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# Review

# Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria

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**Abstract.** The cell wall of Gram-negative bacteria is essential for the integrity of the bacterial cell but also imposes a physical barrier to trans-envelope transport processes in which DNA and/or proteins are taken up or secreted by complex protein assemblies. The presence of genes encoding lytic transglycosylases in macromolecular transport systems (bacteriophage entry, type II secretion and type IV pilus synthesis, type III secretion, type IV secretion) suggests an important role for these specialised cell-wall-degrading enzymes. Such enzymes are capable of locally enlarging gaps in the peptidoglycan meshwork to allow the efficient assembly and anchoring of supramolecular transport complexes in the cell envelope. In this review, current knowledge on the role and distribution of these specialised murein-degrading enzymes in diverse macromolecular transport systems is summarised and discussed.

**Key words.** Bacterial transport systems; macromolecular transport; bacterial cell wall; cell-wall-degrading enzymes; lytic transglycosylase; pathogenicity; virulence; bacteriophage infection.

## Introduction

The cell wall (or murein or peptidoglycan sacculus) of Gram-negative bacteria is a complex cross-linked, largely monolayered, biopolymer [for a recent review see ref. 1]. The cell wall determines the cell shape and encases the whole cytoplasmic membrane of the bacterial cell. It is an exo-skeleton protecting the cell from mechanical stress factors like the cellular osmotic turgor [1]. During cellular growth and especially cell division, the peptidoglycan sacculus needs to be enlarged and finally separated, to allow the formation of two complete daughter cells. Several enzymes, likely organised in enzyme complexes [2], are required to perform the complicated task of replicating the stress-bearing macromolecule. Both peptidoglycan-degrading and peptidoglycan-synthesising enzymes are involved in this process and act in a highly concerted fashion [3]. Disturbing this coordinated machinery can lead to a loss of cellular integrity. Specifically, blocking murein-synthesising enzymes by penicillin or penicillin derivatives leads to disruption of the cell wall of growing cells and bacterial cell death [4].

Despite the inherent elasticity and flexibility of the cell wall [5], in addition to inner and outer membranes, the largely single-layered peptidoglycan sacculus of Gram-negative bacteria has been proposed to act as a permeability barrier [6]. The murein meshwork permits the free passage of only small proteins. However, transport of macromolecules in Gram-negative bacteria requires the assembly of envelope-spanning protein complexes [7]. Thus, specialised enzymes were proposed that facilitate a temporally and spatially controlled opening in the peptidoglycan layer [6]. The proposal was based on several findings in trans-envelope transport systems that indicated the involvement of a specific peptidoglycan rearrangement, and the identification of lytic transglycosy-

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lase (LT) homologues in diverse transport systems [6, 8, 9]. These specialised enzymes need to act at the right place and time, a feature which can be achieved by physically coupling the cell-wall-degrading enzyme to the cognate transporter complex. Recent findings in different transport systems strongly support this notion.

This review is intended to give a comprehensive overview of the progress that has been made in understanding the role of specialised cell-wall-degrading enzymes in bacteriophage infection, uptake of naked DNA and various bacterial secretion systems, many of which are needed to transform a non-virulent bacterium into a potent pathogen. According to the fact that most of these enzymes belong to the LT family of proteins and because they are dedicated to a function apart from cell growth and division, I propose to call this group of enzymes specialised LTs.

## The cell wall of Gram-negative bacteria

Gram-negative bacteria are usually rod shaped and contain a largely single-layered peptidoglycan sacculus which is embedded in the periplasmic space. In peptidoglycan, disaccharide sugar units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) are linked by $\beta$-1,4 glycosidic bonds. Protruding from the helically arranged disaccharide chains are short peptides containing a both dibasic and dicarboxylic amino acid (meso-diaminopimelic acid, A2pm, in the bacterium *Escherichia coli*) which enables the formation of the peptide bond cross-linking the glycan strands. The length of the glycan strands is extremely variable: analyses have revealed a broad distribution of the number of disaccharide units between 2 and 60. The predominant lengths of the glycan strands were 5 to 10 GlcNAc($\beta$-1,4)MurNAc disaccharide units [10].

