety of different topological states (42) (Fig. 12). Bi- and polytopic membrane proteins have at least one stretch of hydrophobic amino acids embedded in the lipid bilayer, with one extremity exposed on each side of the membrane. These transmembrane segments are not subject to sequence constraints other than minimal length (>12 residues) and hydrophobicity (110), and there appear to be no major differences between them and signal peptides. Monotopic proteins are only partially embedded in the lipid bilayer. This section is devoted to how these proteins are translocated and inserted into the membrane and how their topology is established.

Bitopic Membrane Proteins

Bitopic membrane proteins are divided into three classes, depending on their orientation and whether they are made as precursors. The carboxy terminus of type Ia and Ib membrane proteins is in the cytoplasm, whereas that of type II membrane proteins is in the periplasm (Fig. 12).

Type Ia bitopic membrane proteins are made as signal peptide-bearing precursors that are processed by LepB-type signal peptidase. A second hydrophobic region (stop transfer/membrane anchor signal) anchors them in the cytoplasmic membrane. Export of the most extensively character-

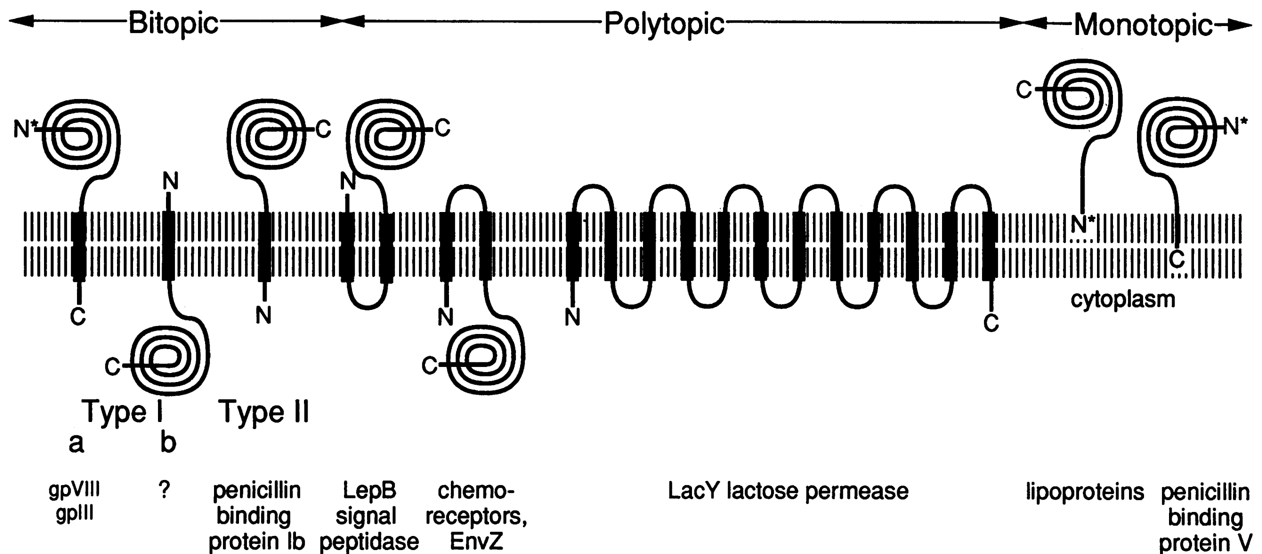

FIG. 12. Examples of the topologies adopted by bacterial cytoplasmic membrane proteins. Bitopic membrane proteins have a single hydrophobic segment (thick line) that may span the membrane in either orientation depending on the polarity of the initial insertion event and on the processing of the signal peptide. The hydrophobic segment need not be located at the extremities of the proteins. Polytopic membrane proteins span the membrane at least twice and are usually not processed by signal peptidase. Monotopic proteins are peripherally associated with the membrane by anchors located at one or other extremity (as shown) or possibly elsewhere in the protein. N, amino terminus; N*, processed amino terminus (after cleavage by signal peptidase); C, carboxy terminus.

ized type Ia membrane protein, gpVIII, is probably Sec-independent (see the section on Sec-independent translocation, above). Export of other natural type Ia membrane proteins, such as filamentous phage protein gpIII (109, 110) or of artificial type Ia membrane proteins generated by inserting stop transfer signals within the mature segments of periplasmic proteins, is assumed to be Sec dependent because the translocated segment between the signal peptide and the membrane anchor is usually longer than in pre-gpVIII. The initial stages in the working model for the Sec-dependent export of these type Ia bitopic membrane proteins (Fig. 13) are identical to the export of soluble presecretory proteins, but continued translocation eventually leads the stop transfer signal into the translocation channel, which is proposed to open laterally to allow it to diffuse into the lipid bilayer. This event, which would be the reverse of that which initially induces lateral channel opening, is probably induced by the entry of a region of high overall hydrophobicity into the water-lined translocase channel.

Type Ib membrane proteins have a single, unprocessed stretch of hydrophobic amino acids (the insertion signal) at

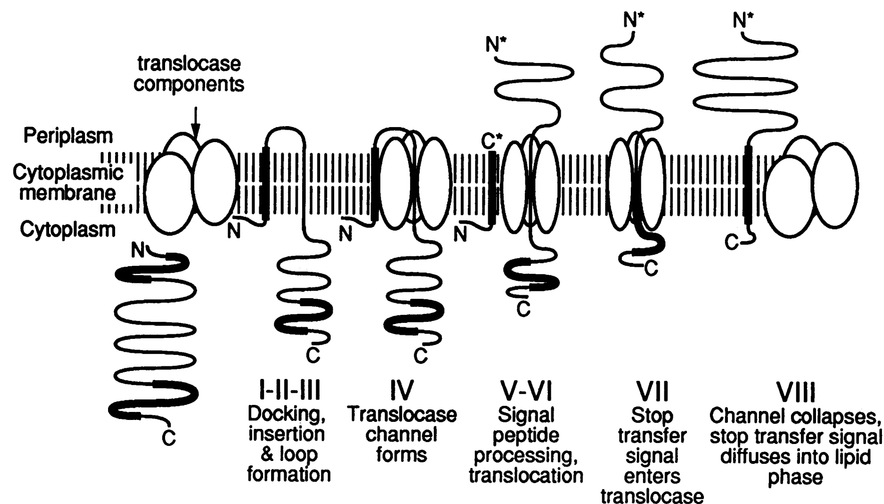

FIG. 13. Model for the Sec-dependent insertion of type Ia bitopic membrane proteins into the cytoplasmic membrane. Initial insertion (I to III) is followed by lateral opening of the translocase channel to engulf the export initiation domain. Translocation continues with simultaneous signal peptide cleavage (V and VI) until the second hydrophobic segment (stop transfer/membrane anchor signal; thick line) enters the channel (VII) to provoke its lateral opening, allowing the anchor to diffuse into the lipid phase of the membrane (VIII). SecA and chaperones are omitted for clarity. N, amino terminus; N*, processed amino terminus; C, carboxy terminus; C*, processed carboxy terminus.

their extreme amino terminus. This inserts and remains embedded in the lipid bilayer in a head-first orientation. The translocated segment is normally at the amino-terminal end of the insertion signal. *E. coli* does not appear to have natural type Ib membrane proteins (the classical example is cytochrome P-450 of the eukaryotic endoplasmic reticulum [479, 539, 540]), but the amino-terminal segment of LepB signal peptidase is a useful model (LepB, a polytopic membrane protein, can be considered to be a hybrid composed of type Ib [amino-terminal] and type II [carboxy-terminal] segments [Fig. 12]). Head-first insertion is caused by the presence of a cluster of basic amino acids at the carboxy-terminal end of the insertion signal (313, 575) (see the section on recognition and insertion, above) and is Sec independent, in line with the fact that very few hydrophilic amino acids are translocated through the membrane (318). The ability of the insertion signal to promote the translocation of an extended upstream region to the periplasmic side of the membrane does not appear to have been examined.

Type II membrane proteins are similar to periplasmic proteins except that the signal sequence (often called signal sequence-membrane anchor, or simply signal-anchor) is not cleaved, presumably because recognition sequences for LepB cleavage are either absent or incorrectly positioned. Depletion of LepB signal peptidase or abolition of LepB cleavage sites from periplasmic proteins causes them to remain embedded in the membrane in type II configuration (see the section on signal peptidases, above). Type II proteins differ from type Ib proteins only in the orientation in which they insert. The signal sequence-membrane anchor of most bacterial type II membrane proteins is usually located close to the amino terminus.

Polytopic Membrane Proteins

Polytopic cytoplasmic membrane proteins span the membrane at least twice via segments of mainly, although not necessarily exclusively, hydrophobic amino acids. Each of these segments is potentially capable of targeting the nascent polypeptide to the cytoplasmic membrane and of initiating its insertion by interacting with membrane lipids in the same way as signal peptides (see the section on signal peptides, above). This provides the basis from which to address the following questions. (i) Does each transmembrane segment interact independently with the cytoplasmic membrane, or is one or a limited number of them specifically required? (ii) Is the export of translocated segments of cytoplasmic membrane proteins Sec dependent? (iii) How is the orientation of each transmembrane segment determined?

The export of complex polytopic cytoplasmic membrane proteins is far less well documented than that of soluble presecretory proteins because they are generally present in only small amounts, are not processed by signal peptidase, and are difficult to study kinetically. Several methods, including gene fusions and protease accessibility, have been used to determine the topology of membrane proteins (Fig. 14). When interpreted with caution, such analyses provide an accurate picture of the overall topology of polytopic membrane proteins but little direct information on how proteins are assembled in the membrane. Analyses of residues in and around known or predicted transmembrane segments revealed that there were significantly more positively charged amino acids in the cytoplasmic loops of integral membrane proteins than in periplasmic loops, especially when the cytoplasmic loops were relatively short or when only the first few amino acids at each end of the hydrophobic segment were considered (573, 576, 578). The "positive inside rule," which is based on these observations, proposed that positively charged amino acids determine the orientation of the adjacent transmembrane segment(s), as they do with signal peptides (see the section on membrane phospholipids, above).

Because the number of proteins that have been analyzed is small (mainly the LacY lactose permease, the MalF component of the maltose permease, and the SecY component of the secretory protein translocase of *E. coli*), models for insertion and topogenesis with be presented first.

Working models. The "sewing-machine model" for Sec-dependent membrane protein topogenesis proposes that the first, amino-proximal segment that has a net positive charge at its amino-terminal end is an uncleaved signal sequence (Fig. 15). The trailing part of the polypeptide is translocated until the second hydrophobic segment with basic amino acids at its carboxy-terminal end (stop transfer signal/membrane anchor) enters the translocase, causing it to open laterally. This part of the model is identical to that proposed earlier for the Sec-dependent export of type Ia membrane proteins (see the section on bitopic membrane proteins, above), except that the signal sequence is not cleaved. Proteins that do not have other hydrophobic segments will remain embedded in the membrane by the two hydrophobic segments, with their carboxy-terminal tail in the cytosol (Fig. 12). A third, downstream hydrophobic segment with basic residues at its amino-proximal end (secondary signal sequence) will, however, reinitiate translocation, which continues until another stop transfer signal/membrane anchor enters the channel. Polytopic membrane proteins are thus built up by the independent, sequential action of a series of signal sequences and stop transfer signals (42) whose activity depends their position and on the distribution of charged residues at either end.

The alternative, Sec-independent model (Fig. 16) proposes that polytopic membrane protein topogenesis is initiated by the spontaneous insertion into the membrane of pairs of hydrophobic segments or of miniloops that are eventually resolved so that each hydrophobic segment is flanked on its cytoplasmic side by positively charged amino acids. This model is an extension of that proposed for the export of gpVIII (Fig. 11) (see the section on Sec-independent translocation, above), except that processing by LepB signal peptidase does not occur. The molecular events involved may be similar to those proposed to occur as colicin A penetrates into the cytoplasmic membrane from the periplasmic side in susceptible bacteria (397, 558).

The data. Different segments of the same polytopic membrane protein may have different modes of insertion. For example, short periplasmic loops may be translocated by the Sec-independent pathway, whereas export of longer periplasmic loops may be Sec dependent. This confuses the interpretation of the limited experimental data that are available. Since transmembrane segments of polytopic membrane proteins are generally highly hydrophobic, it is not surprising that many of them can function as signal sequences when fused to a suitable reporter protein. However, only "odd-numbered" transmembrane segments of LacY, which normally have their amino terminus on the cytoplasmic side of the membrane, function as efficient signal sequences when tested in this way (76) (Fig. 14B). Thus, the orientation of the transmembrane segments seems to be respected when they are taken out of context. Since the even-numbered transmembrane segments are often followed by basic amino acids near their carboxy-terminal ends, they

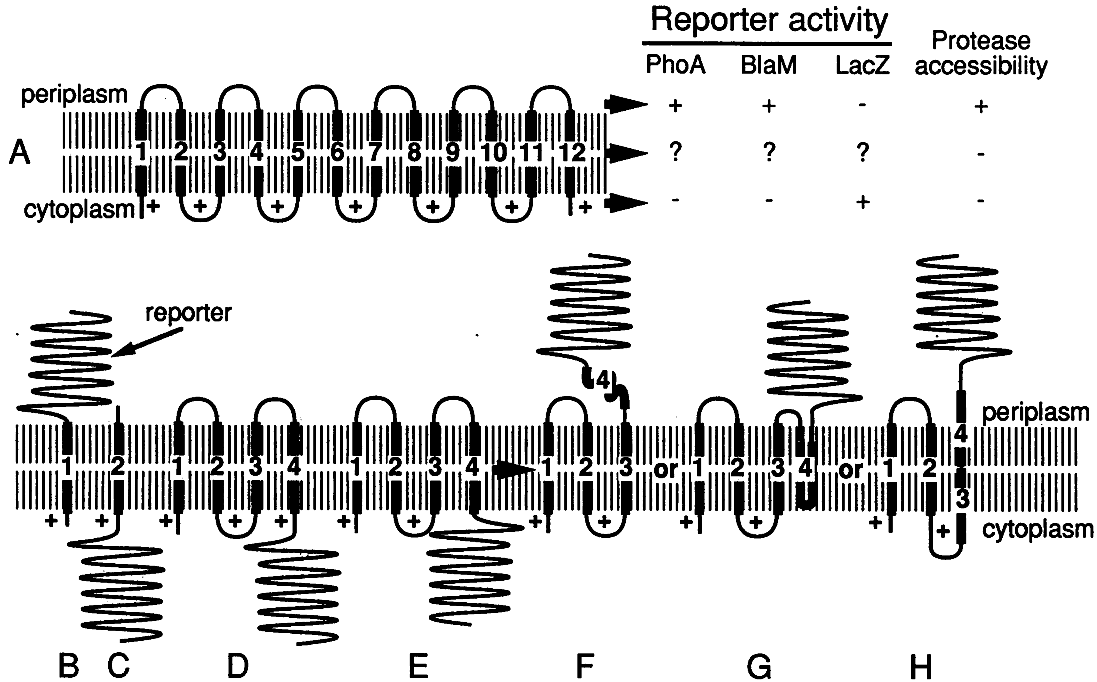

FIG. 14. Schematic representation of the use of reporter proteins and protease accessibility to determine the topology of cytoplasmic membrane proteins. Example A is a typical polytopic membrane protein such as LacY. The 12 transmembrane segments (numbered) are all oriented such that the hydrophilic loops with the greatest positive charge (or strongest insertion polarity signal; +) are exposed on the cytoplasmic side of the membrane. Alkaline phosphatase reporter (PhoA) has enzymatic activity only when fused to periplasmic loops of the protein. Likewise, β-lactamase reporter (BlaM) is active, and therefore able to confer high-level resistance to penicillins, only when fused to periplasmic loops, whereas β-galactosidase (LacZ) is active only when fused to cytoplasmic loops. The behavior of reporter proteins fused to the transmembrane segments is less well defined. Only periplasmic loops of the protein are cleaved by proteases when the cells are converted into spheroplasts. Examples B to H represent topologies of truncated variants of the protein shown in panel A in which the reporter is fused after different transmembrane segments. Examples B and C contain only transmembrane segments 1 or 2. D is a typical example of the topology of a hybrid protein in which the reporter is fused after the positively charged residues following an even-numbered transmembrane segment. If the reporter is alkaline phosphatase, the hybrid will not be enzymatically active. If, however, the hybrid is constructed in such a way that the junction between the membrane protein and reporter is upstream from the positively charged residues, it will have enzymatic activity and must therefore adopt one of the topologies represented in F to H.

should insert in a "head-first" orientation in the same way as the insertion signals of type Ib membrane proteins (Fig. 14C) (353).

In hybrid proteins in which the reporter protein is preceded by several membrane-spanning segments, the reporter is usually exported only when fused to periplasmic loops (428), but Beckwith and his colleagues found that a reporter could be exported when fused to a predicted cytoplasmic

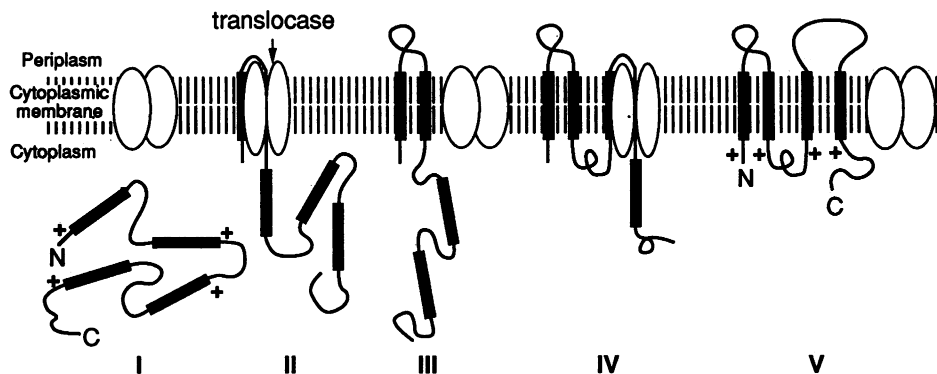

FIG. 15. Sewing-machine model for the Sec-dependent export and topogenesis of a polytopic cytoplasmic membrane protein. The secretory protein shown has four hydrophobic segments (thick lines) and is presumed to be exported posttranslationally (I). Initial interaction between the N-terminal signal sequence and the membrane leads to opening of the translocase channel and to translocation (II). The entry of the second hydrophobic segment (stop transfer/membrane anchor signal) into the channel provokes its lateral opening, allowing the anchor segment to diffuse into the lipids (III). The cycle is reinitiated by the third hydrophobic segment (secondary signal sequence) and so on until the final topology is established (IV and V). +, regions likely to contain several positively charged residues.

