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MINIREVIEW

Peptidoglycan as a Barrier to Transenvelope Transport

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INTRODUCTION

The bacterial envelope can be considered a combined mechanical and permeability barrier. This duality of function protects the cell against detrimental environmental influences and allows the maintenance of a high internal osmotic pressure. Although these properties imply rigidity, the barrier should also be flexible, permitting morphogenetic changes during growth and division and allowing for transport out of and into the bacterial cell. The mechanical integrity and permeability characteristics of the envelope are usually assigned separately to the two major constituents of the envelope: peptidoglycan is considered to be responsible for the mechanical integrity, whereas the membrane(s) constitute(s) the permeability barrier. Although this functional division is for the most part valid, it overlooks the possible contribution of peptidoglycan to the permeability characteristics of the envelope. For gram-positive bacteria, this issue was discussed in some early studies (30, 88), and the fact that the multilayered peptidoglycan, with its high degree of cross-linking and the presence of teichoic acids, might act as a permeability barrier is also the key feature of the recently proposed concept of gram-positive periplasm (68). For gram-negative bacteria, on the other hand, the permeability characteristics of peptidoglycan were overshadowed by those of the outer membrane (72). In fact, whereas low-molecular-weight compounds probably diffuse readily through peptidoglycan, the size of its pores may well be a limiting factor for the passage of larger proteins and protein complexes. In particular, the penetration of peptidoglycan by large multisubunit protein complexes that span the entire envelope may pose considerable steric problems. The presence of these envelope-spanning structures has been demonstrated or postulated for a variety of transfer processes such as the secretion of toxins, the assembly of flagella and fimbriae, conjugation, and transformation. However, when the putative structure or function of these large multiprotein transfer machineries is described, the issue of peptidoglycan penetration is not usually taken into consideration. This is illustrated by the failure to depict the peptidoglycan layer in several schematic representations of these large putative envelope-spanning structures. The presence of peptidoglycan pores large enough to accommodate these structures is often taken for granted. On the other hand, some indirect evidence has led to speculation about possible peptidoglycan rearrangements during the assembly of these structures or during the actual transfer process (19, 26, 27, 36, 66).

This minireview takes this speculation further by looking at the phenomenon of envelope spanning from a dimensional perspective: we focus on the correlation between the structural and functional features of peptidoglycan and the envelope-spanning protein complexes. The observed dimensional discrepancies, combined with an examination of the reported links between the assembly of envelope-spanning complexes and peptidoglycan metabolism, strongly suggest the involvement of specific peptidoglycan-processing enzymes in this assembly. This hypothesis is supported by the recent identification of peptidoglycan hydrolase homologs in several cross-envelope transfer systems.

PEPTIDOGLYCAN STRUCTURE

The peptidoglycan sacculus is a heteropolymeric macromolecule that serves as a protective casing for the bacterial cell. Although the chemical composition of the polymer might vary between different bacteria, the basic architecture is conserved. It consists of relatively short glycan strands, composed of disaccharide subunits, that are cross-linked by peptides. The multilayered peptidoglycan of gram-positive bacteria is located on the outside of the cell and can be up to 10 times as thick as the peptidoglycan of gram-negative bacteria, which is located in the periplasm. Using neutron and X-ray small-angle scattering measurements, Labischinski showed that about 75 to 80% of the peptidoglycan of *Escherichia coli* consists of a single layer and about 25 to 30% has three layers and that it has a thickness of about 2.5 nm per layer (57). Over the years, the exact location of peptidoglycan in the periplasm has been a matter of debate. The classical view of the periplasm as a largely empty compartment with the peptidoglycan associated as a thin layer with the outer membrane has been replaced by the concept of periplasmic gel (37). This periplasmic gel was proposed to derive its gel-like properties from a hydrated peptidoglycan that fills most of the periplasmic space. These features are, however, hard to reconcile with the above-mentioned thickness (101). One study discusses the possibility of peptidoglycan being partially responsible for the extremely slow diffusion of proteins across this periplasmic gel (8). The gel-like properties of peptidoglycan are supported by structural data. It was shown that, in contrast to the chemically closely related chitin, peptidoglycan is not a crystalline structure (55). Rather, its mechanical properties are more those of a viscous gel (95), the elastic properties of which reside within the conformational freedom of the peptide cross-link, that can be stretched up to fourfold (56). The sugar strands are in a far more rigid helical arrangement, with four to five disaccharide units per turn. This implies that in a single-layer arrangement, every fourth peptide pair can form a cross-link between two neighboring chains, whereas in multiple layers a much higher degree of cross-linking is sterically possible. This explains the relatively low degree (25 to 50%) of cross-linking in gram-negative bacteria (29) compared with the high degree of cross-linking (70 to 90%) in gram-positive bacteria (58).