In *E. coli* peptidoglycan and the outer membrane are physically linked by A2pm residues covalently attached to the outer membrane protein LPP [11]. Another protein of the outer membrane, peptidoglycan-associated protein (PAL), has been reported to be involved in interactions with peptidoglycan [12], thereby contributing to the observed close association of the outer membrane with the peptidoglycan sacculus. Association of peptidoglycan with the inner membrane at certain contact zones, although already observed in the late 1960s [13], is less well characterised. PAL-TolB interactions have been proposed to play a key role in the formation of contact sites between the inner and outer membranes of *E. coli* [14], but other trans-envelope transport complexes (like those referred to in this review) are also regarded as likely candidates to form sites of adhesion.

A large number of enzymes is involved in the synthesis and turnover of peptidoglycan. Among the six synthesising enzymes in *E. coli* [1], bifunctional proteins that are both transglycosylases and transpeptidases play a major role. The transpeptidases cross-link parallel glycan strands and are the targets of $\beta$-lactam antibiotics such as penicillins. The growth and division of the Gram-negative bacterium also requires growth and rearrangements in the peptidoglycan sacculus, a task that is achieved by the combined and coordinated activity of murein-synthesising and murein-degrading enzymes. In *E. coli*, murein-degrading enzymes – amidases, endopeptidases, carboxypeptidases and lytic transglycosylases – by far outnumber the murein synthases [1], indicating the importance of peptidoglycan-degrading enzymes. Multiple deletions in murein hydrolases revealed that predominantly amidases but also endopeptidases and lytic transglycosylases are responsible for cleavage of the septum during cell division [15]. Several models have been elaborated to explain how the stress-bearing peptidoglycan sacculus can grow and divide without compromising the integrity of the cell. The 'make-before-break' [16], the 'three-for-one' [1] and the 'nona-muropeptide stretch' [17] models have been proposed. Recently, a model has been presented that describes growth of the murein of both Gram-positive and -negative bacteria by the activity of murein transferases [18].

## Macromolecular transport and the penetration of the peptidoglycan meshwork

Transport of macromolecules (i.e. DNA and/or proteins) requires the assembly of a multiprotein complex that selectively transports a substrate macromolecule from the interior of a bacterial cell into the exterior. In many systems, this secretion step is coupled to a translocation event in which effector molecules (i.e. proteins) are transported across the membrane of a target host cell [19]. In naturally competent bacteria, envelope-spanning protein complexes facilitate the uptake of naked DNA from the environment [reviewed in ref. 20]. Bacteriophages need to get across the cell envelope in order to successfully initiate the infectious life cycle [reviewed in ref. 21]. All these processes have to cross the two membranes of Gram-negative bacteria and need to overcome the peptidoglycan permeability barrier as has already been noted by Dijkstra and Keck [6]. Despite this fact and the accumulating evidence in the literature which will be elaborated and discussed here, the cell wall in Gram-negative bacteria as a hindrance to trans-envelope transport is in many cases not taken into consideration. Why is there a need to deal with the cell wall in these cases? The answer is relatively simple and comes from structural constraints imposed by the murein sacculus. The permeability characteristics of the murein net have been studied, for example by determining the effective pore sizes. Presuming no binding to cell wall components, the size of a globular hydrophilic molecule that can pass freely through the meshwork of an un-

stretched sacculus of *E. coli* was found to be roughly 25 kDa. Proteins with higher molecular masses than 50 kDa were estimated as unable to pass freely through the peptidoglycan [22]. A similar result was obtained by measuring the thickness and elasticity of Gram-negative peptidoglycan by atomic force microscopy [5]. The authors of that study came to the conclusion that the natural spacing between glycan strands in cells would be 1.6–2.0 nm, and if large macromolecules of a diameter greater than 2 nm are to be secreted through this layer, the local ordering of the peptidoglycan must somehow be disrupted. The question of the porosity of the network has also been investigated by computer modelling. The conclusion there was that holes exist in the form of slits running along the long axis of the cells, that large molecules can penetrate the peptidoglycan and that the hypothetically existing slits might be made larger by the bacterium [23]. Another result that supports the suggested in vivo sieving role of the murein fabric is the fact that only proteins with a molecular mass smaller than 100 kDa are released from osmotically shocked *E. coli* cells [24]. Taken together, the results of these investigations strongly suggest that the single-layered peptidoglycan of Gram-negative bacteria forms a permeability barrier not allowing the passage of large proteins (globular proteins with molecular masses of greater than 50 kDa) or protein complexes. Consequently, local rearrangements in the peptidoglycan net are required to allow the assembly and insertion of protein complexes in the cellular envelope of Gram-negative bacteria. Enzymes that are capable of locally opening the cell wall and making specific holes in a temporally and spatially controlled fashion are described below.