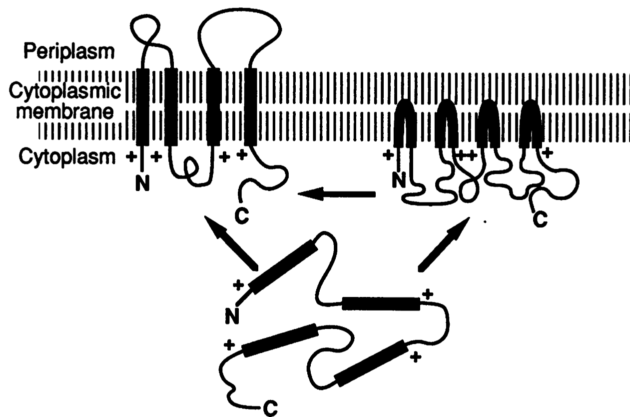

FIG. 16. Model for Sec-independent export and topogenesis of a polytopic cytoplasmic membrane protein. The protein is shown as inserting posttranslationally into the membrane, with all four transmembrane (thick lines) segments inserting simultaneously as loops (right) which are then resolved so that regions rich in positively charged residues (+) remain predominantly on the cytoplasmic side of the membrane. Alternatively, the transmembrane segments may pair up prior to insertion (left). N, amino terminus; C, carboxy terminus.

segment of the MalF protein (58). This particular hybrid lacked a basic residue normally present in the cytoplasmic loop, and this altered the behavior of the preceding hydrophobic segment (Fig. 14E to H) (57, 353). Interestingly, the reporter in this hybrid reached the periplasm more slowly than when it was fused to an authentic periplasmic loop (554), implying that basic residues in cytoplasmic loops prevent the preceding hydrophobic segment from slipping through the membrane or from forming a double loop (Fig. 14E to F) after the initial topology has been established. Alkaline phosphatase export is normally Sec dependent. Thus, at least part of the observed kinetic delay in alkaline phosphatase export in this hybrid might be explained by the slow recruitment of translocase to complete the export of a hybrid whose initial insertion and export were Sec independent. Similar studies with SecY-alkaline phosphatase hybrids (618) confirmed the importance of positively charged residues in cytoplasmic loops of periplasmic proteins as topological determinants.

Some membrane-spanning segments have less than optimal hydrophobicity owing to the presence of polar residues that are essential to protein function. For example, transmembrane segment 9 of LacY, which has an arginine residue (R302) near its center, has relatively low signal sequence activity when fused to a reporter protein (76). This would appear incompatible with the idea that it acts as an independent signal sequence to promote the translocation of the following hydrophilic segment, as it would in the Sec-dependent model. In the Sec-independent model, however, this segment could insert together with transmembrane segment 10, which has a negatively charged glutamate residue that could pair with and neutralize R302 (76). This provides the second line of evidence against the sewing-machine model for membrane protein export and topogenesis.

In the Sec-dependent model, the first transmembrane segment plays a pivotal role, both promoting the initial interaction with the membrane and establishing the orientation of all other transmembrane segments. Removal of this segment might therefore cause the stop transfer/membrane anchor signal that normally follows it to function as a signal sequence, which would result in a complete reversal of cytoplasmic and periplasmic loops and necessitate translocation of positively charged cytoplasmic loops to the periplasmic side of the membrane (Fig. 17B). The first 22 residues of LacY (including the first half of the first transmembrane segment) are not essential for export and function (35), and deletion of the first transmembrane segment of MalF does not prevent correct assembly (145). The topology of these LacY and MalF variants is presumably similar to that shown in Fig. 17C, in which the now amino-proximal hydrophobic segment (originally the second hydrophobic segment) functions as an independent insertion signal in the same way as the insertion signal of LepB signal peptidase (see the section on bitopic membrane proteins, above). A similar situation occurs when internal segments are removed (Fig. 17D to F). Removal of an even number of transmembrane segments should not alter the overall topology (Fig. 17D), but removal of an odd number of hydrophobic segments might be expected to reverse the orientation of upstream or downstream segments (e.g., Fig. 17E). This does not always occur, however (36, 353), implying that at least one of the normally transmembrane segments can adopt an unusual configuration to allow all other membrane-spanning segments to adopt their normal, positive-inside orientation (Fig. 17F to H). Even individually synthesized, overlapping segments of LacY assemble into a partially functional permease (34, 613). This implies that internal segments of the polypeptide not only direct export and topogenesis but also can assemble correctly in the membrane, as do subunits of multicomponent permeases such as MalF-G.

Besides providing strong experimental support for the idea that hydrophilic, generally positively charged cytoplasmic loops are important determinants of polytopic membrane protein topogenesis, these results also tend to support the Sec-independent model for membrane protein insertion. They can be accommodated, however, by a variant of the Sec-dependent model in which odd-numbered transmembrane segments act independently of their neighbors to promote the export and assembly of downstream polypeptide segments up to and including the next stop transfer signal. Clearly, more direct methods are required to measure the Sec dependence of polytopic membrane protein export. Unfortunately, reporter proteins cannot be used because they represent a considerable proportion of the total length of the hybrid polypeptide and may have completely different requirements from those of the membrane protein to which they are fused. Furthermore, direct studies of the Sec dependence of LacY insertion gave apparently conflicting results. In one study, *E. coli* mutants with reduced levels of functional SecY protein accumulated more LacY in the soluble cell fraction than the parent strain did (245); this indicates Sec-dependent export. In another study, however, a membrane-associated, nonintegrated form of LacY produced by cells with multiple copies of the *lacY* gene could be extracted in soluble form and reconstituted into liposomes to give an active lactose permease (465), implying that LacY can insert into membranes in the correct configuration in the absence of *sec* gene products. SecY can also integrate in functional form into inverted *E. coli* cytoplasmic membrane vesicles in which resident SecY has been inactivated (538). Furthermore, recently published results by McGovern and Beckwith suggest that MalF protein requires neither SecA nor SecY for its membrane insertion (352a).

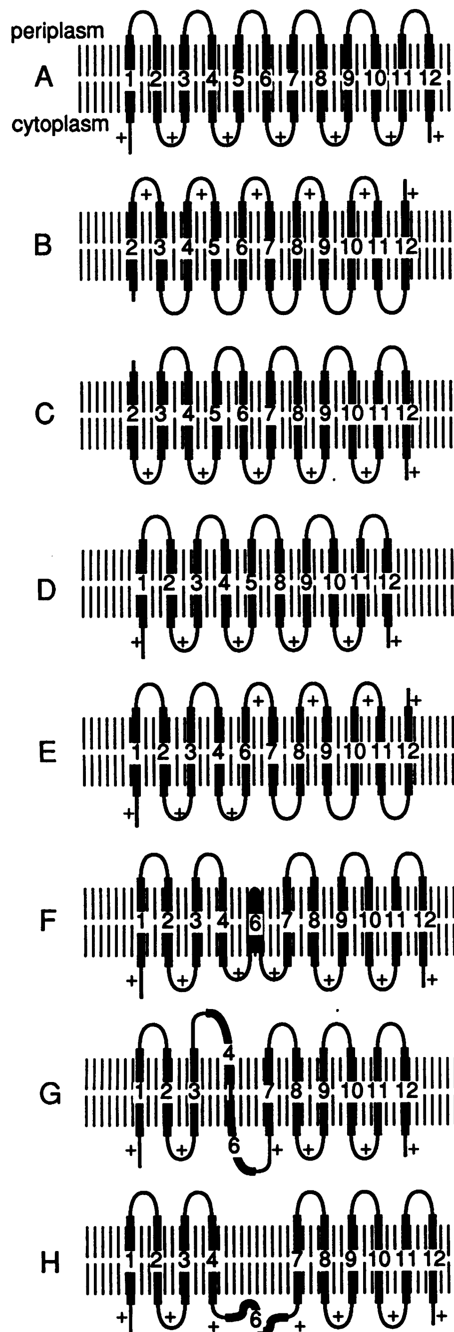

FIG. 17. Possible effects of deletions that remove hydrophobic transmembrane segments on the organization of a polytopic cytoplasmic membrane protein. (A) Wild-type protein. (B and C) Possible conformations resulting from removal of the first transmembrane segment. Conformation C is the only one compatible with experimental observations (see the text). (D) Effects of removing two internal transmembrane segments. (E to H) Possible conformations adopted following the removal of a single internal transmembrane segment. Conformations F to H are compatible with experimental observations (see the text). +, hydrophilic segments with abundant positively charged amino acids.

Some circumstantial evidence also supports the Sec-independent insertion model. For example, polytopic membrane proteins would have to recruit and then dispense with translocase components several times during their progressive, Sec-dependent insertion into the membrane, which is difficult to accept in view of the limited number of translocation sites (approximately 500) in the membrane. On the other hand, how can SecA protein be prevented from binding to signal sequences and downstream regions in large polytopic membrane proteins unless they are inserted cotranslationally? Perhaps SecA recognizes only long hydrophilic translocated sequences (see the section on Sec-independent translocation, above), in which case most polytopic cytoplasmic membrane proteins may not be bound by SecA because they have very short periplasmic loops. In this respect, the reported Sec independence of MalF insertion (352a) is particularly surprising because there are no precedents for the Sec-independent export of polypeptide segments as large as the ca. 180-residue periplasmic loop between the third and fourth transmembrane segments of this protein (58, 175, 353) (see the section on Sec-independent translocation, above).

In conclusion, the available evidence, although mainly in favor of Sec-independent insertion, does not distinguish between the two possible modes of polytopic membrane protein export. Novel polytopic membrane proteins engineered to contain short, protease-susceptible inserts in periplasmic loops could be used to determine protease accessibility on the periplasmic side of the membrane to provide a way of comparing export kinetics in wild-type and Sec$^-$ cells. As mentioned above, further studies of pregpVIII protein might provide important clues to the mechanism of assembly of more complex polytopic membrane proteins. For example, it might be possible to fuse several pregpVIII genes to create pregpVIII dimers or higher oligomers with alternating signal peptides and membrane anchors that might be processed by signal peptidase at internal sites. The kinetics and Sec dependence of cleavage at these internal sites could then be measured. The possible involvement of cytoplasmic molecular chaperones, and particularly SecB, and energy requirements for insertion also remain to be investigated.

Monotopic Membrane Proteins

Bacteria have three types of monotopic proteins. Peripheral membrane proteins such as SecA (60, 74), MalK (108, 507), and HisP (269) are anchored to the cytoplasmic side of the cytoplasmic membrane. Although they are thought to be at least partially embedded in the lipid bilayer (60, 269), their membrane association is probably strengthened by interactions with other, integral membrane components of the secretory translocase or the permeases of which they are part. They do not have signal sequence-like regions of high overall hydrophobicity, and their membrane association is probably spontaneous and accompanied by conformational changes induced, for example, by nucleotide binding (60, 556a).

Proteins with amphipathic helical anchors. In theory, secretory proteins that are released into the periplasm could reassociate with the cytoplasmic membrane if they have suitable N- or C-terminal membrane-penetrating anchors. The membrane anchor of one such protein, *E. coli* penicillin-binding protein 5/carboxypeptidase (DacA), is an 18-residue, C-terminal, amphipathic helix (247, 248, 425, 426). When mixed with cytoplasmic membrane-derived vesicles, DacA appears to exist in one of two states: one, embedded in the

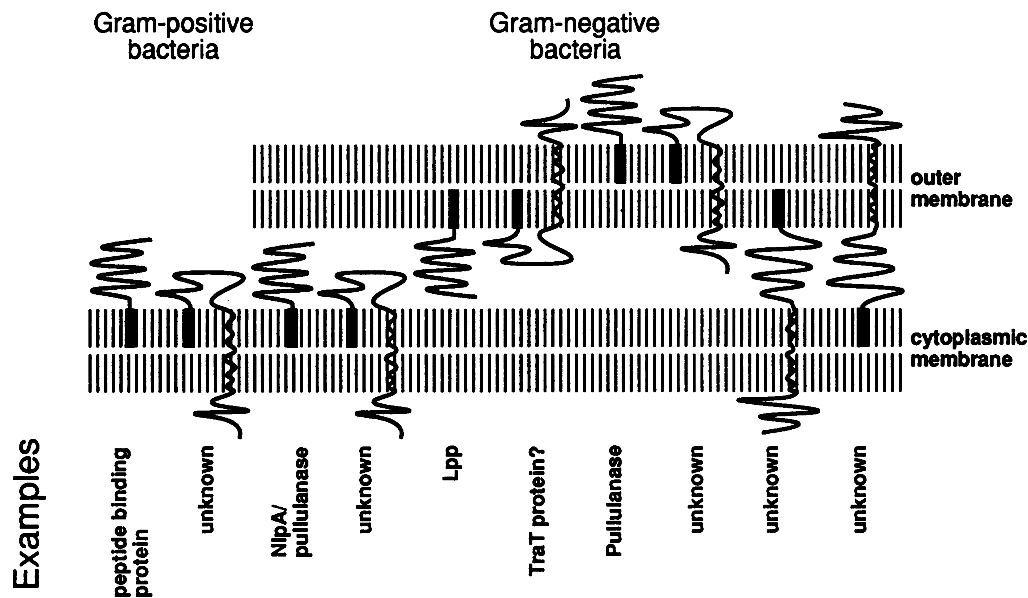

FIG. 18. Possible topologies of lipoproteins in gram-positive and gram-negative bacteria. Fatty acids are indicated by thick black lines.

lipid bilayer, that cannot be stripped off the membranes with urea, and a second, more loosely associated, urea-extractable form which can be shifted to the firmly embedded form by lowering the pH (409). The overall positive charge of the membrane anchor means that it could associate electrostatically with negatively charged phospholipid head groups that face the periplasm, and its amphipathic nature (one face of the helix is entirely hydrophobic) should allow it to penetrate into the lipid bilayer. Penicillin-binding protein 6 appears to have a similar carboxy-terminal anchor (247, 425).

Neither the physiological role of this novel method of membrane association nor its advantages relative to C-terminal, classical stop transfer/membrane anchors, have been determined. There is also no obvious reason why these proteins should insert into the cytoplasmic membrane rather than the outer membrane, which has a similar phospholipid composition. Furthermore, the gram-positive bacterium *B. subtilis* also has proteins with similar amphipathic C-terminal anchors (69, 593). The release-and-reinsertion model proposed for DacA cannot apply to these proteins, which would presumably be rapidly secreted into the external milieu. One possibility is that proteins with carboxy-terminal amphipathic helical anchors form part of a macromolecular complex at specific sites in the cell envelope to which they associate prior to their reinsertion into the membrane. Alternatively, the amphipathic membrane anchor may be sufficiently hydrophobic to cause the lateral opening of the translocation channel to allow it to penetrate directly into the lipid bilayer in the same way as a classical stop transfer signal while providing other features such as greater lateral mobility or a polar face necessary for its interaction with other envelope components.

Lipoproteins. Lipoproteins are the most abundant post-translationally modified bacterial secretory proteins. Their characteristic features are a thiol-linked diacylglyceride and an amine-linked monoacyl group on the cysteine that becomes the amino-terminal residue after signal peptide cleavage by LspA signal peptidase (see the sections on signal peptides and on processing, above). All bacterial secretory lipoproteins are probably modified in exactly the same way as the archetype lipoprotein, the major or Braun lipoprotein (Lpp) of *E. coli* (reviewed in reference 458). Fatty acylation almost certainly occurs on the periplasmic side of the cytoplasmic membrane, and the three fatty acids probably intercalate into the lipid bilayer (Fig. 18).

The main current interest in lipoproteins centers around how they reach their final destination. In gram-positive bacteria, lipoproteins are almost certainly embedded in the outer leaflet of the cytoplasmic membrane (Fig. 18), where many of them perform the same functions as freely diffusing periplasmic proteins in gram-negative bacteria (e.g., as binding proteins for cytoplasmic membrane permeases [402]). Lipoproteins appear to be inserted at any one of three distinct sites in the membranes of gram-negative bacteria (Fig. 18). They cofractionate either with dense outer membrane vesicles or with cytoplasmic membrane vesicles or unique vesicles of intermediate density (55, 238). Lipoproteins can also be spontaneously released from membranes when cells are lysed (55, 440), suggesting that fatty acids are relatively weak membrane anchors. Some lipoproteins may have polypeptide segments that span or interact peripherally with one or other of the membranes in the cell envelope. They could even be anchored in one membrane by fatty acids and in another by a typical membrane anchor (Fig. 18) (187).

For the present, I will concentrate on how peripheral membrane lipoproteins are selectively inserted into the cytoplasmic or outer membranes. The major contribution to this study came from Inouye's group, who noted that the cytoplasmic membrane lipoprotein NlpA has an aspartate residue at position +2 (i.e., immediately after the fatty-acylated cysteine residue), whereas other, mostly outer membrane lipoproteins have different amino acids in this position (55, 617). The only other lipoprotein with an Asp residue at +2 is the enzyme pullulanase of *Klebsiella oxytoca*, which, like NlpA, is not outer membrane associated when its structural gene is expressed in *E. coli* (440, 441) (see the section on the main terminal branch of the GSP, below). To determine the significance of the correlation between the absence of the Asp residue at +2 and outer membrane

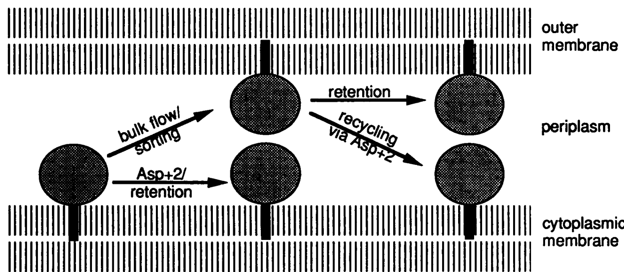

FIG. 19. Possible mechanisms for Asp-2-determined lipoprotein sorting in the cell envelope of gram-negative bacteria. Fatty acids are shown as thick lines.

association, Inouye's group constructed a number of hybrid lipoproteins with a β-lactamase reporter, as well as mutants in which the Asp residue at +2 of NlpA and the Ser residue at +2 of outer membrane-anchored Lpp were exchanged (617). Analyses of these lipoproteins showed quite conclusively that Asp at +2 is the lipoprotein-sorting signal. Asp at +2 has the same function in pullulanase (438) and cannot be replaced by the similarly charged glutamate or the structurally similar asparagine or displaced toward the C terminus without substantially impairing sorting to the cytoplasmic membrane (55, 186, 438). These results seem to rule out the possibility that the charge of the aspartate residue alone determines its sorting activity (186).