A large number of enzymes has been implicated in the peptidoglycan metabolism. Apart from the enzymes involved in synthesis, numerous hydrolytic enzymes which have been

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shown to cleave a range of different bonds in the polymer have been identified (91). Of these hydrolytic enzymes, those that are able to totally degrade polymeric peptidoglycan are called autolytic enzymes. As the peptidoglycan has to withstand a high osmotic pressure, uncontrolled action of these hydrolases would cause the formation of holes through which the cytoplasmic membrane would start bulging out, eventually resulting in lysis. Several models have been proposed to explain the fact that apparently, new peptidoglycan can be inserted during growth without serious structural or morphological consequences. A common feature of most of these models, including the make-before-break (49), three-for-one (38), and hernia (73) models, is that new material is covalently attached before any bonds in the stress-bearing layer are cleaved by hydrolases. This means that new material can be inserted without holes having to be created.

HOLES IN THE PEPTIDOGLYCAN

In one of the few cases in which holes in the peptidoglycan of gram-negative bacteria were described, their appearance was correlated with the expression of fimbriae by *Fusiformus nodosus* (34). These holes are about 6 nm in diameter and were reported to be surrounded by a collar of 15 nm, which the researchers proposed to be a "grommet-like" reinforcement of the wall. If these holes were specifically made to accommodate fimbriae, does that imply that peptidoglycan does not normally contain pores of this size? If so, what is the maximum size of pores in the peptidoglycan?

Koch proposed that the single-layer peptidoglycan is built up of fundamental units composed of two neighboring chains with two cross-links that are four disaccharides apart (50). On the basis of data on the three-dimensional structure of peptidoglycan, the maximal diameter of this unit was calculated to be approximately 5 nm, allowing the passage of globular proteins with a maximal size of 55 kDa (93a). This value is in excellent accordance with experimental data that have recently been published by Demchick and Koch (11). On the basis of their measurements of the penetration of isolated intact sacculi by fluorescein-labeled dextrans, they concluded that the cut-off value for the passage of proteins through the peptidoglycan is about 50 kDa. Unlike former assumptions, this cut-off value was found to be almost identical for *E. coli* and *Bacillus subtilis* peptidoglycan. Furthermore, their data suggest that the peptidoglycan meshwork is devoid of gross imperfections, with only a few holes with a diameter larger than 4 nm.

FLAGELLAR ASSEMBLY

The bacterial flagellum is one of the largest external organelles, and its morphology has been studied extensively, especially in gram-negative bacteria (44, 64). The flagellum is composed of a 5- to 10-μm-long helical filament that is connected via a flexible hook to the basal body, which is embedded in the envelope and which, together with less well-defined components, constitutes the actual motor. The basal body is the only envelope-spanning structure for which detailed information is available as to its architecture and mode of assembly. Its basic structure is that of a rod with two pairs of rings. The MS pair of rings is associated with the cytoplasmic membrane, while the LP ring pair forms the outer cylinder, the L ring being embedded in the outer membrane. The P ring presumably interacts with the peptidoglycan. Apart from being a structurally important part of the flagellum, the basal body also functions as a signal sequence-independent protein export system (40). The P- and L-ring subunits are the only flagellar proteins possessing signal sequences, whereas the other components are transported in a signal sequence-independent way through a central channel in the growing flagellum. This results in a proximal-to-distal assembly of the rod, hook, and filament. The minimum size of the hole in the peptidoglycan necessary to accommodate the basal body would be the size of the rod, which has an estimated diameter of about 13 nm. As the penetration of the peptidoglycan by the rod is thought to precede the assembly of the P ring, the putative embedding of the P ring into the peptidoglycan would require a further widening of the initial hole to a diameter of 26 nm (92). Whether the basal body just penetrates the peptidoglycan or specifically interacts with it is still unclear.