## Bacterial LTs

LTs are peptidoglycan-degrading enzymes that cleave the $\beta$-1,4 glycosidic bond between MurNAc and GlcNAc residues. In contrast to lysozyme, the substrate is not hydrolysed using a water molecule, rather, the reaction results in the formation of a 1,6 anhydro-bond in the MurNAc moiety [25]. The lack of a catalytic aspartate residue in the LTs (position 501 in the sequence alignment in fig. 1) that is present in the c-type lysozymes is


Figure 1. Sequence alignment of selected representatives belonging to the soluble LT (SLT) family of proteins. The sequences of the specialised LTs are aligned to GEWL and Slt70, the structures of which are shown in figure 2. The colour coding is the same as in figure 2. Sequences corresponding to structures that are not shown in figure 2 are in black letters.

proposed to reflect the different reaction mechanism [26]. However, in a subset of sequences identified here as belonging to the LT family of proteins, an aspartate is present (fig. 1). The catalytic activity in the LT family is thought to be conferred by a glutamate residue (position 478 in fig. 1) in the active centre of these enzymes [26]. Database searches and sequence comparisons showed that this glutamate residue is invariantly conserved within the family of LTs [6, 8, 9, 27–30]. A reaction mechanism has been proposed based on biochemical, mutational and structural analyses of two LTs (Slt70 and Slt35) of the bacterium *E. coli* in which this glutamate residue (Glu478 in Slt70 and Glu162 in Slt35) plays a central role [31]. The product of the late bacteriophage λR gene is λ lysozyme (LaL). LaL displays LT activity but has no obvious sequence similarity to the soluble LT (SLT) family of proteins (see below). However, the recently solved structure of LaL in complex with an oligosaccharide substrate [32] revealed a three-dimensional structure with similarity to the bacterial LTs Slt70 and Slt35. From the structure it can be inferred that Glu19 in LaL acts as the catalytic residue and that the reaction mechanism is similar to the other LTs [32]. A characteristic feature of the LTs that have been crystallised so far is that they contain the 'goose-type lysozyme-like' domain (see fig. 2) involved in binding and cleavage of peptidoglycan [31–35]. The name for this domain is derived from the structure of the prototype GEWL protein (goose egg white lysozyme, also called g-type lysozyme) from the goose *Anser anser anser* [36]. Due to structural similarities, yet despite the lack of any significant sequence homology, GEWL has been proposed to belong to a glycohydrolase superfamily [comprising plant chitinase, bacterial chitosanase and lysozymes from hen (HEWL), goose (GEWL) and phage (T4 lysozyme)] and arose from a common ancestor by divergent evolution [37]. However, the clearly present sequence similarity between the bacterial LTs and GEWL (fig. 1) and the almost identical structural core elements of Slt70, Slt35 and GEWL (fig. 2) argue for the view that GEWL is evolutionary related to the SLT family of proteins.

Proteins belonging to the LT family (based on sequence comparisons) are ubiquitous in Gram-positive and Gram-negative bacteria; they are not present in bacteria that do not possess a typical cell wall like the Chlamydiaceae and the Mycoplasmataceae [38]. By database searching and sequence comparisons, Blackburn and Clarke [30] identified 127 amino acid sequences encoded in bacteria with significant similarity to known LTs from *E.coli*, *Pseudomonas aeruginosa* and λ phage. They grouped the sequences into four families, with family one representing a superfamily sharing three conserved sequence motifs typical for the goose-type lysozyme-like domain: ES – GLMQ – AYNAG [30]. Despite the structural similarities mentioned above, the four identified families vary considerably in their sequence. According to this classification scheme, Slt70 belongs to family one, Slt35 (MltB) to family three and LaL to family four [30]. All members of superfamily one, termed SLT, presently comprising more than 200 sequences, can be viewed in the NCBI Conserved Domain Database (cd00254; http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=cd00254) [39]. This CDD entry overlaps with the protein family (PFAM) database [40] entry PF01464 (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc? PF01464) and the Interpro [41] entry IPR000189 (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR000189).