How does the lipoprotein-sorting signal function? All outer membrane lipoproteins transit via the cytoplasmic membrane, where the glyceryl- and acyltransferases are located (458). Thus, a lipoprotein-sorting signal (any amino acid other than aspartate) may actively direct lipoproteins to the outer membrane or prevent them from returning to the cytoplasmic membrane (Fig. 19), whereas retention in the cytoplasmic membrane may be passive. Alternatively, the sorting signal (aspartate) may cause lipoproteins to remain anchored in the cytoplasmic membrane instead of joining the bulk flow of lipoproteins to the outer membrane (Fig. 19). Another possibility is that the sorting signal is part of the recognition domain for fatty acyl transferases, in which case lipoproteins with Asp at position +2 may be modified differently from other lipoproteins. Interestingly, cytoplasmic membrane-anchored lipoproteins of gram-positive bacteria do not have an aspartate residue at position +2, and at least one of them, the BlaZ β-lactamase, is sorted to the outer membrane when produced in *E. coli* (55, 213).

Lipoprotein transport from the cytoplasmic membrane to the outer membrane poses unique mechanical problems. Lipoprotein fatty acids could remain embedded in the membrane lipids (Fig. 20A and C), associate with other fatty acids in lipoprotein micelles (Fig. 20B), or be shielded from the aqueous periplasm by lipoprotein carriers (Fig. 20D). The possible existence of membrane contact sites permitting intermembrane fatty acid transfer is still poorly documented and hotly contested, but one of the best arguments against their existence is that extracted peptidoglycan sacculi do not appear to have pores large enough to permit direct membrane contact or the diffusion of large lipoprotein micelles (268).

These models for lipoprotein sorting imply the existence of an envelope component, possibly the hypothetical lipoprotein carrier depicted in Fig. 20D, that recognizes the sorting/retention signal. It should be possible to select mutations affecting this "receptor" to cause misrouting of lipoproteins to the wrong membrane, where they may be inactive or acquire new functions. *E. coli* may not be able to tolerate the misrouting of all of its approximately 20 outer membrane lipoproteins, but misrouting of the 3 or 4 cytoplasmic membrane lipoproteins is less likely to adversely affect viability.

PERIPLASM

This section will examine the fates of proteins that are translocated into the periplasm, irrespective of their final destination. The periplasm is the site where secretory proteins adopt extensive tertiary and quaternary structure. As we shall see here and again later, proteins that are sorted into terminal branches of the GSP are usually already in or close to their final conformation during transit through the periplasm.

Protein Folding in the Periplasm

The extrusion of secretory polypeptides from the cytoplasmic membrane translocase is similar in many ways to the emergence of nascent polypeptides from ribosomes. ATP-dependent molecular chaperones catalyze the folding of nascent polypeptides by binding to them as they emerge from the ribosome, thereby preventing premature degradation by housekeeping proteases or improper interactions that might produce aggregates or other misfolded products (see the section on translocation competence, above). Despite the potential for misfolding, aggregation, or premature proteolysis, periplasmic proteins were assumed to fold spontaneously into their correct conformation, but few, if any, experiments were undertaken to put this to the test. There are, however, several well-documented examples of molecular chaperones that act specifically on translocated proteins

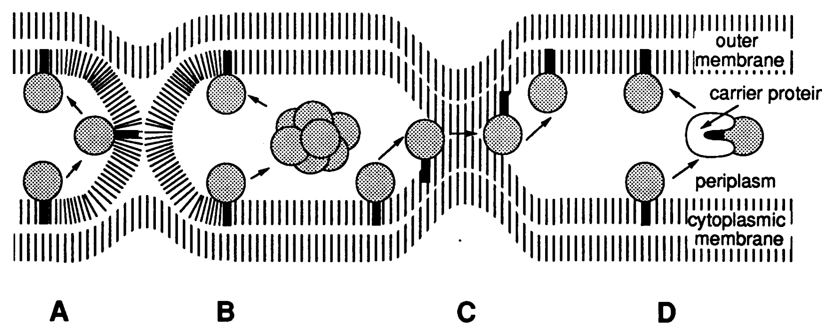

FIG. 20. Possible pathways for the sorting of lipoproteins to the outer membrane of gram-negative bacteria. Fatty acids are indicated by thick lines.

in the endoplasmic reticulum, in mitochondria, and in chloroplasts that set precedents for the existence of similar proteins in prokaryotes, and tests for proteins that might influence posttranslocation protein folding in bacteria are now beginning to produce interesting results.

Disulfide bond formation and isomerization. Many cytoplasmic proteins have free cysteines that cannot form disulfide bonds in the reducing environment of the cytoplasm. The periplasm is not a reducing environment, and, not surprisingly, many periplasmic proteins, as well as extracellular proteins, have disulfide bonds. Indeed, disulfide bond formation occurs so quickly that reduced intermediates cannot be detected even in very short pulse-labeling experiments, suggesting that it occurs during translocation (414), but spontaneous disulfide bond formation is relatively slow and error prone.

The prototype enzyme affecting disulfide bonds in translocated proteins is protein disulfide isomerase (PDI) in the lumen of the endoplasmic reticulum. Like general molecular chaperones, PDI binds nonspecifically to exposed polypeptide backbones, although its affinity is increased by the presence of cysteine residues. The enzyme catalyzes the isomerization of intramolecular disulfide bonds and can promote the correct folding of several proteins in vitro. Its absence from isolated endoplasmic reticulum-derived vesicles reduces the correct folding of an in vitro-translocated polypeptide (70, 381), and its activity is essential for yeast viability (198, 306). In addition, PDI is a subunit of several endoplasmic reticulum enzyme complexes, many of which are involved in the folding or modification of translocated proteins (380, 599).

A gene coding for an *E. coli* periplasmic enzyme with superficially similar properties to those of PDI was identified by two different strategies. Beckwith and his colleagues (26) observed that mutations in a gene they called *dsbA* increased the low enzymatic activity of a hybrid protein formed by fusing β-galactosidase to a periplasmic loop of a polytopic cytoplasmic membrane protein. This hybrid usually becomes permanently jammed as a translocation intermediate and cannot tetramerize or become active because of the formation of a disulfide bond in a translocated β-galactosidase segment. The *dsbA* mutations limited the formation of these disulfide bonds and allowed the enzyme to tetramerize. The second strategy, used by Ito and his group (259), was to look for mutations that prevented the activation but not the synthesis of periplasmic alkaline phosphatase. Mutations in the single gene identified, *ppfA* (*dsbA*), considerably reduced disulfide bond formation, which is essential for alkaline phosphatase activity and stability.

The main activity of DsbA appears to be the catalysis of disulfide bond formation, but, like PDI, it can also catalyze disulfide bond exchange reactions (26, 401). In all, disulfide bond formation in approximately 20 different endogenous *E. coli* periplasmic and outer membrane proteins, including β-lactamase and outer membrane protein OmpA (26, 259), is adversely affected by *dsbA* mutations or by growth in the presence of the reducing agent dithiothreitol. In some cases, such as β-lactamase and OmpA, the normally undetectable reduced forms persist for 1 to 2 min, whereas in other cases they persist indefinitely (433). Other proteins, such as alkaline phosphatase, are rapidly degraded in the absence of DsbA (26, 259, 433, 434). The broader consequences of mutations in *dsbA* in *E. coli* or in related genes in other gram-negative bacteria include reduced extracellular secretion by the main terminal branch of the GSP (432), failure to assemble F pili in *E. coli* (26), inability to produce correctly folded type IV pili in *Vibrio cholerae* (401), and failure to assemble or secrete heat-labile enterotoxin subunit B and cholera toxin (401, 477, 623). Some of these results are discussed again below. The absence of DsbA does not affect translocation across the cytoplasmic membrane (26), although alkaline phosphatase made in its absence appears to remain associated with the outer face of the cytoplasmic membrane (259), as do some (162) but not all (495) derivatives of β-lactamase that lack one or both of the cysteines that normally form the intramolecular disulfide bond. Despite this broad spectrum of activities, DsbA is not an essential enzyme (26, 433).

Proline isomerization. Peptidylprolyl-*cis-trans*-isomerases (rotamases) catalyze the isomerization of peptide bonds in which proline is in the second position and accelerate slow, rate-limiting steps in the refolding of denatured proteins in vitro. *E. coli* has two rotamases, one of which is periplasmic (94, 212, 332) and thus might influence the folding of exported proteins. However, although the gene for periplasmic rotamase (*rot*) has been cloned (332), the consequences of mutations affecting its expression on the activity of secretory proteins have not yet been reported. Similar genes have been identified in other bacteria, but only for the intracellular parasite *Legionella pneumophila* have mutations been characterized. Surprisingly, the *Legionella* gene, called *mip* (150, 161), codes for a cell surface, rather than periplasmic, protein (151) that either potentiates uptake of the bacterium by macrophages or increases intracellular survival. This phenotype could be due to the misfolding or instability of other virulence determinants in the absence of the Mip rotamase.

General molecular chaperones. The best-characterized examples of general molecular chaperones affecting the folding of translocated proteins are BiP, a protein found in the endoplasmic reticulum, and heat shock proteins belonging to the GroEL-GroES-like Hsp60-Hsp10 complex and the DnaK-like HSP70 in mitochondria and chloroplasts (469). BiP (Kar2) is an essential yeast protein (383) whose absence reduces translocation of presecretory proteins (374, 476), implying that it is part of the translocation machinery. However, other studies show that BiP binds tightly to misfolded or aggregated presecretory proteins in the lumen of the endoplasmic reticulum in mammalian cells, implying that it is part of a housekeeping or proofreading system that causes defective secretory proteins to be retained in the endoplasmic reticulum (165, 430). Aggregates of misfolded proteins in the lumen of the endoplasmic reticulum contain mixtures of covalently linked proteins synthesized by different polysomes together with noncovalently associated BiP (345). BiP may thus bind transiently to all nascent chains as they emerge into the lumen of the endoplasmic reticulum and permanently to incorrectly folded polypeptides (280, 339).

Like most other general molecular chaperones, BiP is an ATP-binding protein (261), which probably hydrolyzes ATP during polypeptide release and folding (132). However, it is unlikely that more than trace amounts of ATP could be present in the periplasm because it would either diffuse out of the cell through the outer membrane pores or be actively transported into the cytoplasm. Furthermore, part of the endoplasmic reticulum pool of BiP is ADP-ribosylated and phosphorylated (174) as a result of reactions which are again unlikely to occur in the periplasm. These observations indicate that an enzyme with exactly the same properties as BiP is unlikely to exist in the periplasm. There are as yet very few indications of the existence of *E. coli* proteins with similar properties to those of BiP. For example, there are no

reports on the effects of periplasmic extracts on the folding of in vitro-synthesized or denatured mature secretory proteins, and none of the variants of outer membrane proteins (OMPs) that remain in the periplasm (see the section on outer membrane proteins, below) or of the inactive or overproduced and aggregated periplasmic proteins studied in *E. coli* have been reported to be associated with a periplasmic protein. Furthermore, none of the *sec* genes code for periplasmic proteins (see the sections on the essential elements and on translocation, above), although the membrane-bound SecD and SecF proteins could be involved in translocation channel clearance and protein folding (see the section on release from the translocation channel, above).

Evidence that environmental conditions can influence the folding of exported proteins is provided by the observation that the release and activation of extracellular *B. subtilis* levan sucrase are strongly promoted by Fe^{3+} even though the ion is not found in the mature enzyme. Iron starvation seems to prolong the existence of a normally transient cell-associated secretion intermediate (404). Fe^{3+} has been shown to influence levan sucrase folding in vitro (79), and mutations affecting specific residues within the mature part of the polypeptide affect protein folding and secretion in the same way as iron depletion does (405). Thus, iron-induced conformational changes may coincide with and induce clearance of the translocation channel or release from the cell surface in vivo (78).

These effects of Fe^{3+} do not seem to be chaperone dependent. However, studies of pleiotropic secretion-defective mutants of *B. subtilis* (282) indicate that the product of at least one gene, *prsA*, is specifically required for efficient extracellular secretion of several proteins. PrsA seems to be a lipoprotein (see the section on lipoproteins, above) which is probably anchored to the outer leaflet of the cytoplasmic membrane with the entire polypeptide chain exposed on the cell surface (281). The product of the *prtM* gene of the gram-positive *Lactococcus lactis*, which is required for activation (but not secretion) of an extracellular protease (200, 580), exhibits significant amino acid sequence similarity to PrsA (281) and also appears to be a surface-exposed lipoprotein (201). This raises the intriguing possibility that many gram-positive bacteria possess a cell surface-anchored molecular chaperone that catalyzes the folding of translocated secretory proteins and, by analogy with the effect of Fe^{3+} on levan sucrase, is required for posttranslocation release of secreted proteins or for prevention of their aggregation at the cell surface (where they would be trapped by the cell wall). It will now be important to determine whether proteins with similar activities exist in gram-negative bacteria. These putative general secretory chaperones presumably act during or immediately after translocation and together with other enzymes affecting protein folding. They might therefore exist as a complex or be associated with translocase.

Specific molecular chaperones. Molecular chaperones can be defined in several different ways. One widely accepted definition emphasizes their ability to prevent polypeptides from adopting their final conformation for as long as the complex persists. An alternative definition places less emphasis on this and, instead, emphasizes their ability to prevent proteolysis or illicit intra- or intermolecular interactions while facilitating favorable reactions such as oligomerization or translocation. One class of proteins that fits well within the latter definition contains specific periplasmic molecular chaperones involved in the morphogenesis of certain types of cell surface pili or fimbriae in *E. coli* and related bacteria.

Subunits of most pili (pilins) of enteric bacteria are produced as signal peptide-bearing precursors that are translocated across the cytoplasmic membrane, are processed by LepB-type signal peptidase, and then transit through the periplasm before being transported across the outer membrane, where they are assembled and remain anchored (see the section on enterobacterial pili, below). Genes coding for pilins are linked to another gene encoding a protein that binds strongly to periplasmic pilin secretion intermediates (236, 294, 365). Mutations in this gene prevent pilus assembly and cause pilins to aggregate (294) or to be degraded by nonspecific periplasmic proteases (20, 328, 365). This periplasmic pilin chaperone is probably released when the chaperone-pilin complex docks at the outer membrane site where pilus assembly occurs (see the section on enterobacterial pili, below). However, chaperone-complexed pilins retain their ability to bind to their receptor ligand (235), have disulfide bonds (236), and are recognized by many but not all monoclonal antibodies that bind to the native pilus (20). All of these results suggest that chaperone-complexed pilins are in or are close to their final conformation before they are transported across the outer membrane and assembled.

The crystal structure of a pilin-specific chaperone (PapD) reveals a boomerang-like structure, reminiscent of immunoglobulins, which might capture and retain pilins within its cleft (226). Recent unpublished studies reviewed by Hultgren et al. (236) show that a mutation that changes a residue within the cleft reduces piliation. Furthermore, chaperones that are specific for different pilins share a considerable degree of sequence identity and are all predicted to fold into the same conformation as PapD (236), but they are not interchangeable (20). Pili are usually composed of at least four different subunits (see the section on enterobacterial pili, below), all of which are recognized by the same molecular chaperone. Their C-terminal regions show a degree of sequence conservation that is considerably higher than that of other segments. The cleft region of the pilin chaperones might therefore recognize sequences within this region. It would be interesting to see whether mutations that affect the chaperone cleft can be suppressed by mutations affecting the pilin C terminus.

In summary, periplasmic, pilin-specific molecular chaperones have several unique features, including their shape, their independence of ATP for release, and the fact that their ligands are in a tightly folded conformation. The kinetics of their interaction with pilins has not been determined, but Hultgren et al. (235) reported that processing of prepilin by signal peptidase is enhanced by pilin chaperone. This suggests that the chaperone interacts with the nascent precursor during translocation and may therefore catalyze the correct folding of pilus subunits.

Endochaperones. Most exported proteins are not subjected to proteolytic processing beyond the removal of part or all of a signal sequence, but some have additional peptide segments that are removed from the amino- or carboxy-terminal ends (pro- or apopeptides, respectively) or, less frequently, are cleaved into two segments. In most cases, these additional processing steps, which are probably performed by nonspecific envelope or extracellular proteases, do not affect the activity of the protein concerned, and the peptides that are removed are generally very short. Only in a few cases are pro-, endo-, or apopeptides known to perform any biological function. One such example is the internal cleavage of a bifunctional cytochrome precursor by a LepB signal pepti-

dase (see the section on signal peptidases, above). Other examples concern proteases that are secreted by bacteria via the GSP. Many of these have long (77- to >200-residue) propeptides between the signal peptide and the mature segment that are autocatalytically removed once the proprotease has crossed the cytoplasmic membrane. The propeptide-bearing precursors (zymogens) are capable only of autoprocessing; they do not have measurable proteolytic activity on any other protein substrate. Thus, the propeptides prevent activation of the proteases until they have been exported, thereby avoiding any harmful effects they may have prior to export (583).

Two well-documented examples of propeptide-bearing proteases serve to illustrate the effects of these aminoterminal extensions on protein folding, secretion, and activation. The simplest of these systems comprises a family of structurally similar alkaline serine proteases, the subtilisins, produced by gram-positive bacilli. Maturation of preprosubilisin is blocked by mutations that alter the catalytic site of the enzyme, but maturation can occur if the producing strain produces another, catalytically active, *trans*-acting subtilisin (424). Surprisingly, the signal sequence of the inactive preprosubtilisin is also not processed, leading to the accumulation of membrane-associated, full-length precursor (424). Similar results were obtained in studies on another subtilisin derivative in which four residues had been deleted from the substrate-binding cleft (494). Thus, in this exceptional case, cleavage of the propeptide rather than the signal sequence seems to be required for release from the cytoplasmic membrane. This unusual phenomenon appears to be specific to the subtilisin signal sequence, however, since studies with prosubtilisin sequences fused to other signal peptides showed that signal peptide processing was sufficient to release the proenzyme from the cytoplasmic membrane (241, 562), although the subtilisin signal sequence itself can be cleaved at a consensus LepB-type signal peptidase cleavage site in vitro by a mixture of *B. subtilis*-derived membrane vesicles and detergent (611).

A crucial breakthrough in understanding the multiple functions of these propeptides came with the demonstration by Inouye and his colleagues that mutations that affect the sequence of the propeptide, or direct fusion of the mature subtilisin sequence to a signal peptide, resulted in the synthesis of an inactive enzyme that remained inactive after denaturation and renaturation (240, 241). Thus, the propeptide is required not only to maintain the protease in an inactive form but also to catalyze the formation of the active protease after release. These studies culminated in the demonstration that the propeptide could catalyze the refolding of denatured mature subtilisin in *trans* (625) as well as in *cis* (240), confirming its role as a normally endogenous molecular chaperone.