Along with the production of degradative enzymes and the development of competence (see "Competence" below), flagellation is one of the pleiotropic processes that seems to be linked to the levels of autolytic peptidoglycan hydrolases in *B. subtilis* (2, 20). Fein was the first researcher to propose that these linked phenotypic traits may reflect the necessity for the localized breakdown of peptidoglycan in order to incorporate the flagellum into the cell wall (19). The mutants, on which he based his hypothesis, were later determined to be regulatory mutants, rather than strains with mutations in the autolytic enzymes (77, 89). Nevertheless, combined with observations such as the increased autolysin level of hypermotile mutants (77) and the impaired motility of amidase-deficient mutants (65), the above-mentioned coregulation makes a strong case for the direct involvement of autolytic peptidoglycan hydrolases in flagellar assembly (59).

A very interesting direct link between flagellar assembly and peptidoglycan hydrolases is the homology of a *Salmonella typhimurium* flagellar protein, FlgJ, to autolytic muramidases from *Enterococcus hirae*, *Streptococcus faecalis*, and *Lactococcus lactis* (9, 45, 46). The function of FlgJ in flagellar assembly is unknown, but it is essential for the formation of the rod (53). Combining the homology of FlgJ to peptidoglycan hydrolases with the fact that it is the last gene in an operon containing the basal body structural genes, it is tempting to speculate that FlgJ is responsible for the penetration of the peptidoglycan by the rod. Furthermore, as FlgJ lacks a typical signal sequence, it might be transported through or be an integral part of the rod, in analogy to the flagellar structural proteins. This would provide a very elegant means of topological restriction of putative peptidoglycan hydrolase activity, whereby only the bonds obstructing rod penetration would be cleaved.

PROTEIN EXPORT

The secretion of degradative enzymes, toxins, and other virulence factors is an important feature of many pathogenic bacteria. In contrast to transport across the cytoplasmic membrane, which is governed by one uniform system, transport across the entire envelope is a far less uniform process, involving several systems. However, over the last few years it has become clear that these export systems were less heterogeneous than was at first assumed, and common mechanistical principles are emerging.

The most prominent of these principles seems to be so widespread among a range of unrelated gram-negative bacteria that it has been referred to as the main terminal branch of the general secretion pathway (79). The first system of this type (also referred to as type II export systems) described was that responsible for the export of pullulanase by *Klebsiella oxytoca*. The export of this lipoprotein was shown to proceed through two distinct steps (80), the first of which is mediated by the Sec system and the second of which was shown to involve the gene

products encoded by *pulS* and the *pul* operon. Export systems similar to the pullulanase system have now been identified in a broad range of gram-negative bacteria and seem to be involved in the export of a variety of proteins, most of which are virulence related. An intriguing finding was the similarity of components of these protein export systems to proteins involved in the biogenesis of type IV fimbriae (pili) (36). These fimbriae protrude from the surfaces of several pathogenic bacteria and are thought to be important mediators of attachment. The similarity between the protein export and pilus assembly mechanisms gave rise to the speculation that a pilus-like structure, called the pseudopilus, might be involved in the latter (81). Although this model is supported by its analogy to the flagellar assembly system, no firm evidence has been obtained for the existence of this structure. The type IV pilus has an outer diameter of 6 nm and an inner channel diameter of 1.2 nm (24). However, it has been reported that the proteins that are exported by the putative pseudopili have considerable tertiary and even quaternary structure (35, 78). As a globular protein of the molecular weight of pullulanase would have a diameter of approximately 8 nm and would therefore not fit into the type IV pilus channel, the putative pseudopilus may have considerably larger dimensions.