One important aspect here is that besides sequence homology (the presence of the characteristic SLT motif or a similarity to other characterised LTs), a careful biochemical analysis is needed to demonstrate that the suspected LT indeed catalyses the proposed transglycosylase reaction that results in peptidoglycan degradation and the formation of a 1,6 anhydro-bond in the MurNAc moiety. A first indication that the enzyme degrades peptidoglycan can be obtained by zymogram analysis, a method that was originally developed to detect autolysins (peptidoglycan-de-

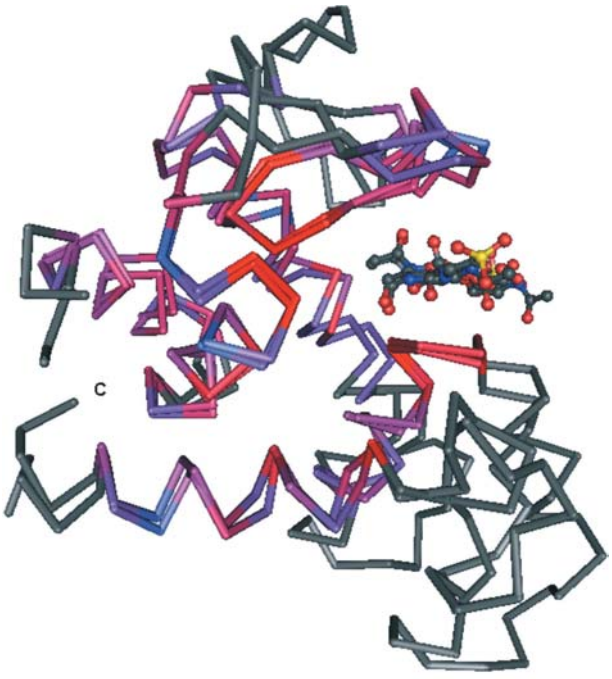


Figure 2. Structural superposition showing the conserved domains (blue) of Slt70 (PDB entry 1SLY) of *E. coli* and GEWL, the egg white lysozyme of the goose *Anser anser anser* (PDB entry 154L). The backbone trace of invariantly conserved amino acids of the sequences shown in figure 1 is shown in red, the trace of less conserved residues is shown in purple. Structures that cannot be superposed in space are shown in grey (loop regions). The shown structures start at position 450 in figure 1 (Pro450 in Slt70) and end at position 621. In the active-site cleft, a ball-and-stick representation of bulgecin, an inhibitor of Slt70 and a trisaccharide bound by GEWL, are shown. The image was generated with the Cn3D software available at the NCBI website [39].

grading enzymes) in Gram-positive bacteria [42]. This method includes the separation of proteins using a modified SDS PAGE in which purified peptidoglycan is copolymerised. If a protein has peptidoglycan-degrading activity, the peptidoglycan in the gel is digested during a renaturation procedure. Subsequent staining with methylene blue should reveal cleared zones devoid of polymeric peptidoglycan indicating peptidoglycan cleavage. This method has been successfully adapted for assaying autolysins in Gram-negative bacteria [43] and has been widely used to demonstrate peptidoglycan-degrading activity. However, cleared zones seen on zymograms do not necessarily indicate a peptidoglycan-degrading activity of the investigated protein but also arise when the protein tightly binds to peptidoglycan [44; and our unpublished observations]. Thus, at least one additional assay needs to be performed to prove a peptidoglycan-degrading activity of a protein. This can be done by using a labelled polymeric peptidoglycan substrate and by measuring the release of soluble fragments from that substrate [45, 46]. Further enzymatic characterisation involves reversed phase HPLC and ESI-MS analysis of reaction products to unequivocally demonstrate the formation of the characteristic 1,6 anhydro muropeptides [10, 47, 48].