This elegant first demonstration of endochaperone activity in an extracellular protease was soon extended to many other proteases, including some produced by eukaryotic organisms. Of particular importance to the present discussion was the almost simultaneous demonstration by Agard and his colleagues that the 174-residue, normally autoprocessed propeptide of the extracellular α-lytic protease of the gram-negative bacterium *Lysobacter enzymogenes* (508–510) catalyzed the correct folding of the 20-kDa mature protease in vivo and in vitro (508). Active protease could also be obtained when the propeptide and mature segments were each produced as signal peptide-bearing precursors that were independently exported to the *E. coli* periplasm. Thus, the endochaperone had been converted into a specific chaperone (see the section on specific molecular chaperones, above) by physically separating it from its normal substrate. The possibility that the separately synthesized endochaperone could act in repeated cycles of protease activation was apparently not examined, although high-level expression of the gene coding for the signal peptide-endochaperone construct appears to have been necessary for maximum activation (508). These studies raise an interesting and hitherto largely ignored aspect of the folding of secretory proteins, namely the possibility that other proteins have endochaperones which, unlike protease propeptides, do not hinder the function of the protein concerned.

OUTER MEMBRANE PROTEINS

The lipids of the outer membrane, which is, of course, unique to gram-negative bacteria, have a polarized organization; the outer leaflet contains only the glycolipid lipopolysaccharide (LPS), while the inner leaflet has a similar phospholipid composition to that of the cytoplasmic membrane and does not contain LPS (376). Integral OMPs approach the outer membrane from the periplasmic face; their insertion into this membrane is therefore superficially similar to the export and assembly of integral membrane proteins at the cytoplasmic face of the cytoplasmic membrane. The major difference is, of course, the fact that OMPs have already been transported across one membrane. Considerable attention has been devoted to several abundant OMPs with the aim of determining their membrane organization and, more recently, the events that lead to their membrane insertion and assembly. The following sections discuss data which suggest that these processes are intimately related and that the insertion of OMPs is largely, if not wholly, determined by their unique structural and conformational properties.

Structure and Topology

At first sight, OMPs seem to be similar to the vast majority of polytopic membrane proteins. They have cell surface-exposed loops that can be specifically recognized as receptor sites by bacteriophages or bacteriocins, cleaved by exogenously added proteases, or bind specific antibodies (81, 123, 275, 366, 484, 485, 560). Other exposed loops face the periplasm and are recognized by antibodies or cleaved by proteases only when the cells are lysed or the outer membrane is permeabilized. Extensive studies with these and other topological probes on a wide variety of OMPs from different bacteria led several groups to propose topographical models in which extramembranous loops thus identified are separated by membrane-spanning segments (Fig. 21) (reviewed in reference 552).

Alignment of these models with the sequences of the proteins concerned reveals the remarkable absence of any regions of high overall hydrophobicity in predicted membrane-spanning segments or, indeed, throughout the entire length of the polypeptides. Although originally considered puzzling, this unusual feature of OMPs is readily explained by the fact that typical hydrophobic transmembrane segments would prevent polypeptide release from the cytoplasmic membrane by acting as stop transfer-membrane anchor signals (341, 505), thereby preventing assembly into the outer membrane. Closer inspection of the putative transmembrane segments reveals the presence of sequences that could form amphipathic β-sheet structures of alternating hydrophobic and hydrophilic amino acids (276, 552). This prediction was in line with the determined presence of

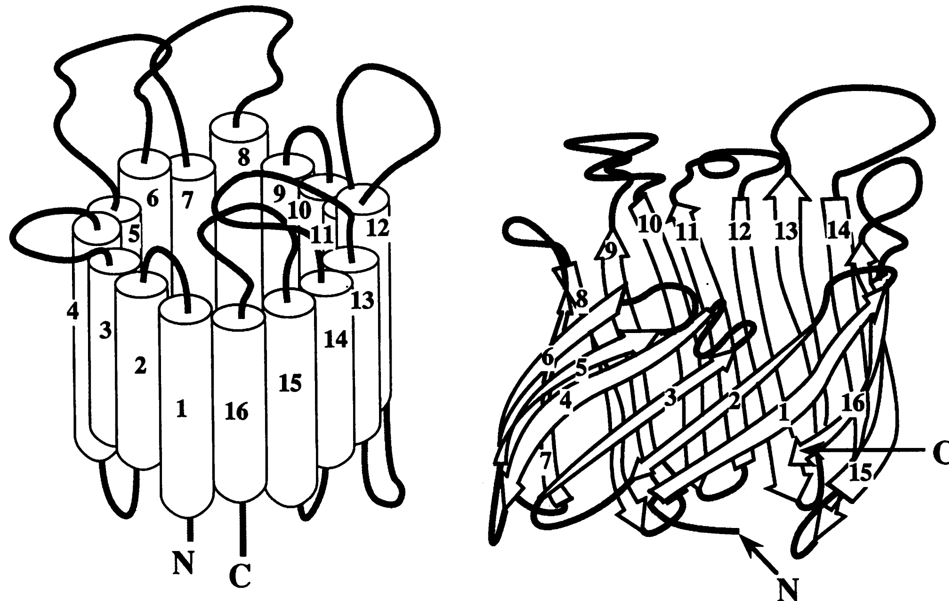

FIG. 21. Possible topologies of a single subunit of an OMP of gram-negative bacteria, as viewed from the outer face of the outer membrane. The 16 transmembrane segments that are typically found in the subunits of trimeric porins are shown as cylinders in the plan on the left (representing hydrophobic α-helices originally thought to be predominant in these proteins) and as arrows in the plan on the right (representing antiparallel amphipathic β-sheets). Other proteins may have fewer transmembrane segments. They are composed of approximately 8 to 16 alternating residues of relatively higher and lower hydrophobicity in a β-sheet structure. Connecting loops are shown as thicker, shaded lines. Both extremities are exposed on or are close to the periplasmic (lower) side of the membrane. The model on the left shows the conventional view in which the transmembrane segments are perpendicular to the membrane. The model on the right shows inclined transmembrane segments, as revealed by high-resolution crystal analysis (see the text). The protein shown has a central channel typical of porins, which usually contain three identical or near-identical subunits with interfacing transmembrane segments. For simplicity, complex structures revealed by X-ray analysis are largely omitted. The various elements are not drawn to scale, and the membrane lipids are omitted for clarity.

abundant β-sheet structure in several OMPs (276, 570). The hydrophobic amino acids of these β-sheet structures are therefore predicted to face outward toward the fatty acids of the outer membrane or toward hydrophobic amino acids in adjacent transmembrane segments, while hydrophilic residues interact with each other or line the walls of a membrane-spanning channel.

This notion of OMP architecture was confirmed by electron and then X-ray diffraction analyses of extracted membranes and crystals of detergent-solubilized OMPs that culminated in the fine-structure determination of first the outer membrane porin from *Rhodobacter capsulatus* (489, 596, 597) and then the OmpF and PhoE porins of *E. coli* (97). These structural analyses revealed another feature of outer membrane architecture that could not have been predicted from topological mapping and structure predictions based solely on knowledge of the primary sequence, namely that the transmembrane segments are not perpendicular to the plane of the membrane but, rather, are inclined, as indicated schematically in Fig. 21 (97, 596, 597).

The determined structures of the three OMPs include a number of interesting features that specifically pertain to their function as porins and therefore need not concern us here. Apart from these specific details, however, it is likely that other integral OMPs, most of which seem to form channels of different sizes and substrate specificities, are structurally similar to outer membrane porins; i.e., they form a barrel-shaped channel linked by antiparallel, inclined amphipathic β-sheets. This forms the basis from which to examine details of their outer membrane insertion.

Periplasmic Intermediates

Two quite different models have been proposed to explain how proteins are inserted into the outer membrane. In the first of these models, OMPs are partially assembled in the cytoplasmic membrane and migrate to the outer membrane via contact sites that are proposed to span the periplasm between the two membranes. Although this model has a number of attractive features, the existence of such contact sites has been challenged (268). Furthermore, although putative cytoplasmic membrane intermediates representing early folding intermediates have been identified in the export of several OMPs (56, 100, 173, 525), most experimental data argue in favour of the release of OMPs from the cytoplasmic membrane, transit through the periplasm, and spontaneous insertion into the outer membrane as a result of a series of conformational changes and possibly interactions with other outer membrane components.

The first evidence that OMPs have periplasmic intermediates was the observation by Metcalfe and Holland (356) that newly synthesized OmpF protein is released into the medium by spheroplasts derived from *E. coli*. Pulse-chase studies by the same group with another OMP also suggested the existence of a periplasmic assembly intermediate (19, 249). The release of soluble OmpF from spheroplasts was confirmed by Sen and Nikaido, who further showed that the released protein was monomeric (the outer membrane form is usually trimeric) and that it could trimerize and insert into *E. coli*-derived membranes when mixed with a very small amount of the detergent Triton X-100 (499). Similar results

were obtained by Tommassen and his colleagues, who showed that in vitro-synthesized mature PhoE protein (but not prePhoE) could trimerize and spontaneously associate with outer membrane fragments under similar conditions (111, 113), and by Eisele and Rosenbusch, who found that trimeric OmpF extracted from the outer membrane could be denatured to monomers and then renatured and inserted in a functional (presumably trimeric) form into artificial lipid bilayers (147).

The obvious conclusion from these studies is that OmpF and PhoE proteins can, and presumably do, insert into the outer membrane via a soluble periplasmic intermediate in a spontaneous process determined by the conformation of the protein itself and independent of exogenous factors such as molecular chaperones or membrane contact sites. One feature of these reconstitution experiments that remains puzzling is the observed strict requirement for low levels of detergent. Tommassen's group observed that omission of detergent from the reconstitution experiment prevented insertion into the membrane in a protease-resistant form without affecting trimerization and association with the membrane fragments (111, 112). Therefore, in this case at least, the detergent does not seem to induce a dramatic conformational change but, rather, to assist insertion into the membrane. One possibility is that the detergent acts in the same way in vitro as a lipid or other molecule such as a molecular chaperone with which OMPs might associate during transit through the periplasm. Studies discussed below suggest that de novo-synthesized LPS might perform the same function as Triton X-100 in vivo.

Conformational Changes and Role of LPS

Monoclonal and other, epitope-specific antibodies are among the most powerful probes of protein conformation. Several groups have developed such probes to study the topology of OMPs (see above) and used them to monitor the conformational changes that occur during their export and assembly. These studies reveal that certain monoclonal antibodies recognize structures that appear early during porin assembly, prior to oligomerization, whereas other epitopes appear later as the proteins pass through a metastable trimeric intermediate stage (metastable trimers dissociate in sodium dodecyl sulfate at lower temperatures than stable trimers) and still others appear only when the trimers become heat stable or even later in the native, fully inserted outer membrane trimer (167, 460). The last two steps in the assembly pathway require concomitant LPS and lipid biosynthesis, suggesting that the transition from metastable to stable trimer and full insertion into the outer membrane occur only after a critical porin-LPS association step (46, 167, 459). It is interesting that although highly purified porins appear to have at least one tightly associated LPS molecule (464) and high-resolution electron-microscopic studies seemed to suggest the presence of LPS in porin trimers (225), the determined X-ray crystal structures do not contain a suitable binding site for LPS (97). Thus, several LPS molecules, rather than a single, tightly bound LPS molecule, could exert detergent-like properties to stimulate the correct folding of OMPs or to stimulate association with or insertion into lipid bilayers.

Association with the lipid A portion of LPS has also been reported to shift the conformation of a cytoplasmic membrane-associated OmpA export intermediate toward that of the integral outer membrane-embedded form (173). Denatured OmpA can, however, be renatured and reconstituted into lipid vesicles in the absence of LPS (133). It has been known for some time that mutants of *E. coli* and of the closely related *Salmonella typhimurium* which produce LPS molecules with truncated saccharide segments display pleiotropic phenotypes that sometimes include altered levels of several OMPs, most notably the porins but less frequently OmpA (13, 459, 557), but the significance of these observations has remained elusive. Furthermore, the LPS-induced trimerization of OmpF protein that is secreted by spheroplasts is strictly dependent on the presence of at least the glucose residue that is normally linked to the lipid A part of *S. typhimurium* LPS through two tandem heptoses and several molecules of 3-deoxy-D-*manno*-octulosonic acid (500). This observation supports the proposal, based on in vivo studies with mutants defective in LPS biosynthesis, that sugar residues at the outer extremities of the LPS core, as well as lipid A, are involved in outer membrane-porin interactions that are necessary for porin assembly and insertion.

Unfortunately, it is difficult to synchronize the rapid conformational changes that accompany outer membrane insertion, and hence it is difficult to determine the site at which they occur (460). Furthermore, simple tests such as solubility in the detergent Sarkosyl, as used by some authors (see, e.g., reference 56), may not indicate the true location of assembly intermediates in the cells. OmpF monomers were found associated with outer membrane-derived vesicles in one study (25), and OmpF monomers secreted by spheroplasts remain monomeric until they are mixed with cell envelopes (499). Both of these studies suggest that trimerization occurs at the surface of or within the outer membrane, possibly via partially folded monomeric and dimeric forms (111, 112, 460). Likewise, a monomeric assembly intermediate of a variant LamB protein that is encoded by a *lamB* gene carrying a temperature-sensitive assembly mutation associates with the outer membrane as a monomer at the nonpermissive temperature. This assembly intermediate can be chased into trimers when the cells are switched to the permissive growth temperature, indicating that they are probably similar to bona fide intermediates in the normal assembly pathway (362). In the future, it will be essential to determine the stage at which porin trimerization occurs since this, together with other factors, may be the crucial conformational change that induces outer membrane insertion. More thorough investigation of the influence of LPS (or detergent-like lysolipids) on trimerization and insertion might not only resolve the apparent differences in the reported data but also distinguish between the requirement for de novo-synthesized LPS (e.g., porins) and that for LPS that is already in the outer membrane (e.g., OmpA protein?). The possibility that interaction with LPS ensures specific insertion into the outer membrane rather than the cytoplasmic membrane should also be seriously considered. It is worth pointing out that a better understanding of the ways in which phospholipids and LPSs are transported to and inserted into the outer membrane would provide important clues to the mechanisms of OMP insertion into this membrane. Furthermore, the once popular idea that OMPs interact with a specific receptor on the periplasmic face of the outer membrane still cannot be totally discounted.

Sequence Alterations Compromise Outer Membrane Insertion

We saw above that polytopic cytoplasmic membrane proteins are sometimes remarkably tolerant of even quite

dramatic sequence alterations such as the removal of one or more transmembrane segments (see the section on polytopic membrane proteins, above). However, this is manifestly not the case with OMPs, which often, although not always, fail to assemble in the outer membrane as a result of even relatively minor tinkering with the sequences of the transmembrane segments (49–52, 171, 175, 277–279). Instead, OMP derivatives with altered transmembrane segments accumulate in the periplasm, as would be expected if their insertion into the outer membrane is determined by their assembly and associated conformational changes that normally occur during transit through this compartment. Rather surprisingly, duplications of internal segments of OMPs seem less likely to reduce outer membrane insertion than do corresponding deletions of the same segments (290).

Although the fact that a wide variety of structural alterations abolish OMP assembly makes the idea that OMPs have a specific outer membrane insertion signal rather unlikely, certain segments of OMPs may be more crucial than others because they nucleate the folding reactions that eventually lead to the formation of the insertion-competent state (278) or interact with LPS or other envelope components that promote assembly. Furthermore, Tommassen and his colleagues have noted the remarkable fact that the last residue of the last transmembrane segment of virtually all OMPs is a phenylalanine (534). Their own studies (50, 534), as well as those of others (77, 217), show that removal of this last transmembrane segment or, more specifically, replacement of this Phe residue by unrelated amino acids causes a dramatic reduction in outer membrane insertion without affecting porin trimerization. Thus, this particular amino acid is crucial for OMP assembly even though it probably does not act as an outer membrane insertion signal.

Sequence changes affecting the extramembranous segments of OMPs appear to be relatively well tolerated. Comparisons of the sequences of the same protein from different species reveal that the majority of the sequence differences affect surface-exposed loops (59, 559). Likewise, amino acids can be removed from or inserted into these extramembranous segments without affecting outer membrane assembly, although there appears to be an upper limit to the length of the sequence that can be inserted without compromising insertion, assembly, and stability (2, 51, 53, 80, 169, 278). Likewise, only some of the mutations that replaced turn-inducing residues in the short periplasmic loops of PhoE protein (400) dramatically affected trimerization or outer membrane insertion (114).

These results suggest that OMPs retain their ability to insert and assemble in the outer membrane provided that their characteristic barrel-like structure is not drastically perturbed either by removing transmembrane segments (the barrel staves) or by introducing additional, strong folding constraints by, for example, considerably expanding one of the extramembranous loops or disrupting sharp turns in two closely spaced transmembrane segments. However, one recent report indicates that the entire sequence of a normally periplasmic protein, β-lactamase, can be transported to the cell surface with relatively high efficiency when grafted onto the end of a truncated OmpA (168). Although this hybrid protein has several special features (e.g., the first two of the normally eight transmembrane segments are replaced by a lipoprotein fatty acyl anchor) that may alter its behavior relative to that of normal OMPs, it is still remarkable for two reasons. First, the transport of a polypeptide segment as large as β-lactamase (259 amino acids) through the outer membrane is almost totally unprecedented. It is also difficult to reconcile with the idea that insertion into the membrane occurs after folding in the periplasm, implying that the β-lactamase segment crosses the outer membrane as a fully folded, disulfide-bonded molecule (see the section on protein folding in the periplasm, above). Second, this particular hybrid was constructed in such a way that the last transmembrane segment, including the critical phenylalanine residue that is essential for outer membrane insertion, is no longer present. There are, however, certain similarities between this hybrid and immunoglobulin A (IgA) protease secretion by *Neisseria gonorrhoeae* that will be discussed below.

Although we now have a good impression of the molecular architecture of OMPs, we are still a long way from understanding how they are inserted and assembled in the outer membrane. The complex processes of folding and, for porins, trimerization appear to occur on the periplasmic face of the membrane before complete insertion. This must mean that information for outer membrane association is presented by the monomeric form of the protein and that this is replaced at a later stage by information for outer membrane insertion. Presumably, a folding intermediate may expose a hydrophobic face that causes partitioning into the membrane, possibly accompanied in some cases (e.g., porins) by LPS insertion. Whatever the precise mechanisms involved, they apparently do not involve energy coupling or pilot proteins and are thus quite distinct from those proposed to be involved in protein insertion into the cytoplasmic membrane (see the section on polytopic membrane proteins, above).