Whereas a need for localized peptidoglycan hydrolysis during assembly of (pseudo)pili has never been demonstrated (nor discussed), Hobbs and Mattick described an intriguing connection between the pullulanase and type VI fimbria export systems and peptidoglycan synthesis (36). They reported that in *Haemophilus influenzae* (see "Competence" below), *K. oxytoca*, *Pseudomonas aeruginosa*, and *E. coli*, genes coding for high-molecular-weight penicillin-binding proteins (PBPs) or their homologs, are found upstream of members of the *pulD* family that encode the proposed outer membrane portholes of the (pseudo)pili. It was further speculated that a fimbrial operon of *Dichelobacter nodosus* might contain a PBP-like gene (*fimD*), although this homology is less obvious. The high-molecular-weight PBPs are responsible for the polymerization of peptidoglycan. Moreover, a gene encoding a homolog of the cell division gene *ftsA* was recently found in an operon involved in pilus assembly in *P. aeruginosa* (66).

An alternative type of export is signal sequence independent and involves the so-called type I systems (87), of which the best-studied example is the system responsible for the export of *E. coli* hemolysin. The machinery involved in the secretion of hemolysin is composed of three proteins: HlyB, HlyD, and TolC. HlyB is a cytoplasmic membrane protein and is connected to the outer membrane porin TolC by HlyD, the latter supposedly spanning the periplasmic space. HlyD is related to a protein family, designated the membrane fusion protein family (86). Fusion of the membranes, as also proposed for the adhesion sites described by Bayer (4), is a controversial phenomenon though, and the presence of peptidoglycan calls for a clear distinction between the membranes being in close proximity or actually being fused. Kellenberger challenged the existence of Bayer junctions and argued that the peptidoglycan does not contain holes large enough to allow for the membranes to fuse (48). Rather, in the closest contacts observed, both membranes are separated by approximately 5 nm, enough distance to accommodate at least a single layer of peptidoglycan (4). Furthermore, it was shown that the separated membrane fraction that was proposed to contain the adhesion sites not only contains peptidoglycan but also harbors the peptidoglycan synthetic machinery (42). Therefore, rather than fusing the membranes, HlyD-type proteins and other types of transenvelope complexes described in this minireview might bring the two membranes in close proximity by interacting with both membranes directly or via other proteins, thereby penetrating the peptidoglycan. Peptidoglycan penetration might also be an important issue in other transfer processes for which an involvement of adhesion sites has been discussed, such as phage and colicin entry (involving the Tol and TonB transport systems) or the transfer of outer membrane components (e.g., lipopolysaccharide, phospholipids, and lipoproteins). The fact that some gram-positive type I export systems also have HlyD-like components underscores the importance of peptidoglycan penetration for this type of export (17).

A new family of virulence-related export systems seems to combine features of several of the established systems (87, 97). The prototype for this proposed third general type (type III) of protein secretion is the secretion of Yops (*Yersinia* outer proteins) by virulent *Yersinia* species. Secretion of these plasmid-encoded proteins is essential for pathogenicity. Although export of these proteins is signal sequence independent and YopB shows homology to hemolysin, the machinery involved is reminiscent of the terminal branch of the general secretion pathway in that it involves a large number of gene products and that one of these is homologous to PulD (69). Similar types of export systems were shown to be involved in the secretion of hypersensitivity response-inducing proteins from plant pathogens and secretion of Ipas (invasion plasmid antigens) from *Shigella* species (97). An interesting new type of surface structure was shown to be expressed by *S. typhimurium* during the invasion process (28). These surface appendages are morphologically distinct from pili or flagella and are about 60 nm in diameter. Whether they span the envelope and would therefore require a hole in the peptidoglycan of the same size is presently unknown. Proteins involved in the assembly and disassembly of these structures are homologous to proteins that are involved in the above-mentioned type III systems. Surprisingly, this whole family of virulence-related export systems shows extensive homology to several of the proteins involved in flagellar assembly in *E. coli*, *B. subtilis*, *Caulobacter crescentus*, and *S. typhimurium* (14, 28). As information on the architecture of the type III systems is lacking, no estimation can be made on the size of the hole in the peptidoglycan that would be needed to accommodate them. However, recently a *Shigella flexneri* gene that clusters with and might even be part of the same operon as genes that are involved in Ipa secretion was found to show convincing homology to a peptidoglycan hydrolase (see below). This finding suggests that peptidoglycan rearrangements may be needed for the assembly of this export system. A more speculative link between type III export and peptidoglycan metabolism is the observed regulation of the levels of the *Yersinia pestis* penicillin-binding proteins by the virulence plasmid (21).