## Specialised LTs

Although the term 'specialised' has not been used in the literature, I would like to introduce it here to indicate that certain LTs do carry out a defined task which is not related or connected to normal peptidoglycan turnover during cell growth and division. The genes encoding the specialised LTs are typically found in bacteriophage genomes, other mobile genetic elements like conjugative plasmids, and pathogenicity islands. The encoded proteins share the SLT motif described above and shown in figure 1, are completely dispensable for cell growth and division but are required for the function or greatly enhance the efficiency of macromolecular transport systems.

## Entry systems

The entry of DNA or RNA into bacterial cells is either forced by a parasitising element like a phage or is mediated by an uptake system in naturally competent bacteria. In both cases, the bacterial cell wall imposes a barrier that has to be crossed.

### Bacteriophage entry

Besides the 'true' lytic enzymes that are used by bacteriophages at the end of the life cycle to escape from the infected bacterium (these enzymes are not considered here), many bacteriophages also encode a LT which has been proposed to be involved in the entry of the phage at the beginning of the infectious cycle [28]. In the *E. coli* phage T7, the 144-kDa internal virus protein D (gp16) encoded by gene *16* [49] has been shown to contain the SLT motif [8] at its N-terminal end. Gp16 is not involved in cell lysis at the end of the developmental cycle [50]; rather, it was ejected together with two other proteins during infection into the host cell, probably forming an extension of the short T7 tail. That T7 tail extension is thought to form a channel in the bacterial cell envelope through which the DNA is translocated [51]. Extensive mutational analysis of gp16-E37 (corresponding to the invariantly conserved glutamate in the SLT family) demonstrated that this amino acid in gp16 was not essential in the infection process in exponentially growing cells above 30 °C. However, T7 phage particles containing a mutant gp16-E36 protein failed to efficiently infect cells that were growing at 20 °C or were at the late exponential growth phase at 30 °C, suggesting that the muralytic part of the protein was required only when peptidoglycan was highly cross-linked [52]. Unlike bacteriophage T7, T4 has a long contractile tail that can fully penetrate the bacterial cell envelope during infection. Recently, the structure of the cell-puncturing device of bacteriophage T4 was reported [53]. It consists of a complex of gp5 (63 kDa) and gp27 (44 kDa) of T4 and forms a torch-like structure that is connected to the tail plate of T4. This complex has also been proposed to form the conduit for DNA translocation [53]. Similar to bacteriophage T7 gp16, T4 gp5 possesses a domain with muramidase activity; in this case, however, with high similarity to T4 lysozyme. Superimposition of the gp5 structure with T4 lysozyme indicated that a typical peptidoglycan-binding pocket is formed, suggesting that the lysozyme domain of gp5 can indeed locally degrade peptidoglycan in the process of T4 infection [53]. Interestingly, bacteriophage T4 seems to encode a third muramidase encoded by *vs.1* which has been identified by sequence searching. The function of open reading frame (ORF) *vs.1* carrying the SLT motif is unknown [29]. Bacteriophage PRD1 is a broad-host-range bacteriophage that infects a variety of bacteria harbouring N-, P- or W-type conjugative plasmids. The capsid encloses the DNA genome and an internal membrane which contains P7, a protein containing the SLT signature that has been shown to possess glycolytic activity [54]. Mutant PRD1 particles lacking P7 showed delayed asynchronous DNA replication and lysis indicating that P7 plays a role in an early infection step of the bacteriophage PRD1 life cycle [54].

### Uptake of naked DNA

Naturally competent bacteria are capable of 'eating' DNA that is present in the environment. The DNA that is taken

up by a process called transformation is usually degraded and its components are used as nutrients. Very rarely, the foreign DNA is not degraded and is maintained in the new host, which in some cases endows the bacterium with novel properties. Besides DNA transfer via transducing phage particles and bacterial conjugation, uptake of naked DNA is a major route of horizontal gene transfer in bacteria and an important driving force of bacterial evolution [55]. Some bacteria like *Haemophilus influenzae* and *Neisseria gonorrhoeae* are perpetually competent for DNA uptake, whereas others like *Bacillus subtilis* and *Streptococcus pneumoniae* develop competence in a certain physiological state in their life cycle [20]. In the strictly human pathogen *N. gonorrhoeae*, two proteins have been identified that bind to peptidoglycan: ComL, the competence lipoprotein [56], and Tpc, the tetrapac protein [57]. Both ComL and Tpc contribute to competence in a manner that is unrelated to the function of the type IV pilus in DNA uptake by *N. gonorrhoeae* and they have been suggested to be involved in remodelling peptidoglycan [58]. However, there is no evidence for a peptidoglycan-degrading activity of these proteins.