TERMINAL BRANCHES OF THE GSP

To reach the cell surface of a gram-negative bacterium, extracellular enzymes, toxins, and the subunits of cell surface appendages must cross the two lipid bilayers of the cell envelope. Although a large proportion of extracellular proteins of gram-negative bacteria are made as signal peptide-bearing precursors, little effort has been made until recently to study their secretion, largely because many of them are not normally made by the laboratory *E. coli* strains in which the *sec* genes were discovered and their products characterized. Recent studies are beginning to redress this imbalance. They reveal the existence of several terminal branches of the GSP in many gram-negative bacteria. In some cases, the cloning and expression of genes coding for components of these terminal branch pathways in *E. coli* have greatly facilitated their characterization.

Enterobacterial Pili

The first terminal branch of GSP to be characterized was that for cell surface pili (fimbriae) of *E. coli* and closely related bacteria. These pili are generally rigid, rod-like appendages, composed mainly of approximately 1,000 copies of a single ca. 18-kDa polypeptide (pilin), that protrude beyond the outer membrane and allow bacteria to adsorb to specific receptors on other cell surfaces. They are usually up to 2 μm long and 5 to 10 nm in diameter. One of the most extensively characterized pili, the Pap or P pilus of certain uropathogenic *E. coli*, will be used here as a model to illustrate various aspects of pilin secretion and assembly. References to studies on other, related pili will be included where appropriate, but the reader is referred to the excellent review by Hultgren et al. (236) for a more in-depth treatment of the entire subject, including information on structural or

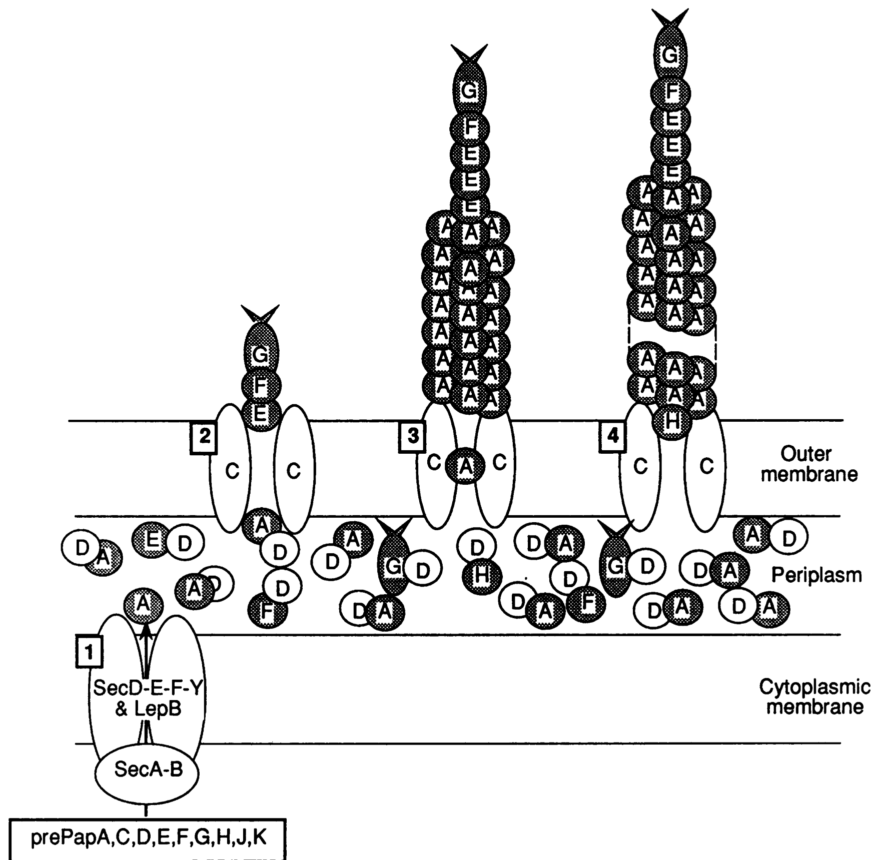

FIG. 22. Schematic representation of events leading to the secretion of Pap pilin subunits and their assembly into pili on the surface of *E. coli* cells expressing the entire cluster of *pap* genes. Step 1 corresponds to the export of pilin subunits (shaded) and accessory secretion/assembly factors (PapC, PapD; not shaded) via the early stages of GSP and processing by signal peptidase (LepB). Direct involvement of all the components of this section of GSP has not been demonstrated experimentally. Pilin subunits emerging on the periplasmic side of the cytoplasmic membrane are complexed by PapD until they arrive at the assembly platform (PapC). Pilus growth is initiated by the insertion of PapE, PapF, and PapG, which is the adhesin subunit (step 2). These subunits form a thin filament at the end of the pilus. The major subunit, PapA, is then continuously incorporated (step 3) until a PapH-PapD complex arrives at the assembly platform. The incorporation of PapH at the base of the growing pilus prevents further incorporation of other subunits, and the pilus remains anchored to the assembly platform (step 4).

morphogenic features of other enterobacterial pili that differ from those presented below for the Pap pilus.

Pap pili are composed of a main subunit, PapA, together with one or a few copies of several minor pilins, including PapG (adhesin), which is responsible for the specific recognition of the Pap pilus receptor (α1-4-linked digalactoside) (327, 336, 337); PapE and PapF, which are located together with PapG at the tip of the pilus; and PapH, which is located at the base (Fig. 22). All of these subunits have a relatively high degree of sequence similarity, and their genes, as well as genes required for their expression and for pilus assembly, are clustered in the same part of the genome (Fig. 23). The effects of mutations in these genes are illustrated in Fig. 24.

Since all of the various pilins are made as precursors with typical signal peptides, they are probably exported across the cytoplasmic membrane by the Sec-dependent part of the GSP. Removal of the signal peptide by LepB signal peptidase releases pilin subunits into the periplasm (467), where they are found complexed with the specific pilin chaperone PapD (235, 328) (see the section on specific molecular chaperones, above). The pilin-PapD complex persists until it reaches the "assembly platform" formed by the integral outer membrane protein PapC (134, 382). PapD must then be released (328), presumably as a result of conformational changes induced by PapC in PapD or, perhaps, in the pilins. This implies that PapD is specifically recognized by PapC and therefore that PapD is both a molecular chaperone and a pilot protein and that PapC is its receptor.

Pap and related pilins arrive at the site of transport across the outer membrane in or close to their final conformation (see the section on specific molecular chaperones, above) and are presumably not extensively unfolded before they cross the membrane. The exact site at which the subunits are assembled has not been determined, although, by analogy with other pili (334), Pap pili probably grow from the base (Fig. 22). If assembly occurs on the outer face of the membrane, PapC must be a translocator that transports individual pilin subunits across the outer membrane to the outer face, where they displace a previously assembled subunit. Alternatively, assembly may occur on the periplasmic side of the membrane, with the pilus structure spanning the membrane to be gradually pushed through it as more subunits are added. It should be relatively easy, using topological probes, to determine whether PapC is exposed on the cell surface and to determine whether it has channel-

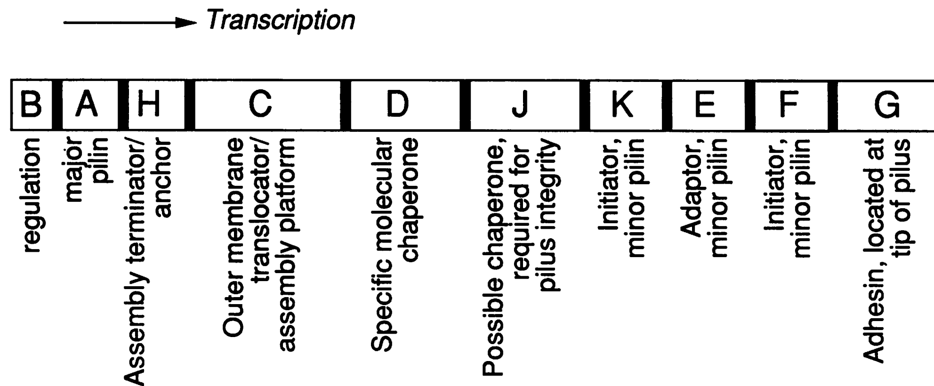

FIG. 23. Schematic representation of genes in the *pap* operon. The genes are shown in the order in which they appear in the operon, which is transcribed from left to right. The presence of sequences coding for a typical signal peptide is indicated by black boxes. The functions attributed to the gene products are indicated. A transcription terminator (attenuator) located between *papA* and *papH* is in part responsible for the lower level of expression of genes beyond *papA*.

forming properties. Overproduction of PapC not only increases the number of pili on the cell surface, presumably by providing more sites for pilin secretion and assembly, but also is lethal to the cells, which spontaneously release periplasmic β-lactamase and are sensitive to lysozyme (382). Outer membranes of bacteria producing large amounts of a similar protein from another pilus morphogenesis system are permeable to vitamin B_{12} (463). This increase in outer membrane permeability could be due to toxic effects of unusually high levels of PapC in the outer membrane or to the presence in the membrane of large numbers of open channels formed by PapC.

The sequential events of initiation, incorporation of pilins, elongation, and termination are tightly regulated. Assembly seems to be initiated by the insertion of PapE, PapF, PapG, and possibly PapK into the assembly platform (Fig. 22). None of these subunits appears to be essential for pilus assembly, but PapF appears to increase its efficiency and PapE may be the adaptor which links the G-subunit pilin to the main-subunit pilin (327) (Fig. 22). The PapCEFG complex can then accept PapA or PapH subunits. Interestingly, the PapE-containing distal end of the pilus seems to be a short filament with a diameter approximately one-third of that of the main filament (Fig. 22) (236, 293a). This implies that PapE packs into a structure that differs from that formed by PapA. Indeed, the packing arrangement of PapE may be more similar to that of the K88 pilus, which is a long filament, whereas the structure of the rest of the Pap pilus is closer to that of the type 1 pilus, which is a right-handed helix with a central axial hole (63, 236, 293a).

The incorporation of PapH into the growing pilus blocks all further subunit incorporation, presumably by displacing the previously assembled A subunit (as proposed above) and becoming permanently anchored to PapC. The ratio of PapA to PapH subunits in the periplasm determines pilus length: high levels of PapA (relative to PapH) result in elongated surface-anchored pili, whereas unusually long pili are released into the medium by cells completely devoid of PapH (Fig. 24). On the other hand, cells producing unusually high levels of PapH produce stubby pili, presumably because elongation is prematurely terminated as subunits are stochastically incorporated into the growing pilus (Fig. 24).

Mutants lacking another pilin-like protein (PapJ), which does not appear to be located in the pilus, produce fragile pili that snap off and are released into the medium (Fig. 24). The

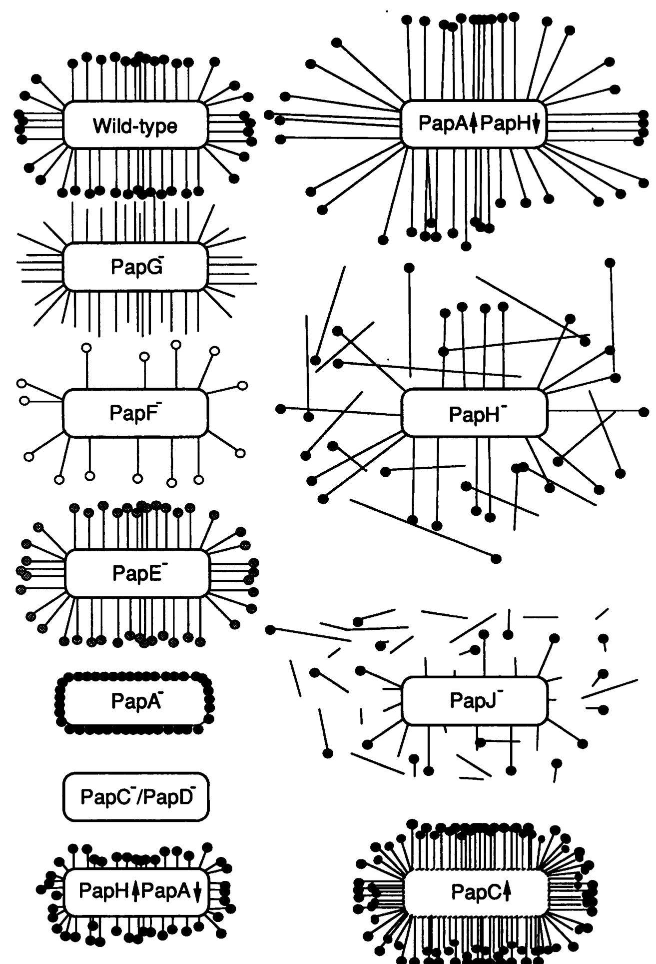

FIG. 24. Schematic representation of phenotypes of *E. coli* mutants carrying the entire *pap* gene cluster with or without mutations therein that alter the levels of the proteins indicated. Pili are represented as long rods. The circles at their ends represent the adhesin. Solid circles indicate fully functional adhesin; open circles indicate that the PapG adhesin subunit is present but that the cells do not bind to the digalactose receptor; and shaded circles indicate that the adhesin is active in intact cells but not in purified pili. Arrows indicate that the protein is overproduced or underproduced relative to normal levels. Overproduction of PapC protein (the pilus assembly platform) causes the envelope to become leaky and is lethal to the cells.

Organism	Protein	Sequence (PPP ↓ after first G; SP sites marked in figure)	References
Neisseria gonorrhoeae		MNTLQKGFTLIELMIVIAIVGILAAVALPAYQDYTARAQV---	358
Neisseria meningitidis		MNTLQKGFTLIELMIVIAIVGILAAVALPAYQDYTARAQV---	423
Bacteroides nodosus		MKSLQKGFTLIELMIVVAIIGILAAFAIPAYNDYIARSQA---	148
Pseudomonas aeruginosa		MKA-QKGFTLIELMIVVAIIGILAAIAIPQYQNYVARSEG---	399
Moraxella lacunata		MNA-QKGFTLIELMIVIAIIGILAAIALPAYQDYISKSQT---	470
Moraxella bovis		MNA-QKGFTLIELMIVIAIIGILAAIALPAYQDYISKSQT---	470
Vibrio cholerae		MQLLKQLFKKKFVKEEHDKKTGQEGMTLLEVIIVLGIMGVVSAGVVTLAQRAIDSQNM---	154, 501
Klebsiella oxytoca	PulG	MQR-QRGFTLLEIMVVIVILGVLASLVVPNLMGNKEKADR---	461
	PulH	MR--QRGFTLLEMMLILLLMGVSAGMVLLAFPASRDDSAA---	461
	PulI	MKK-QSGMTLIEVMVALVVFALAGLAVMQATLQQTRQLGR---	461
	PulJ	MIRRSSGFTLVEMLLALAILAALSVAAVTVLQNVMRADTL---	461
Bacillus subtilis	ComG.3	MNE--KGFTLVEMLIVLFIISILLLITIPNVTKHNQTIQK---	8

FIG. 25. Alignment of the amino acids (single-letter code) in the amino-terminal segments of type IV prepilins with those of the corresponding segments of four Pul proteins that form part of the main terminal branch of the GSP in *K. oxytoca* and with ComG.3, one of the prepilin-like proteins required for transformation competence in *B. subtilis*. All of the sequences begin at the initiator methionine. The prepilin peptidase cleavage site (PPP) and potential cleavage sites for signal peptidase (SP) are indicated.

idea that PapJ could be a periplasmic ATP-dependent chaperone (545) can probably be discounted on the grounds that ATP is unlikely to be present in the periplasm. The presence of a consensus ATP-binding site in PapJ may therefore be purely coincidental, but the role of PapJ, as well as that of PapK, remain to be determined.

Type IV Pili

At first sight, type IV pilins produced by *Pseudomonas aeruginosa*, *N. gonorrhoeae*, and several other gram-negative bacteria, including some pathogenic *E. coli* strains (191), appear similar to Pap and other pilins (Fig. 3). Unlike Pap pilins, however, their apparently typical signal sequences are processed on the amino-terminal side of the hydrophobic region rather than at the consensus signal peptidase cleavage site(s) on its carboxy-terminal side (Fig. 3 and 25). The position and sequence of the cleavage site are well conserved in different type IV pilins; only that in the major prepilin of *V. cholerae* (TcpA), which is preceded by an unusually long hydrophilic segment containing several positively charged residues, is slightly different (Fig. 25) (154, 501). Truncated pilins lacking the positively charged extreme amino terminus of the signal sequence are exported normally, at least in *E. coli* (398), but they are not assembled into pili when produced in *P. aeruginosa* (531).

Processing and modification. Lory and his colleagues were the first to characterize a type IV prepilin peptidase. They identified the product of the *P. aeruginosa pilD* gene (384) as the peptidase by showing that the purified enzyme could cleave the *P. aeruginosa* type IV prepilin in vitro (385). As discussed above in the section on signal peptidases, type IV prepilin peptidase cleaves after the first glycine residue in the prepilin signal sequence. Replacement of this glycine residue dramatically reduces prepilin cleavage and abolishes piliation (283, 531). Since the phenylalanine residue that immediately follows this glycine is also highly conserved (the only other amino acid found in this position in other type IV prepilins and related proteins is methionine), one would also expect its substitution to prevent processing and pilus assembly. In fact, however, only two of the nine different substitutions tested by Lory and his colleagues (glycine and, to a lesser extent, aspartate) prevented assembly of type IV pili in *P. aeruginosa* (531), implying that this residue is not essential for substrate recognition by prepilin peptidase.