Although gram-positive bacteria do not have an outer membrane, this does not necessarily mean that transport over the cytoplasmic membrane directly results in secretion into the medium. It has been shown that exported degradative enzymes can be retarded by the thick peptidoglycan layer (30). The proposed existence of an enzyme pool between the membrane and the peptidoglycan wall may be explained by the limited availability of translocation pores large enough to allow for passage of these proteins. The fact that autolytic-deficient mutants of *B. subtilis* and *Streptococcus pneumoniae* show a decreased permeability of the wall suggests that peptidoglycan hydrolases are involved in the formation of these portholes (100).

COMPETENCE

Several bacteria are naturally competent for transformation (15). The ability to take up transforming DNA is in most cases tightly regulated and dependent on the physiological state of the bacterium. Of the different bacterial competence systems, that of *B. subtilis* is the best characterized. A large number of genes have been implicated in the development of competence in this bacterium and were classified into the so-called early genes, which are thought to have a regulatory role, and the late genes, the products of which seem to be involved in the actual transformation process. The proteins encoded by several of the late genes identified so far show a striking homology to members of the pullulanase family of secretory systems (99). On the basis of these homologies, there is reason to believe that the uptake of transforming DNA is mediated by a pseudopilus analogous to the pullulanase system (15). The greatest difference between these two proposed pseudopili would be that, in the case of gram-negative bacteria, the pseudopilus is anchored between two membranes, whereas in the case of the gram-positive bacteria, it would protrude from the cytoplasmic membrane into the peptidoglycan wall.

There are several indications that the assembly of the competence apparatus requires rearrangements within the peptidoglycan. In the early 1960s, a correlation between autolytic activity and competence of *B. subtilis* had already been demonstrated, which led Young to propose that the autolytic enzymes might produce local gaps in the peptidoglycan through which the DNA could be taken up (102). The same correlation was also shown for group H streptococci (82). Later it was demonstrated that the development of competence is coregulated with the expression of autolytic activity and flagellar assembly (33). An interesting indirect correlation between competence and peptidoglycan metabolism in *S. pneumoniae* was the identification of a signal transducing pathway that seems to control both the activity of the PBPs and the development of competence (32).

The mechanism of competence differs between gram-negative and gram-positive bacteria in several aspects. Nevertheless, the systems seem to be related, as illustrated by the similarity between competence genes of *H. influenzae* and *B. subtilis* (63). A very interesting link between gram-negative competence and peptidoglycan metabolism is the mapping of the *H. influenzae* PBP1a gene within a cluster of open reading frames shown to be involved in transformation. Insertional inactivation of this gene results in a 25- to 50-fold decrease in transformation efficiency (96). An even more convincing link is found in the DNA uptake system of *Neisseria gonorrhoeae*. The natural transformation competence of this species is tightly coupled to piliation; several genes encoding proteins known to be associated with or involved in the assembly of type IV pili are also required for transformation. This involvement of a type IV pilus in DNA uptake supports the above-mentioned concept of a pseudopilus playing a role in *B. subtilis* competence. Recently, two additional proteins were identified in *N. gonorrhoeae* that are essential for transformation: the *tpc* gene product and the peptidoglycan-linked lipoprotein ComL (26, 27). Because *tpc* mutants are defective in cell division, it was postulated that Tpc is a peptidoglycan hydrolase. Tpc and ComL are thought to be involved in the transfer of the DNA across the peptidoglycan layer. Moreover, Tpc also plays a role in the cellular invasion process and might be required for the efficient export of virulence factors, comparable to the LytA peptidoglycan hydrolase of *S. pneumoniae* (7).