## Secretion systems

Whereas evidence for a direct involvement of specialised muralytic enzymes in DNA uptake or 'secretion in reverse' [59] is still lacking, most, if not all, the type III (TTSS) and type IV (T4SS) secretion systems described so far contain one gene that based on sequence comparisons belongs to the class of specialised LTs (see table 1). Both in type III and type IV secretion, large envelope-spanning protein complexes are involved in the transport process, the transported substrate being protein or DNA or protein and DNA. In type IV pilus biogenesis which is related to type II secretion and competence systems, LT homologues are present as well (table 1). For both type I and type V secretion, however, there are no clear indications that peptidoglycan-degrading enzymes are involved in these processes. For a recent review on bacterial secretion systems see Thanassi and Hultgren [7]; an important note on the classification and nomenclature of bacterial secretion systems is given in Henderson et al. [60].

### Type II secretion and type IV pilus biogenesis

Type IV pili are surface appendages assembled by many Gram-negative pathogenic bacteria and are required for the attachment of the bacterial cell to a specific receptor on the respective host cell. Therefore, type IV pilus systems are important virulence determinants [61 and references cited therein]. Type II secretion is widely distributed among Proteobacteria and is responsible for the extracellular secretion of toxins and hydrolytic enzymes, many of which contribute to pathogenesis in both plants and animals [62]. Sequence similarities suggest that type II secretion pathways, type IV pilus synthesis and DNA uptake systems are closely related [59, 63, 64]. A secretion apparatus is also encoded by filamentous phage; however, there is only limited sequence similarity to type II secretion genes [65]. Recently, the thin pilus encoded by the IncI plasmid R64 has been identified as a type IV pilus [66]. In this case, the pilus is required for conjugative DNA transfer in liquid media. Twelve of the 14 *pil* genes encoded by R64 have been identified to be essential for conjugation [67], among them *pilT*. The PilT protein belongs to the SLT family of proteins (see table 1) and is located in the periplasm [68]. Thus it could function as a specialised LT in type IV pilus biogenesis. *bfpH* is another LT gene present in the gene cluster encoding the bundle-forming pilus (BFP) which is required for localised adherence (LA) of enteropathogenic *E. coli* (EPEC strain E2348/69; O127:H6). However, the plasmid-encoded *bfpH* gene (table 1) was found to be neither required for pilus assembly nor for LA, but there was a modest but significant reduction seen in the ability of the *bfpH* mutant to autoaggregate [69]. Interestingly, the locus of enterocyte effacement (LEE, encoding a type III secretion system) from the same enteropathogenic *E. coli* strain contains *rorf3*, a gene encoding a BfpH homologue [70] (table 1). Therefore, a redundancy in the genetic information of the organism may have enabled cross-complementation and rescue of the *bfpH* mutant by *rorf3*. A third chromosomally encoded specialised LT gene is present on both the sequenced *E. coli* strains EDL 933 and MG1655 (table 1). The respective genes, *Z4175* and *ORF_o138,* are contained within the colinear backbone sequences that are conserved in both strains [71]. Strikingly, a similar genetic redundancy can be seen in the case of enterohaemorrhagic *E. coli* (EHEC; O113:H21) in which two specialised LT genes associated with type III secretion genes are present in the chromosome (*Z4175* and *Z5131*). A third specialised LT homologue, *pilT*, is encoded by the large virulence plasmid pO113. Plasmid pO113, also isolated from Shiga-toxigenic *E. coli* strains (STEC), encodes putative DNA transfer genes and a type IV pilus operon [72]. Another type IV pilus gene cluster encoding a colonisation factor has been recently identified in enterotoxigenic *E. coli* (ETEC). Not surprisingly, on that plasmid-borne gene cluster, a specialised LT gene, *cofT*, is present [73].