Mutations that abolish the activity of PilD prepilin peptidase prevent piliation (384, 385). Likewise, mutations in the *tcpJ* gene of *V. cholerae* prevent processing of the endogenous type IV prepilin and also abolish piliation (264). The predicted *tcpJ* gene product sequence is 50% identical to PilD (264). Genes coding for other members of this family of peptidases were recently found in bacteria that are not known to have type IV pili (Fig. 26), where they are required for extracellular protein secretion by the main terminal branch of the GSP and cleave prepilin-like precursor proteins that are believed to form part of the secretion machinery (see the section on the main terminal branch of the GSP, below). These proteins all display >50% sequence identity to PilD and TcpJ, and one of them, PulO from *K. oxytoca*, correctly cleaves *N. gonorrhoeae* type IV prepilin (prePilE) in *E. coli* (143). We have repeatedly observed that *N. gonorrhoeae* type IV prepilin is partially processed when synthesized in *E. coli* in the absence of an introduced prepilin peptidase structural gene (143), suggesting that the

Bacterium/plasmid	Role	Name																	References
Klebsiella oxytoca	Secretion: pullulanase	Pul	S	(B)	(A)	C	D	E	F	G	H	I	J	K	L	M	N	O	120, 121, 122, 421, 442, 461
Erwinia chrysanthemi	Secretion: pectate lyases, cellulase	Out	S	B		C	D	E	F	G	H	I	J	K	L	M		O	95, 214, 291, 329
Erwinia carotovora	Secretion: pectate lyases, cellulase	Out				C	D	E	F	G	H	I	J	K	L	M	N	O	475
Pseudomonas aeruginosa	Secretion: toxin A, lipases, proteases etc	Xcp(Pdd/Pil)				Q	P	R	S	T	U	V	W	X	Y	Z		A	23, 24, 160, 384, 386
Xanthomonas campestris	Secretion: cellulase, protease	Xps					D	E	F	G	H	I	J						142, 231
Aeromonas hydrophila	Secretion: toxins, protease, acyltransferase	Exe					D	E	F	G									253
Vibrio cholerae	Secretion: cholera toxin, protease	Eps						E											478
Yersinia enterolytica	Secretion: YOPs	Ysc					C												360
Shigella flexneri	Secretion: IPAs	Mxi					D												9
Pseudomonas aeruginosa	Piliation	Pil(Xcp)						B	C	A	A	A	A					D	384, 385, 399
Vibrio cholerae	Piliation	Tcp							E	A	A	A	A					J	154, 264, 501
Bacillus subtilis	Transformation	Com						G1	G2	G3	G4	G5	G6					C	8, 364
Haemophilus influenzae	Transformation	ORF					E												551
pRK2	Conjugation	KilB						1											367
Agrobacterium tumefaciens pTi	Conjugation	VirB						11											88, 585
Filamentous phage	Morphogenesis	gp					IV												64

FIG. 26. Proteins related to those encoded by the *pul* genes from *K. oxytoca* identified in other bacteria in which they perform functions related to the movement of macromolecules across the cell envelope. The Pul proteins are ordered at the top of the checkerboard according to the order of their genes in the *pul* gene cluster (see Fig. 27). The reported identification in another bacterium of a gene that could encode a protein with >20% overall sequence identity to a *pul* gene-encoded protein is indicated by a black box, and their functions are listed on the left. In cases in which the overall sequence identity is <20% but relatively long stretches of sequence are more highly conserved, the box is shaded. These regions encompass the carboxy-terminal half of proteins similar to PulD, the central region between the two sequences (Walker boxes A and B) involved in nucleotide binding in proteins related to PulE, and the signal sequences (and especially the prepilin peptidase cleavage site) of proteins related to PulG, PulH, PulI, and PulJ. All of these regions are similar to the signal sequences of the precursors of type IV pili in *P. aeruginosa* and *V. cholerae*. Names given to the entire group of proteins in the different bacteria are listed on the left, and the specific names given to each protein are indicated in the boxes. Three different names have been attributed to proteins involved in protein secretion in *P. aeruginosa*. The name Xcp takes historical precedence. XcpA and PilD are the same protein. Putative functions for some of the proteins are indicated at the top of the checkerboard panel. Note that YOPs and IPAs are secreted by GSP-independent pathways (see the text and references for details).

uncharacterized gene that, according to its sequence, could code for type IV prepilin peptidase (600) is indeed active. The *E. coli* K-12 chromosome also has several other uncharacterized genes that could code for proteins with type IV prepilin cleavage sites (600).

The kinetics and site of processing by prepilin peptidase are interesting in several respects. PulO cleaves prePilE and its endogenous substrate (prePulG) within 15 to 30 s (437). This is close to the time required to synthesize these ca. 17-kDa polypeptides, suggesting that cleavage is cotranslational. In contrast, TcpJ apparently cleaves *V. cholerae* type IV pilin (TcpA) much more slowly (264), although this may have been due to special features of the system used, including high-level expression of *tcpA* and *tcpJ* in a heterologous host (*E. coli*). On the basis of studies with prepilin-alkaline phosphatase hybrid proteins, prepilins are predicted to be type II bitopic membrane proteins, with their extreme N-terminal regions that include the cleavage site exposed in the cytosol (144). Therefore the catalytic site of prepilin peptidase, which is a polytopic cytoplasmic membrane protein, is presumably exposed on the cytoplasmic side of the membrane. Indeed, processing could even occur before complete insertion into the cytoplasmic membrane. This might explain the observed Sec independence of preTcpA processing by TcpJ in *E. coli* (264).

The Phe residue of processed type IV pilins is N-methylated, implying that bacteria producing these pili have prepilin *N*-methyltransferase. Other amino acids placed at position +1 can also be methylated, but methylation does not appear to be essential for pilus assembly (531). The totally conserved glutamate residue at position +5 is required for efficient methylation but not for cleavage by prepilin peptidase (531). Intriguingly, the amino-terminal phenylalanine of PilE processed in *E. coli* by PulO prepilin peptidase is apparently methylated (143), implying either that PulO is the *N*-methyltransferase or that *E. coli* has such an enzyme. Further studies of prepilin peptidase and mutations affecting its activity should distinguish between these two possibilities.

Other assembly factors. Two other *P. aeruginosa* genes, *pilB* and *pilC*, coding for presumed cytoplasmic and polytopic cytoplasmic membrane proteins, respectively, are required for pilin secretion and assembly (384). A homolog of *pilC* has been implicated in Tcp pilus assembly in *V. cholerae* (265) (Fig. 26), and, as we shall see below, genes coding for proteins with relatively high levels of sequence identity to PilB and PilC have been identified in many extracellular protein-secreting bacteria (Fig. 26) (see the section on the main terminal branch of the GSP, below) (421). The precise roles of these proteins remain to be determined. The PilB and PilC homologs of *K. oxytoca*, PulE and PulF, may be involved in the assembly of type IV prepilin-like proteins into a cell envelope structure that facilitates extracellular protein secretion (421, 431). At least one of the subunits of this "pseudopilus" is processed by the PulO prepilin peptidase, and it is therefore logical to presume that PulE and

PulF perform the same function as PilB and PilC in pilus assembly.

PilB and its homologs all have a consensus nucleotide-binding site composed of the so-called Walker boxes A and B that are typically found in ATPases (431, 581). Highly conserved amino acids in Walker box A of PulE are essential to its function in extracellular protein secretion (420). This, together with the relatively high degree of overall sequence similarity to the VirB11 protein of *Agrobacterium tumefaciens*, an autophosphorylating ATPase involved in conjugation (88, 585), suggests that PilB might either couple energy released by ATP hydrolysis to pilin export or pilus assembly or that it might phosphorylate and thereby activate another component of the assembly machinery. Prepilins and prepseudopilins are exported and are processed normally by prepilin peptidase when their genes are expressed in *E. coli* in the total absence of other proteins required for their assembly (143, 144, 437, 530). This implies that ATP hydrolysis by PilB homologs is not required to drive prepilin export across the cytoplasmic membrane. The pseudopili of protein-secreting bacteria do not appear to reach the cell surface, however, implying that PilB and PilC are not involved in transporting pilin subunits across the outer membrane either. Their function may therefore be to catalyze the assembly of pilin or pseudopilin subunits on the periplasmic side of the cytoplasmic membrane. Processed pilins retain most of their signal sequence-membrane anchors and consequently probably accumulate in the cytoplasmic membrane (143, 144). However, processed pilin subunits might be less firmly anchored in this membrane than the precursors since all of the positively charged amino acids are removed by prepilin peptidase (Fig. 25) and the positively charged amino group at the amino terminus of mature pilin is methylated and thus neutralized. Thus, ATP hydrolysis by PilBC may be used to expel processed pilins from the cytoplasmic membrane to facilitate their assembly, which may involve hydrophobic interactions between the remaining segments of their signal sequences. This would seem to exclude the possible occurrence of a freely diffusing, chaperone-bound periplasmic intermediate of the type seen in the assembly of Pap and related pili (see above). It should be possible to use high-resolution electron microscopy to see where the pilus is anchored to the cell envelope and to determine whether PilC forms an assembly platform or whether subunits at the base of the pilus remain anchored to the cytoplasmic membrane by their hydrophobic anchors.

pilA, *pilB*, *pilC*, and *pilD* are clustered in the *P. aeruginosa* chromosome, but the fact that their simultaneous expression in *E. coli* does not result in piliation implies that at least one important element in the assembly pathway is missing (384). Further analysis of mutations in *P. aeruginosa* that block piliation might lead to the identification of genes coding for these missing components (254), as might further analysis of the *V. cholerae tcp* gene cluster, which contains several genes required for type IV pilus assembly (265). At the very least, one would expect to identify a gene coding for an OMP similar to the PilC proteins of *N. gonorrhoeae*, which appear to be involved in type IV pilus assembly as well as in the control of the frequent antigenic variations which are characteristic of type IV pili in this bacterium (256, 257).

An unusual phenomenon that so far seems to be specific to type IV pili of *N. gonorrhoeae* is the extracellular secretion of aberrantly processed pili. These so-called S pilins are produced as a result of processing after the end of the hydrophobic signal sequence region by an uncharacterized peptidase. Cells do not normally produce S pilins, but they can be induced to do so by mutations that block cleavage by prepilin peptidase (283) or that block piliation for other reasons (203, 256, 257). These results imply that transport of pilin subunits through the outer membrane is independent of their assembly into pili and that it must involve an outer membrane component distinct from *N. gonorrhoeae* PilC. Further studies of the secretion of S pili might provide important clues to the identity and function of these proteins. Of course, the eventual aim will be to reconstitute the entire pathway for type IV pilus assembly in *E. coli*, which would greatly facilitate attempts to understand the mechanisms involved. Reconstituted systems could conceivably be composed of proteins derived from different bacteria, since not only are the prepilin peptidases at least partially interchangeable (24, 143) but also type IV pilins from one bacterium can be assembled in a heterologous, type IV pilus-producing host (30, 148, 351).

The possibility that additional minor pilin subunits are required to initiate type IV pilus assembly and to anchor pili to the cell envelope should also be seriously considered. There is as yet no evidence for genes coding for minor type IV pilins except for the fact that the *V. cholerae tcpA* gene is immediately upstream of another gene that could code for a type IV prepilin (154). The *N. gonorrhoeae* and *N. meningitidis* chromosomes carry a large number of pilin structural genes. Only one, or at most two, of these genes (*pilE*) are expressed at high levels, while the others (*pilS*) are all assumed to be silent because they lack promoter regions and other elements essential for their expression (202, 204, 358, 498). However, the possibility that some of these genes are expressed at very low levels should be reexamined, especially since isolated gonococcal pili are reported to contain a number of minor proteins (395) that might be minor pilin subunits.

To conclude this section, it is worth mentioning another unusual phenomenon concerning type IV pili that was reported by Whitchurch et al. (600). *P. aeruginosa* cells exhibit twitching mobility as a result of the movement of their type IV pili. This twitching mobility is abolished in mutants carrying a mutation in a gene, *pilT*, whose product includes a potential ATP-binding site and exhibits a high level of sequence similarity to PilB. Although the molecular explanation for the twitching mobility remains to be elucidated, one attractive idea is that it is caused by PilT-ATP-dependent depolymerization of the pilus filament (600).

Main Terminal Branch

One of the most remarkable and somewhat unexpected recent developments in studies of protein export and secretion in bacteria was the discovery that several quite unrelated species of gram-negative bacteria possess what has already become known as a main terminal branch of the GSP, which they use to secrete extracellular toxins and hydrolases. In fact, it was known for some years that a large proportion of the extracellular proteins secreted by gram-negative bacteria were made as precursors with typical signal peptides (429, 443). Beyond this, however, relatively little was known except that certain chromosomal mutations in many of these protein-secreting bacteria prevented extracellular secretion while exerting little or no effect on cell envelope proteins (see, e.g., references 17, 194, 227, 285, and 612). This suggested that the extracellular release of these proteins depended on specific gene products, as well as components of earlier stages in the GSP, but relatively little

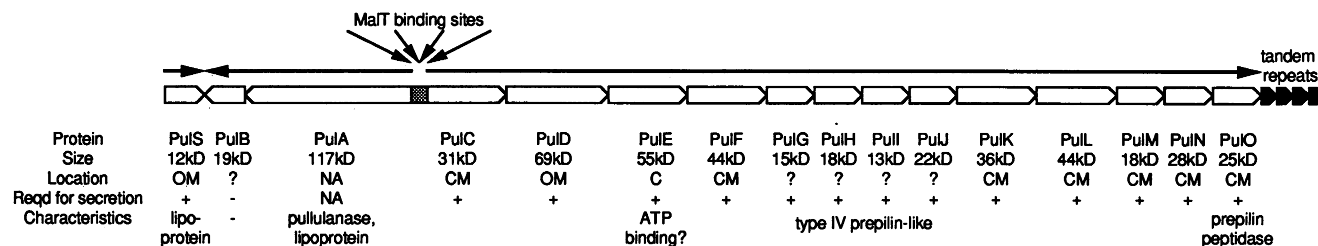

FIG. 27. Organization of genes in the *pul* gene cluster of *K. oxytoca* and some properties of the DNA of the encoded proteins. Four binding sites for the positive transcription activator protein MalT are found in the promoter regions between *pulA* and *pulC*. These control the transcription of the two long operons (*pulAB* and *pulC* to *pulO*), whose expression is induced in the presence of maltodextrins. The third transcript shown in specific for *pulS* and is not induced by maltodextrins or MalT dependent. The location of the *pul* gene products was determined by subcellular fractionation of cells producing β-galactosidase or alkaline phosphatase hybrid proteins or the products of the genes themselves under the control of a strong promoter, and generally in the absence of most other *pul* gene products. The PulG, PulH, PulI, and PulJ proteins were initially found to be located in the cytoplasmic membrane, but subsequent studies showed them to be associated with light outer membrane vesicles derived from osmotically lysed cells. See the text and references 120, 122, 421, 437, 442, and 461 for further information. NA, not applicable.

progress was made toward their identification and characterization because the bacteria concerned were not as amenable as *E. coli* to genetic analysis.

At about the same time as work on these protein-secreting bacteria was beginning, it became clear that the specific extracellular secretion of some proteins by certain gram-negative bacteria required very few, if any, cell components in addition to those required for the export and assembly of cell envelope components (see the section on other terminal branches of the GSP, below). It was therefore rather surprising when our own studies on the genes required for the secretion of the cell surface enzyme pullulanase by *K. oxytoca* and *K. planticola* (reviewed in reference 436) and simultaneous studies by several other groups working with different bacteria revealed an extremely complex situation.

Pullulanase secretion pathway: an update. Since the results of our own studies on the pullulanase secretion pathway up to April 1990 are the subject of an earlier review (436), only the main features and our more recent results will be presented here.

Pullulanase is a 117-kDa lipoprotein (263, 288, 435) produced by most *Klebsiella* strains. Unlike most other lipoproteins (see the section on lipoproteins, above), the fatty acids of pullulanase anchor the enzyme to the cell surface with the entire polypeptide chain exposed to the medium (286, 287, 417, 440). The cell surface-anchored form is slowly released into the medium (118, 359, 605), where it is found as large complexes (presumably protein micelles held together by the fatty acids). Release from the outer membrane is believed to be a spontaneous event but has not yet been studied in any detail.

To study the earlier steps in the secretion pathway, the structural gene (*pulA*) together with all of the genes required to permit the secretion (cell surface exposition) of its product were cloned and expressed in *E. coli* (122). The cloned ca. 18.6-kb fragment of the *K. oxytoca* chromosome contains 16 complete genes, together with a truncated gene coding for a protein similar to penicillin-binding protein Ib of *E. coli*. One of the 16 genes is *pulA* (288), which is immediately followed by a gene, *pulB*, with no known function (120) (but see the next section). *pulA* and *pulB* form an operon, downstream from which is another gene, *pulS*, which is in the opposite orientation and is absolutely required for secretion (120). Upstream of *pulA* is a long operon of 13 genes (*pulC–O*), all of which are required for pullulanase secretion (121, 421, 442, 461). The promoter-operator of this operon overlaps that of the divergent *pulAB* operon. Both promoters are induced in vivo by maltodextrins and in vitro specifically by maltotriose, and both are dependent on the transcription activator protein MalT (568). This is a crucial feature of the system, since maltodextrins produced by pullulanase action on branched starch molecules (amylopectine, glycogen, etc.) are transported and metabolized by a complex pathway whose components are all encoded by genes whose expression likewise depends on maltotriose and MalT (497). With the sole exception of *pulS*, all of the cloned genes required for pullulanase secretion are expressed only when *pulA* is expressed, and vice versa.