CONJUGATION

Whereas transformation involves the uptake of nonspecific DNA, conjugation couples the export of specific plasmid DNA from the donor cell to import into the recipient cell. Although the process of bacterial conjugation has been extensively studied, the actual mechanism by which the DNA is transported remains elusive. The first conjugative plasmid to be identified was the sex factor F, and the conjugative system by which this plasmid is transferred is one of the best characterized. The *tra* genes, which encode proteins involved in the transfer process, are clustered together in the transfer region. For most of the Tra gene products, information on their subcellular localization and the stage of conjugational transfer in which they are involved is available, although for many of them the precise function is still unclear (41). Several envelope-spanning structures may be envisaged to be present during the different stages of the conjugative process. It was speculated that several Tra proteins may interact together to form the transenvelope pilus assembly machinery (41). The dimensions of this proposed complex are not known but are most probably comparable to those of the exposed part of the pilus, having a diameter of 8 nm (23). Although there have been speculations about the DNA being transferred through the pilus, the generally accepted view is that efficient transfer requires the cells to be in close contact (mating pairs) and that the DNA is transferred through so-called conjugational junctions (16, 74). These junctions are stabilized by the TraN and TraG proteins, and the TraN-TraG complex is thought to span the donor envelope (41). The size of the DNA transport channel would presumably be dependent on what is transported. Whereas in the case of F and F-like conjugation this is probably only naked DNA, for which no large holes in the peptidoglycan would be required, other plasmids are accompanied by large escort proteins which can be up to 210 kDa in size and are cotransmitted in multiple copies (83). Apart from serving as primases, these large escort proteins have been proposed to actually facilitate the transport of the DNA through the envelope. The final transenvelope structure that could be envisaged is one spanning the envelope of the recipient cell. It has been proposed that endogenous pores might be involved in passage of the DNA through the outer membrane and that transport into the cytoplasm might be mediated by sugar uptake systems (16). This however would not explain the cotransport of the escort proteins or the passage of the DNA-protein complex through the peptidoglycan of the recipient cell. The notion that an intact peptidoglycan is necessary for the mechanism of surface exclusion implies that bulk peptidoglycan does not allow passage of the incoming DNA, but a specific uptake system that is blocked during surface exclusion is present (25).

Agrobacterium tumefaciens transfers tumor-inducing genetic material into cells of wounded plant tissues. The transferred DNA (T-DNA) and the genes involved in its transfer are located on the Ti plasmid. The mechanism of T-DNA transfer was proposed to be similar to the process of conjugation (5), and this hypothesis is supported by extensive homologies between nicking sites and transfer gene products of several different conjugative plasmids and Ti plasmid-encoded sequences (47, 61, 75). Of the six *vir* operons on the Ti plasmid, *virB* is the largest, encoding 11 proteins involved in transfer of T-DNA. On the basis of their subcellular localization, the VirB proteins were proposed to assemble into a large complex T-DNA transport channel that spans the bacterial envelope and that is reminiscent of a conjugative pilus (22, 90, 93). This channel would allow one-step transfer of the T-DNA, covalently bound