Some of the determined molecular features of the *pul* gene products are listed in Fig. 27 and are discussed in later sections. For the moment, it is important to note that *pulB* is the only one of them that can be inactivated without blocking pullulanase secretion and causing the enzyme to accumulate at a site which, according to immunoelectron microscopy of thin-sectioned cells (440), corresponds to the cytoplasmic membrane (presumably the outer, periplasmic face) (Fig. 28). When the cells are fractionated, the enzyme appears to be associated mainly with vesicles containing the so-called cytoplasmic membrane lipoprotein NlpA (622). These vesicles are slightly less dense than bulk outer membrane vesicles (55, 438, 441). As discussed above in the section on lipoproteins, only lipoproteins with an aspartate residue immediately after the fatty-acylated cysteine residue are sorted to this site. Pullulanase has an aspartate at this position (288), and, as expected, its replacement by another amino acid causes pullulanase to be sorted to the inner face of the outer membrane in cells lacking the *pul* secretion genes (438) (Fig. 28). Furthermore, this single-amino-acid substitution causes a partial (40 to 70%) defect in secretion when the *pul* secretion genes are expressed (Fig. 28). This secretion defect can be amplified by first expressing the mutated *pulA* gene in the absence of *pulC–O* expression, causing the enzyme to accumulate on the inner face of the outer membrane, and then activating expression of the *pulC–O* operon. Under these conditions, the product of the mutant *pulA* gene (without the lipoprotein-sorting signal) cannot reach the cell surface, whereas wild-type pullulanase that is produced under identical conditions (and that accumulates first on the cytoplasmic membrane) is secreted within 1 to 3 h after induction of *pulC–O* expression (438, 441) (Fig. 29). Thus, the lipoprotein-sorting signal is required for efficient pullulanase secretion, presumably because it

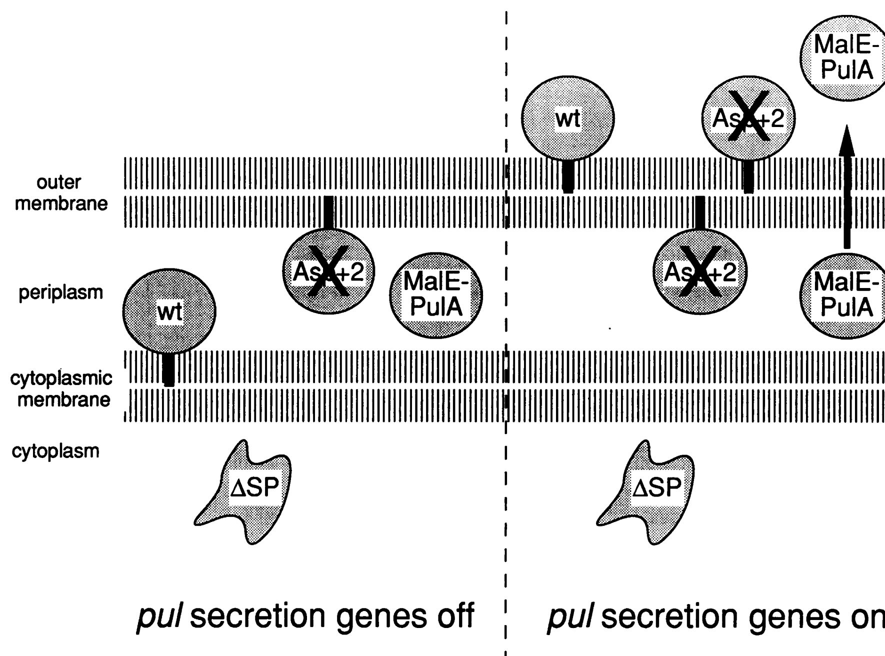

FIG. 28. Subcellular location of wild-type pullulanase (wt) and its variants in strains in which the *pul* secretion genes are either absent (left) or present and expressed (right). Pullulanase is normally located on the cell surface with its fatty acids (thick line) embedded in the outer leaflet of the membrane and the entire polypeptide chain exposed in the medium. Mutations in any 1 of the 14 *pul* genes required for pullulanase secretion cause the enzyme to accumulate at a site that is presumed to be the outer leaflet of the cytoplasmic membrane, with the polypeptide chain in or close to its natural configuration in the periplasm. Mutations in *pulA* that affect or remove the signal peptide (ΔSP) cause the enzyme to accumulate in the cytoplasm in a form that appears to be structurally different from the active polypeptide. Substitution of the aspartate residue at position +2 of the mature part of pullulanase (Asp +2) causes sorting to the inner leaflet of the outer membrane in strains defective for the Pul secretion factors and only partial translocation to the cell surface in secretion-competent strains. Replacement of the pullulanase signal peptide with that from the precursor of periplasmic maltose-binding protein (MalE-PulA) causes release into the periplasm, followed, in secretion-competent strains, by progressive translocation across the outer membrane at a rate that appears to depend on the size of the accumulated periplasmic pool. See the text for further details.

helps to position the enzyme correctly at an intermediate stage in its secretion.

Since the products of so many genes were found to be required for pullulanase secretion, and especially since many of them appeared to be located in the cytoplasmic membrane (Fig. 27), it was pertinent to ask whether some of them might replace functions normally performed in the GSP by the *sec* gene products. This does not appear to be the case, however, since mutations in any of the six *sec* genes block processing of the prepullulanase signal peptide (439). Furthermore, amino acid substitutions or deletions in the pullulanase signal peptide, or its complete removal, cause the enzyme to accumulate in the cytoplasm in a form which is uncharacteristically susceptible to inactivation by carboxymethylation (439) (Fig. 28). Thus, pullulanase secretion requires not only the 14 *pul* gene-encoded secretion factors but also the six Sec proteins together with lipoprotein signal peptidase and the glyceryl and fatty acyl transferase required for lipoprotein modification. Furthermore, recent studies (433) show that, although it is not absolutely required, catalysis of disulfide bond formation in pullulanase by disulfide oxidoreductase increases the rate at which individual pullulanase polypeptides reach the cell surface. Thus, a total of 25 proteins are known to be involved in pullulanase secretion and modification (Fig. 30).

Replacement of the lipoprotein-type signal peptide and the first few amino acids of the mature part of pullulanase (including the fatty-acylated cysteine) by the corresponding segment of the precursor of a periplasmic protein (lacking a cysteine residue that could be fatty acylated) results in cleavage by LepB signal peptidase and abolishes membrane association but not secretion (417). This confirms earlier studies suggesting that fatty acylation was not required for secretion (286, 287, 370). Under certain conditions, the nonacylated enzyme is secreted almost as quickly as the wild-type protein. However, whereas high-level production of the wild-type enzyme does not appear to affect its secretion, secretion of the nonacylated enzyme was severely retarded under the same conditions, implying that the fatty acids improve the efficiency of secretion. Furthermore, the location of the pool of nonacylated pullulanase which accumulates under these circumstances is indistinguishable from that of typical periplasmic proteins (417) (Fig. 28). This interesting observation allowed us to test our hypothesis that the two steps in pullulanase secretion (*sec* and *pul* gene dependent) are completely independent and that none of the *pul* gene products are involved in translocation across the cytoplasmic membrane. This was done by uncoupling the synthesis of the nonacylated pullulanase from expression of the *pulC–O* operon as described above. Under these conditions, the enzyme which accumulated in the periplasm when the *pulC–O* operon was not expressed was subsequently secreted out of the cell when expression of the operon was induced (Fig. 29) (417). Thus, the first two steps in pullulanase secretion are quite independent, and pullulanase that accumulates in the absence of *pulC–O*-encoded secretion

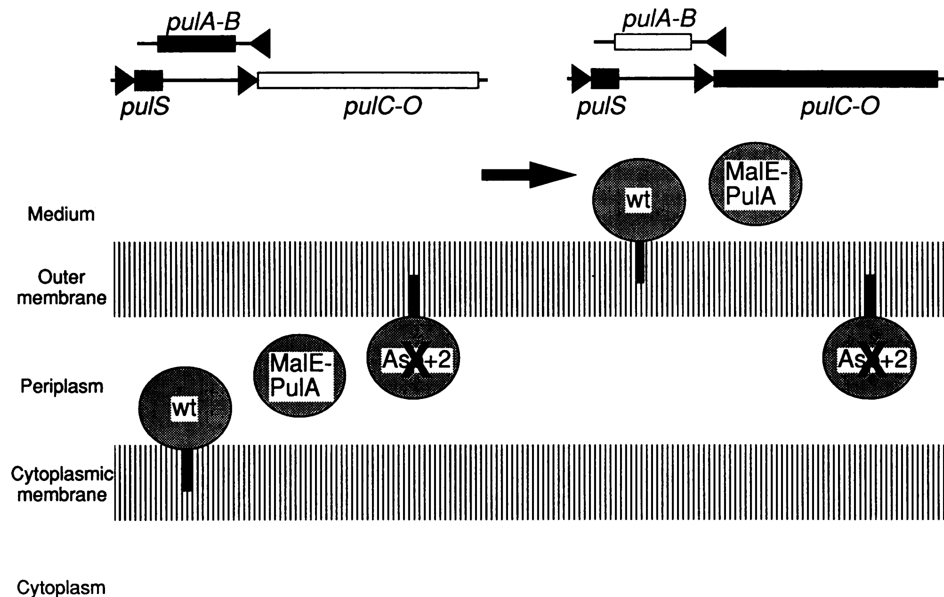

FIG. 29. Effects of mutations affecting the lipoprotein-sorting signal (aspartate residue at position +2) of fatty-acylated pullulanase, or replacement of the pullulanase signal peptide by that from periplasmic maltose-binding protein (MalE-PulA hybrid) on pullulanase secretion under conditions of uncoupled expression of the pullulanase structural gene (*pulA*) and the *pulC-O*-encoded secretion genes. The organization and control of the *pul* genes is illustrated at the top. *pulA* (or *malE-pulA*) is carried by a different plasmid and is under the control of a different promoter from the *pulC-O* operon. *pulS* is on the same plasmid as *pulC-O* but is constitutively expressed. Black and white boxes indicate that the genes are being expressed or not expressed, respectively. *pulA* is initially expressed in the absence of *pulC-O* expression, and then *pulC-O* expression is induced while *pulA* expression is repressed. Arrowheads indicate the positions of promoters. The location of pullulanase is determined by enzymatic assays or detection of radiolabeled protein in subfractionated cells and in the medium. Fatty acids on the pullulanase polypeptide are indicated by a black stalk. wt, wild-type pullulanase; Asp +2, pullulanse lacking the aspartate residue at position +2.

factors remains competent for transport across the outer membrane. Further implications of this important observation are discussed below.

Other bacteria. As mentioned above, genes coding for proteins similar to those required for the second step in pullulanase secretion have now been identified in several other protein-secreting bacteria (23, 24, 95, 142, 160, 214, 253, 329, 384, 386, 475, 478) and seem to be present in yet others (117). A summary of the results from several groups working with different bacteria is presented in Fig. 26.

The degree of sequence identity between similar proteins from different species varies considerably, ranging from nearly 80% (e.g., OutE and PulE) to less than 30% (e.g., proteins similar to PulF). Relatively few tests have been performed to determine whether the entire pathways (or components thereof) from different bacteria are interchangeable, but full interchangeability would not be expected in view of the large number of proteins involved and their sometimes extensive sequence divergence. Even the secretion pathways and secreted proteins from two closely related bacteria (*Erwinia chrysanthemi* and *Erwinia carotovora*) are not interchangeable (447), possibly because only one of them (*Erwinia carotovora*) has an *outN* gene (329, 475). Buckley and his colleagues have, however, reported examples of the secretion of extracellular proteins by heterologous hosts via what appear to be typical main terminal branch GSPs (609, 610). Formal proof that all of the Pul homologs identified so far in other bacteria are actually required for protein secretion has not been obtained. Only the pullulanase secretion pathway and the *out* gene-encoded pathway from *Erwinia chrysanthemi* (214) have been successfully reconstituted in *E. coli*. As with pullulanase secretion in *E. coli* carrying the *pul* secretion genes, secretion of pectate lyase by *E. coli* carrying the *out* gene cluster is also *sec* dependent (215).

The overall organization of the *pul* homologs is similar to that of the *pul* genes themselves. Indeed, the organization of the *pul* genes and the *out* genes in *Erwinia* species appears to be identical except for the absence of a *pulA* homolog from both *Erwinia* species and of *outN* from *Erwinia chrysanthemi* (329). In other species (e.g., *Xanthomonas campestris* and *P. aeruginosa*), the secretion gene operon starts with the *pulE* homolog, although in *P. aeruginosa* at least, the *pulC* and *pulD* homologs are located very close to the *pulE* homolog on the chromosome. The PulO homolog may not always be part of the operon (23, 329, 384). Of particular interest is the recent observation (95) that whereas *pulB* is not required for pullulanase secretion in *E. coli*, the equivalent gene in *Erwinia chrysanthemi* (*outB*) is required for extracellular secretion. This may be because *E. coli* has a *pulB* homolog that can replace the cloned *pulB* gene from *K. oxytoca*.

In *K. oxytoca*, the genes coding for most of the protein components of the main terminal part of the GSP are coregulated with the structural gene for the sole enzyme (pullulanase) that is known to be secreted by this bacterium. Many other gram-negative bacteria use the main terminal GSP branch to secrete a variety of different enzymes whose structural genes appear to be expressed in response to different environmental signals (see, e.g., references 40, 415, and 563). It is therefore pertinent to ask whether the genes coding for components of the main terminal GSP branch in these bacteria are either constitutively expressed or subject to complex regulatory controls by each of the factors that control the genes coding for the secreted proteins. In *Er-*

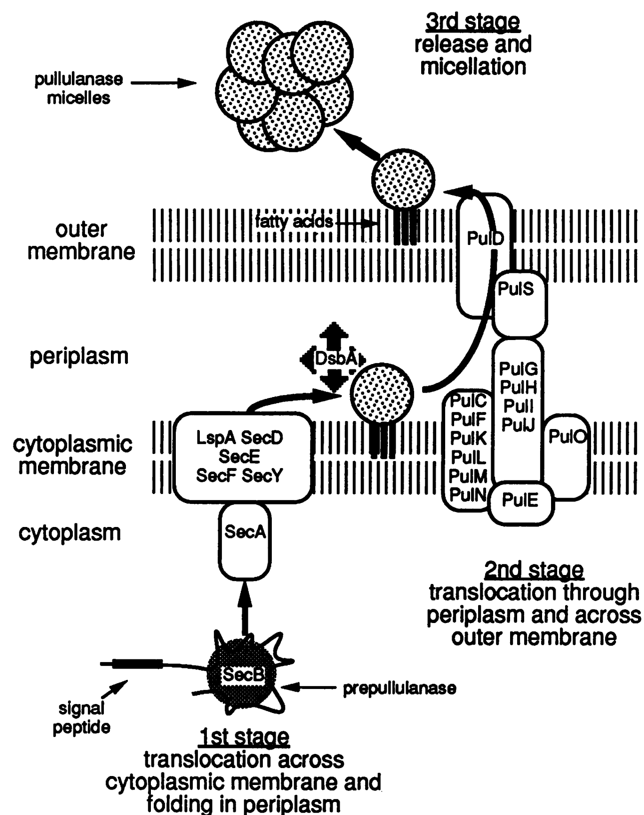

FIG. 30. Model for the three steps in the secretion of fatty-acylated pullulanase by the complete GSP including the *pul* gene-encoded main terminal branch in *K. oxytoca*. Glyceryl and fatty acyl transferase are omitted for clarity. DsbA, disulfide oxidoreductase. PulE is shown associated with the cytoplasmic membrane via other Pul proteins, a complex of PulGHIJ is shown spanning the periplasm, and pullulanase is shown to pass through the outer membrane via PulD. None of these points have been demonstrated experimentally.

winia chrysanthemi, the *outC* operon appears to be regulated by the KdgR protein, which also controls expression of the genes coding for the secreted pectate lyases (95, 329). This situation is highly reminiscent of the regulation of *pul* gene expression, although the pectate lyase structural genes are not adjacent to the *out* genes.

Pullulanase is the only lipoprotein so far shown to be secreted by the main terminal branch of the GSP, although other cell surface and extracellular lipoproteins are produced by certain gram-negative bacteria (251, 352, 523) while others secrete soluble proteins that are made as fatty acid-bearing precursors (232–234). This raises the question of whether the pullulanase secretion pathway includes elements specifically required to translocate the protein-bound fatty acids to the cell surface. The possibility that PulS is required for this purpose (287) can now be discounted because *Erwinia chrysanthemi* has a *pulS* homolog (*outS*) which is required for the secretion of nonacylated proteins (95) and because PulS protein is itself required for the secretion of a nonacylated variant of pullulanase in *E. coli* (417).

Mutations in the *pul* gene homologs of other protein-secreting bacteria cause the normally extracellular enzymes to accumulate in the periplasm (17, 227, 228, 237, 549), as does expression of cloned genes coding for extracellular proteins from these bacteria in *E. coli* (93, 135, 229, 266, 319, 333). Aerolysin that accumulates in the periplasm of *Aeromonas salmonicida* cells expressing the cloned structural genes from *A. hydrophila* can be chased into the medium under certain conditions (609), but secretion intermediates do not usually accumulate for more than a very brief period in these extracellular protein-secreting bacteria (207, 215, 228). Notable exceptions are cholera toxin and the closely related *E. coli* heat-labile enterotoxin, which accumulate and oligomerize in the periplasm before being secreted out of *V. cholerae* cells (223, 224) by a pathway that requires a PulE homolog (478) and may be a typical main terminal GSP branch.

Salmonella and *Shigella* species and, above all, *E. coli* do not naturally secrete extracellular proteins by the main terminal GSP branch and probably lack many, if not all, of the genes coding for components required to do so. It seems probable that these bacteria lost these genes during evolution (although, surprisingly, many of them still produce but do not secrete proteins such as heat-labile enterotoxins and Shiga toxins that are similar to cholera toxin, which is secreted by *V. cholerae*). The pullulanase secretion pathway in *K. oxytoca* may represent the vestiges of a once more catholic terminal branch of the GSP, whose genetic determinants were progressively grouped together with and brought under the same genetic control as the only enzyme (pullulanase) that this bacterium now secretes.

Common elements in other systems. Although the observation that many widely different bacteria secrete extracellular proteins by using secretion factors similar to those needed for pullulanase secretion was striking and unexpected, it did not further our efforts to determine the individual roles of these secretion factors. Major advances were made, however, when it was realized that certain pullulanase secretion factors and their functional and structural homologs in other bacteria were similar to yet other proteins with known activities or functions (Fig. 26).

The most striking of these discoveries was that proteins encoded by four of the *pul* genes (*pulG*, *pulH*, *pulI*, and *pulJ*) all have potential cleavage sites for type IV prepilin peptidase and that a fifth gene, *pulO*, codes for a protein with approximately 50% sequence identity to two proteins, PilD and TcpJ, that are known to have prepilin peptidase activity (264, 385) (see the section on processing and modification of type IV pili, above). PulO is in fact able to cleave a type IV pilin precursor (143) as well as prePulG protein (437). Furthermore, the products of two other *pul* genes (*pulE* and *pulF*) show a relatively high degree of sequence identity to two other proteins, PilB and PilC, that are required for type IV pilus assembly in *P. aeruginosa* (421) (see the section on other assembly factors, above). All of these elements are highly conserved in other bacteria that possess the main terminal GSP branch (Fig. 26); indeed, the gene coding for prepilin peptidase in *P. aeruginosa*, *pilD*/*xcpA* (Fig. 26), is required both for type IV pilus assembly (384) and for extracellular secretion (23, 532). This prepilin peptidase cleaves both prepilin (385) and the products of genes that code for prepilin-like proteins (24, 386). The prepilin-like peptidases from different bacteria are likely to be one of the few components of this terminal GSP branch that can function in heterologous bacteria (24, 143) (see above).

The obvious interpretation of these results is that a pilus-like structure might be required for extracellular enzyme secretion and that at least some of the genes identified as required for secretion encode pseudopilus subunits (*pulG*, *pulH*, *pulI*, and *pulJ*) or proteins required for their assembly

(*pulE*, *pulF*, and possibly others). The most logical location for this pseudopilus would be between the two membranes, where it might serve as a platform or scaffold for the assembly of other components required for secretion or as a sort of ladder to direct proteins to the outer membrane via a cascade of signal-receptor interactions. One intriguing possibility is that a relatively abundant pseudopilin (PulG in the pullulanase system) is the main structural element of the pseudopilus while other components act as receptors for proteins that are to be secreted. As yet, however, there is no evidence for stable interactions between the type IV pilin-like proteins involved in secretion, let alone for the presence of a pilus-like structure. A similar set of genes has been identified in *B. subtilis*, in which they are required for transformation competence and may be directly involved in the uptake of transforming DNA (140). In this case, of course, the putative pseudopilus can be anchored only to the cytoplasmic membrane.

Another important point that merits closer examination is how secreted proteins cross the outer membrane. There is considerable evidence that proteins secreted by this pathway can and indeed may have to fold into complex tertiary or even quaternary structures before they are transported across the outer membrane. For example, a disulfide bond does not prevent (441) and may be required (432) for pullulanase secretion as well as for heat-labile enterotoxin/cholera toxin and protease secretion by *V. cholerae* (401, 623). However, the best evidence that secreted proteins fold before they cross the outer membrane comes from elegant studies by Hirst and Holmgren (223, 224) showing that oligomerization of toxins occurs in the periplasm before they are secreted out of *V. cholerae* cells. This means that outer membrane translocation is fundamentally different from cytoplasmic translocation by the Sec translocase, which can tolerate only limited tertiary structure (see the section on translocation competence, above) and in this respect is similar to enterobacterial pilus assembly (see above).

The transport of such bulky structures as pullulanase or heat-labile enterotoxin across the outer membrane might occur through the lipid phase of the membrane or through a channel formed by one or more specific proteins. Preliminary studies on pullulanase-secreting cells indicate that the outer membrane permeability barrier to normally nonpermeating antibiotics is not compromised (584). The most likely candidates as outer membrane, channel-forming proteins in the main terminal GSP branch are PulD and its homologs, the only integral outer membrane proteins so far demonstrably required for secretion (PulS is probably a peripheral outer membrane lipoprotein). PulD homologs have also turned up in other systems. In particular, proteins with relatively high levels of identity to the carboxy-terminal halves of PulD and other, closely related proteins are required for assembly and secretion of filamentous phages (64), for secretion of extracellular proteins by GSP-independent pathways (9, 360), and for transformation of *Haemophilus influenzae* (551) (Fig. 26). All of these functions relate to the transport of macromolecules (assembled bacteriophages, proteins, or DNA) across the outer membrane, making it even more likely that PulD and its close homologs form channels in the outer membrane.

Attempts to study the channel-forming properties of this group of proteins are now under way, but one important consideration is their size: an extremely large pore diameter reminiscent of that of the pores in nuclear envelopes (430) would be required to allow the passage of complex folded proteins such as heat-labile enterotoxin and pullulanase. Nuclear pores are very complex structures made of several different components (222), and the diameter of the channel they form can be altered to allow the passage of large molecules such as proteins that carry specific nuclear localization signals (430). Although some other components of the terminal GSP branch pathway may interact with the putative channel component in the outer membrane, the channel itself is unlikely to be as complex as the nuclear pore.

Energy coupling. Since secreted proteins are probably translocated in a prefolded form, folding upon extrusion from the cell surface cannot be used to pull the polypeptide through the membrane. Extracellular protein secretion occurs down a considerable concentration gradient (223, 224) and could be independent of additional energy input. However, there are few, if any, precedents for the transport of macromolecules through biological membranes without exogenous energy input at some stage of the process (see the sections on energy requirements and on Sec-independent translocation, above). This is one aspect of protein secretion that has received relatively little attention. Experiments performed by Wong and Buckley to determine the requirement for and the nature of the energy required for the second step in GSP-dependent protein secretion revealed the surprising requirement for a PMF for aerolysin secretion in *A. salmonicida* (607). The authors were not aware at the time that the pathway they were studying was similar to that used for pullulanase secretion (253) and interpreted their data to indicate the existence of an energy gradient across the outer membrane. This seems highly unlikely in view of the fact that this membrane, like the outer membranes of all gram-negative bacteria, probably has permanently open pores to allow the diffusion of small solutes (375), and it seems more likely that their results indicate that the PMF across the cytoplasmic membrane is coupled to aerolysin transport across the outer membrane. A somewhat analogous situation seems to occur during the uptake of iron-siderophore complexes, vitamin B_{12}, and certain colicins across the outer membrane, which are PMF-dependent processes promoted by a cytoplasmic membrane-anchored protein, TonB (422). One intriguing possibility is that one or more of the cytoplasmic membrane-anchored components of the GSP terminal branch couples the PMF to protein transport to the exterior by a similar mechanism (TonB itself is not required for pullulanase secretion [434]). Further examination of this important aspect of protein secretion would be most informative.

The cytoplasmic PulE protein of *K. oxytoca* and its homologs involved in protein secretion in other bacteria have conserved sequences (Walker boxes A and B [581]) that are found in many nucleotide-binding proteins with ATPase or kinase activities (431). Key residues in the more highly conserved of these sites (Walker box A) are absolutely required for PulE activity, as are residues between these two sites that are highly conserved in all of the PulE homologs listed in Fig. 26 (420). This suggests that they all perform related functions. Apart from the proteins with the lowest level of sequence identity to PulE, VirB11 and KilB.1, all of them are involved in protein secretion or DNA uptake (GomG.1) by systems that involve type IV pilin-like proteins or are directly involved in type IV pilus assembly (Fig. 26) (see the section on other assembly factors, below, and the previous section). PulE and its close relatives are therefore more likely to be required for the assembly of a pseudopilus rather than to provide energy for the transport of proteins across the outer membrane.

Selectivity. Another remarkable property of the main terminal GSP branch is that transport across the outer membrane is restricted to one or very few proteins. This implies the presence of "secretion signals that are recognized and decoded by receptors" encoded by secretion genes. Interactions between secreted proteins and their receptors might be amenable to both biochemical and genetic analyses, but very little progress has been made so far toward the identification of secretion signals or their receptors. Sequence comparisons of structurally quite distinct proteins secreted by the same bacterium by the same pathway have so far failed to reveal the presence of any stretches of conserved amino acids that might constitute a secretion signal (434). This suggests that the secretion signal might be composed of several amino acids (a patch signal) that are brought together only in the fully folded polypeptide in the periplasm. This would explain the failure to secrete normally extracellular proteins in which the formation of disulfide bonds is prevented or reduced by the absence of disulfide oxidoreductase (401, 432, 623). It has been proven notoriously difficult to identify patch signals in proteins in other systems (430). It is to be hoped that the secretion signal might be present in a specific domain of the polypeptide that is independent of the domain that carries the catalytic or other activity, in which case knowledge of the fine structure of the protein (see, e.g., reference 516) may be particularly valuable. It would appear unlikely that secretion signals could be detected by random mutagenesis. Indeed, linker insertions have so far failed to produce mutations in *pulA* that specifically block pullulanase secretion (288, 416), but Buckley and his colleagues have identified some key residues in aerolysin that cannot be substituted by site-directed mutagenesis without reducing secretion (608). Deletion mapping (207) and gene fusions (119, 215, 232, 288, 289, 446) might be more useful in attempts to define regions of secreted polypeptides that are essential or nonessential for secretion, but early successes (207, 289) have not been purused and more work is clearly required.

Other Terminal Branches

At this stage of this review, it would be possible to list the many other signal peptide-bearing proteins whose processed form is secreted extracellularly, but only fragmentary information on the molecular details of how most of these proteins are secreted is available and it will probably transpire that many of them are secreted by variants of the main terminal GSP branch discussed in the preceding sections of this review. I have therefore chosen to discuss just three further examples of extracellular protein secretion and to refer the reader to earlier reviews (429, 436, 443) for details of other systems.

***Serratia* spp.: pluripotential protein secretion.** *Serratia* species secrete numerous extracellular proteins by a wide variety of different mechanisms. For example, *Serratia marcescens* secretes a protease by a GSP-independent pathway similar to that used by *E. coli* to secrete α-hemolysin (320), a serine protease by a mechanism similar to that used by *N. gonorrhoeae* IgA protease (see the next section), and a hemolysin that is produced as a signal peptide-bearing precursor that can be secreted by *E. coli* provided that a second gene linked to the hemolysin structural gene is also expressed (487). This second gene codes for an OMP that is also required to activate the hemolysin (390). No other *Serratia* gene product seems to be required for hemolysin secretion in *E. coli*. The structural genes for several other proteins secreted by *Serratia* species have also been cloned and expressed in *E. coli*. Remarkably, many of their products seem to be secreted even though the recombinant bacteria carry at most only one other *Serratia*-derived gene (22, 192, 255). Mutants of *Serratia marcescens* that are defective in extracellular protease secretion have been isolated (221). Surprisingly, none of these mutations affect several different extracellular proteins, implying that each of them, like the hemolysin, has its own particular pathway for transport across the outer membrane and that a pluripotential main terminal GSP branch similar to that found in other bacteria such as *P. aeruginosa* and *Erwinia* species is absent from *Serratia* species. Further examination of the secretion kinetics of these extracellular proteins and of *E. coli* mutants that are blocked in their secretion might reveal useful information regarding possible secretion intermediates, protein folding, and factors required for secretion.

α-Lytic protease. Many of the reports of heterologous protein secretion by *E. coli* carrying the cloned structural gene must be treated with caution because proper controls for nonspecific release of periplasmic or membrane proteins were not performed. Apart from the examples discussed above, two other cases merit further discussion. The first of these concerns the α-lytic protease that is secreted by *Lysobacter enzymogenes* (the other concerns some IgA proteases and a serine protease, which are discussed in the next section). Expression of the α-lytic protease structural gene in *E. coli* at <30°C results in autoprocessing of the amino-terminal propeptide, activation (see the section on endochaperones, above), and periplasmic accumulation followed by apparently specific extracellular secretion (509). Incubation at higher temperatures or mutations that abolish catalytic activity cause misfolding and block secretion (509). Protease that is synthesized without its propeptide is also not secreted. These nonsecreted forms are outer membrane associated, but the propeptide can act in *trans* to catalyze the folding (and thence the secretion) of an inactive protease. This implies that the protease must fold correctly (without necessarily being active) before it can cross the outer membrane (176). Presumably, misfolding causes the protein to abort secretion and to become outer membrane associated (or aggregated), whereas the normal secretion pathway transports a correctly folded periplasmic intermediate. Thus, in this case at least there is good evidence for unassisted transport of a protein across the outer membrane that merits further investigation. *L. enzymogenes* also secretes an alkaline phosphatase which, unusually, has a carboxy-terminal "apo" peptide, but nothing appears to be known about how this protein is secreted (18).

***N. gonorrhoeae* IgA protease.** The archetypical example of an "autosecreted" protein is the IgA protease of *N. gonorrhoeae* (411), which, like the IgA protease of *H. influenzae* (197) and a serine protease of *Serratia marcescens* (363, 621), is secreted via an outer membrane-anchored intermediate. The elegant work of Meyer and his colleagues has shown that the protease is made with a typical signal peptide that directs its transport across the cytoplasmic membrane and is then proteolytically removed (presumably by a LepB-type signal peptidase). The remainder of the polypeptide is then inserted into the outer membrane by mechanisms that appear similar to those used by classical OMPs (see the section on outer membrane proteins, above). Outer membrane integration is determined by the 30-kDa carboxy-proximal part of the protein (Iga_{β}), which is predicted to have eight transmembrane amphipathic β-sheets, typical of this class of membrane proteins (274). As with most OMPs (534), the last residue of the polypeptide is a phenylalanine

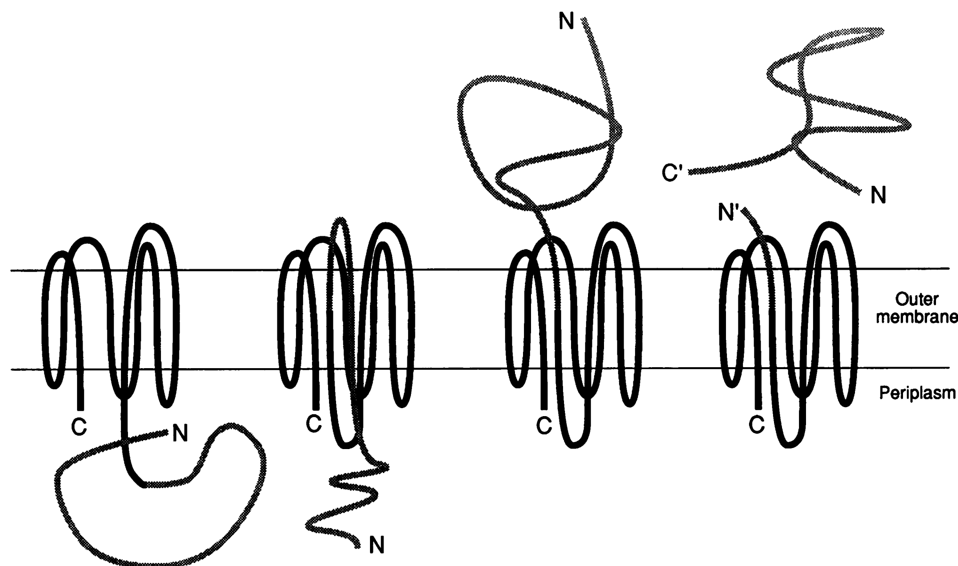

FIG. 31. Model for translocation and release of *N. gonorrhoeae* IgA protease across the outer membrane and its release into the medium, based on work by Meyer and his colleagues (273, 274). For clarity, the protease is considered to be composed of two segments, an integral outer membrane β-barrel domain (black line) and an amino-proximal region containing the catalytic domain and an intermediate segment (shaded line). Translocation and release proceed through four stages depicted (left to right) as insertion, translocation through a central channel in the integral membrane β-barrel, autoproteolysis, and release. N and C, amino and carboxy termini, respectively; N′ and C′, extremities produced after cleavage, which may occur at several closely spaced sites.

(411), but, atypically, the amino-proximal end (Iga_p and Iga_α), which includes the catalytically active part of the protein (Iga_p), is very long, and is initially exposed on the outside of the outer membrane before it is cleaved off and released (compare Fig. 21 and 31). Autoproteolytic processing at three typical IgA cleavage sites in and around Iga_α result in the release of Iga_p into the medium, while the β-domain remains in the membrane. No additional secretion factors are required. Expression of the *iga* gene in *E. coli* results in extracellular protease secretion as in *N. gonorrhoeae* (206, 284), except that release from the cell-associated intermediate can occur even when the IgA protease is inactive, because of the action of *E. coli* outer membrane protease OmpT (274).

Thus, the β-domain acts as an endogenous secretion factor that allows the extracellular part of the protein to reach the cell surface, whence it can be released, but how does the amino-proximal end get to the cell surface? One possibility is that, unlike typical OMPs, the IgA protease precursor spontaneously inserts into the outer membrane with its amino-proximal end facing outward. Although this is generally considered unlikely, the recent demonstration that the β-lactamase segment of an OmpA–β-lactamase hybrid apparently reaches the cell surface (168) (see the section on outer membrane proteins, above) could set a precedent for such a mechanism of polypeptide transport through the outer membrane.

The alternative explanation for the translocation of the amino-proximal end of IgA protease to the cell surface, which seems to be favored by Meyer and his colleagues (274), is that the β-segment of the protease forms a channel through which the amino-proximal end is threaded in much the same way as proteins are proposed to cross the cytoplasmic membrane. Thus, the β-domain performs the same function as that proposed for the outer membrane component of the main terminal GSP branch (see the section on common elements in other systems, above). The best evidence in favor of this model for IgA secretion is the demonstration that the β-domain of IgA protease can promote the translocation of a passenger protein, the B subunit of cholera toxin, to the cell surface (whence it can be released by OmpT proteolysis) (273), but only as long as the passenger protein is prevented from forming an intramolecular disulfide bond (273, 274). Translocation intermediates were found to accumulate when disulfide bonds were allowed to form, but the insertion of the β-domain into the outer membrane apparently occurred normally irrespective of the presence of these disulfide bonds. This was assumed to indicate that outer membrane insertion and translocation of the passenger to the cell surface are independent, sequential events.

Certain features of this interesting system are worthy of further comment. First, the fact that the sole secretion factor specifically required for IgA protease secretion is initially an integral part of the protease precursor obviates the need for receptors that are proposed to specifically select proteins from periplasmic pools for secretion across the outer membrane in more complex systems (see the section on selectivity, above). In fact the IgA protease secretion pathway may facilitate the outer membrane translocation of virtually any polypeptide that is fused to its amino terminus, provided that all other conditions for efficient secretion are met. However, the IgA protease secretion factor can be used only once. Second, the dependence of translocation on the absence of disulfide bonds (and presumably any extensive tertiary structure) is reminiscent of translocation across the cytoplasmic membrane (see the section on translocation competence, above) and is the reverse of that seen in the main terminal GSP branch (see the section on the main terminal branch of the GSP, above). Thus, analogies between the function of the β-subunit in IgA protease secretion and putative OMPs in secretion by the main terminal branch of the GSP do not extend to the mechanisms involved. A periplasmic molecular chaperone may be required to prevent

premature folding of the p domain of the IgA protease or of the passenger protein that replaces it, while allowing the β-domain to fold and insert into the outer membrane. The α-domain does not appear to perform this function (273). Third, as pointed out by Meyer and his colleagues (274), folding of the p domain on the outer face of the membrane might provide the driving force for translocation.

CONCLUDING REMARKS

Since we first reviewed the topic in 1985 (443), studies on protein export and secretion have undergone a progressive broadening of scope to include other aspects besides translocation across the cytoplasmic membrane. This broadening of scope is open to criticism—why diversify into other systems before we completely understand the one we started with? The answer is that our aim is to learn more about how proteins cross membranes, no matter how this is achieved, no matter how exotic the system may appear. A case in point is our own work on pullulanase secretion, which we started by considering as an interesting, albeit highly complex oddity (436), but which is now one of the best-characterized examples of the main terminal branch of the GSP in gram-negative bacteria.

Even in a review of this size, it is impossible to mention all of the work that has been carried out on protein secretion in bacteria. Readers who find that I did not mention their favorite system or organism or, worse still, their own work, will, I hope excuse me. The decision to deal only with the export and secretion of proteins that contain signal sequences meant that many novel secretion pathways were not mentioned at all (see the review by Fath and Kolter [156] for a complete description of one such pathway), but it is perhaps in studies of some of these less well characterized secretion pathways that the greatest progress will be made during the coming years.

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