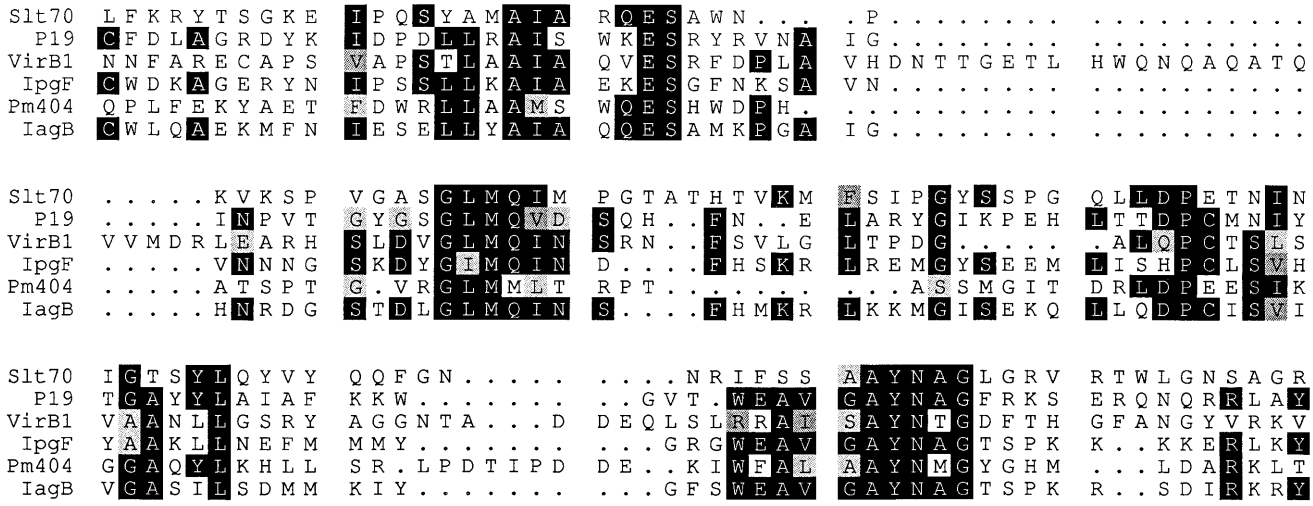

FIG. 1. Multiple alignment of the amino acid sequences of the C-terminal lysozyme-like domain of Slt70 of *E. coli* (residues 483 to 569), protein 19 (P19) of plasmid R1 (residues 22 to 134), VirB1 of *A. tumefaciens* (residues 31 to 169), IpgF of *S. flexneri* (residues 20 to 130), ORF404 of *P. mirabilis* (Pm404) (residues 206 to 312), and IagB of *S. typhimurium* (residues 21 to 131).

to the 56-kDa VirD2 protein and coated with the single-stranded-DNA-binding 60-kDa VirE2.

A very intriguing link between conjugation and protein secretion is the extensive homology that was found between the *A. tumefaciens* VirB proteins and the products of the *Bordetella pertussis ptl* operon that are involved in secretion of pertussis toxin (10, 47, 98). Pertussis toxin is a complex toxin, composed of five subunits. These subunits are proposed to be transported over the cytoplasmic membrane in a Sec-dependent manner, after which the mature toxin assembles in the periplasm and is secreted. The finding that all eight of the Ptl proteins have homologous VirB counterparts, combined with studies on the localization of three of the Ptl proteins (43), gave rise to the speculation that a similar type of transenvelope structure is involved in both export systems. This raises the question as to how the same kind of putative transenvelope channel can be involved in secretion of a multisubunit toxin of about 105 kDa from the periplasmic compartment and the export of a DNA-protein complex from the cytoplasmic compartment.

A process that is often mechanistically compared to conjugative transfer is the assembly of filamentous phage (84). While being concomitantly assembled and released, the phage forms an envelope-spanning complex with the assembling machinery. This export process also seems to be related to other transenvelope trafficking systems, since one of the components of this complex, pIV, is similar to the PulD family of putative outer membrane portholes (83).

LYTIC TRANSGLYCOSYLASE HOMOLOGS IN TRANSFER SYSTEMS

The 70-kDa soluble lytic transglycosylase (Slt70) of *E. coli* is an exomuramidase that degrades polymeric peptidoglycan. Although it cleaves the same bond as the lysozymes, the released muropeptides differ from the lysozyme products in that they contain an internal 1,6-anhydro bond (39). Elucidation of the three-dimensional structure of Slt70 enzyme by X-ray crystallography (94) showed that, in spite of the lack of significant sequence homology with the lysozymes, the amino acid residues involved in the architecture of the active site are conserved between the primary sequences of Slt and those of the swan-type lysozymes to which it is structurally most related (13). This finding prompted us to search the protein databases for related proteins containing the three conserved amino acid motifs that are most prominent in the architecture of the active site: E-478–S, including the catalytic glutamate, G-493–LMQ and A-551–YNAG. The results of this approach, the validity of which was shown by our recent identification of a new *E. coli* lytic transglycosylase (12) were consistent with those of Koonin and Rudd, who described a putative transglycosylase fingerprint (51). However, our approach yielded additional homologs that are noteworthy in the context of this minireview. The first of these is a protein of unknown function that is encoded by an open reading frame conserved in the leading regions of F- and R-like conjugative plasmids. This open reading frame, designated Orf169 in plasmid F, gene 19 in plasmid R1, and gene x in plasmid R100 (18, 31, 62), is located directly adjacent to the origin of transfer, and hence it is the first gene to be transmitted into the recipient. Although the gene was described to be nonessential for transfer, its inactivation resulted in marked inhibition of conjugation of a derepressed R1 variant and therefore is regarded as a transfer gene (3). The presence of a classical signal sequence points to a periplasmic location. Furthermore, its expression was shown to be tightly regulated by RNase III, which was interpreted to be a limitation on the expression of this protein during a specific phase of the conjugational process (52).

The second sequence identified also was that of a protein that is thought to be involved in a conjugational process, namely, VirB1 of *A. tumefaciens*. The VirB1 gene product is a 28-kDa protein with a putative N-terminal signal sequence (54). On the basis of fractionation studies, its suggested location is the periplasmic side of the inner membrane (93). In analogy to protein 19, VirB1 was described as nonessential for transfer of the T-DNA, but deletion of its gene resulted in a marked attenuation of virulence (6). Although the homology between VirB1 and the protein 19 sequence extends beyond the hydrolase motifs (Fig. 1), such homology was not previously reported. The fact that there are other functional and sequential homologies between these two DNA transfer systems (see "Conjugation" above), combined with the presence of homologs in other conjugative systems (3, 76), supports the relevance of the above-described findings.

These newly identified homologies not only suggest the involvement of putative peptidoglycan hydrolases in conjugational processes but also establish a connection to signal sequence-independent protein export. The virulence plasmid of *S. flexneri* encodes a protein, IpgF, that is homologous to the above-mentioned protein 19 homologs (1). The *ipgF* gene is located between the genes encoding the Ipa proteins and the genes involved in the secretion of these proteins, possibly belonging to the same operon as the latter. *ipgF* was reported to be nonessential for invasion as measured by standard virulence assays, but as the researchers question the sensitivity of the assays used, the role of this gene in the natural pathogenic process remains to be clarified, even more so because IagB, a homolog of IpgF that was recently identified in *Salmonella typhi*, was reported to have a role in invasion (70).

A more speculative connection between cross-envelope transport and peptidoglycan hydrolysis is provided by another Slt70 homolog encoded by an open reading frame, ORF404, that clusters with a fimbrial operon in *Proteus mirabilis* (67). The alignment of the above-mentioned homologs with the C-terminal catalytic domain of Slt70 is depicted in Fig. 1.

CONCLUSIONS

In spite of the heterologous nature of the different transenvelope transport systems described in this minireview, a wealth of evidence has emerged during the last few years that shows that they have common aspects and are genetically quite related (36, 60, 79, 85, 97). This is reflected by the homologies of groups of genes that are involved in different systems, for example the similarity of type III protein export to flagellar assembly, or by individual branch points like the already mentioned PulD family or the PulE/PilB family of proteins that are involved in type I protein export and fimbrial assembly and for which homologs play a role in conjugational transfer, gram-positive competence, and T-DNA transfer by *A. tumefaciens* (60, 71, 79). As the dimensions of most of the transfer systems or those of the transported proteins or protein-DNA complexes seem to exceed the maximum size of the holes that are present in bulk peptidoglycan, one of the common aspects of these systems may be the requirement for localized peptidoglycan rearrangements. This assumption is supported by the several links between the different transfer machineries and peptidoglycan metabolism and is underscored by our identification of new peptidoglycan hydrolase homologs, which may function as hole-punching enzymes.

The putative involvement of specific peptidoglycan-processing enzymes in the assembly of transfer systems could account for the observed redundancy of peptidoglycan-metabolizing enzymes. Moreover, as most of the described transfer systems are not constitutively expressed, this may imply that the peptidoglycan-processing activities involved might not be detected in bacteria grown under laboratory conditions.

As the activity of most of the transenvelope trafficking systems seems to be related to virulence, understanding common principles of these systems could be very useful in the development of new antibacterial strategies. One of these common principles, the possible involvement of potentially lytic enzymes, opens the exciting possibility of inducing autolysis by deregulation of these enzymes. As the induction of several of the virulence-related export systems depends on outside signals, interfering with the regulation of the hole-punching enzymes may be more feasible than deregulation of the hydrolases that are involved in overall peptidoglycan metabolism.

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ADDENDUM IN PROOF

The results of a recent study validate the homology between VirB1 of *A. tumetaciens* and peptidoglycan hydrolases by showing that site-directed mutagenesis of conserved amino acid residues results in attenuated virulence (A. R. Mushegian, K. J. Fullner, E. V. Koonin, and E. W. Nester, Proc. Natl. Acad. Sci. USA **93:**7321–7326, 1996).

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