The type II secretion apparatus is composed of at least 12 different gene products that are thought to form a multiprotein complex spanning the periplasmic compartment [64]. Clearly, such a transport apparatus for secretion of folded proteins would require peptidoglycan remodelling. Surprisingly, this aspect has been neglected so far, probably because of the lack of genes encoding muramidases in these systems. However, in the case of the *gsp*

Table 1. Specialised LTs in bacterial type II, type III and type IV secretion systems.

| Organism | System | Plasmid/ chromosome | Gene name/ reference | Accession numbers[a] | Protein data[b] |
|---|---|---|---|---|---|
| **LT genes in type IV pilus assembly and type II secretion** | | | | | |
| *Burkholderia pseudomallei* | type II secretion | chromosome | *orfC* [74] | Q9ZF87 (AAD05172) | 161 (25–143) 119 9.28 |
| *Escherichia coli* | type IV pilus/ conjugation | plasmid R721 (IncI2) | *pilT* | Q9F539 (BAB12641) | 160 (32–157) 126 9.30 |
| *Escherichia coli* ETEC strain | type IV pilus, colonisation factor antigen III synthesis | plasmid | *CofT* [73] | Q93I74 (BAB62896) | 147 (21–139) 7.97 |
| *Escherichia coli* EHEC O113:H21 | type IV pilus gene cluster | plasmid pO113 | *pilT* | Q93AE5[c] AAL18835 | 157 (27–153) 127 9.97 |
| *Escherichia coli* EPEC strain B171 (O111:NM) | localised adherence, bundle-forming pilus | plasmids pB171 and EAF | *bfpH* [109] | Q46781 (BAA84847 AAC44047) | 148 (12–138) 127 8.92 |
| *Escherichia coli* EPEC strain O127:H6 E2348/69 | localised adherence, bundle-forming pilus | plasmid pMar2 | *bfpH* [69] | Q47073 (CAA92335) | 148 (12–138) 127 8.92 |
| *Escherichia coli* STEC O113:H21 | type IV pilus gene cluster | plasmid pO113 | *pilT* [72] | Q93D61[c] (AAL05524) | 175 (45–171) 127 9.97 |
| *Salmonella enterica* serovar typhi CT18 | type IVB pilus – host invason | chromosome | *pilT* [110] | Q9ZIU8 (CAD09326) | 158 (25–151) 127 9.42 |
| *Salmonella enterica* serovar typhimurium *Escherichia coli* | type IV pilus – conjugation | plasmid R64 and ColIb-P9 | *pilT* [68] | O07378 (BAA77980) | 186 (40–167) 128 9.62 |
| **LT genes in type III secretion systems** | | | | | |
| *Burkholderia cepacia* genomovar III | type III secretion genes | chromosome | *virB1* [89] | Q8VP09 | 261 (37–183) 147 9.10 |
| *Burkholderia pseudomallei* | type III secretion | chromosome | *bapC* [75] | n.a. | n.a. |
| *Citrobacter rodentium* | LEE locus, type III secretion genes | chromosome | *rCR1* [111] | Q93FL8 (AAL06358) | 153 (20–140) 121 7.92 |
| *Escherichia coli* RDEC-1 | LEE locus, type III secretion genes | chromosome | *rorf3* [112] | Q9AJ25 (AAK26704) | 152 (19–139) 121 8.53 |
| *Escherichia coli* EHEC O157:H7 (EDL 933) | LEE locus, type III secretion genes | chromosome | *Z51131* [71] | O85639 (AAG58843) | 152 (19–139) 121 8.43 |

Table 1 (continued)

| Organism | System | Plasmid/ chromosome | Gene name/ reference | Accession numbers[a] | Protein data[b] |
|---|---|---|---|---|---|
| *Escherichia coli* EPEC strain O127:H6 E2348/69 | LEE locus, type III secretion genes | chromosome | *rorf3* [70] | O52131 (AAC38373) | 152 (19–139) 121 8.74 |
| *Escherichia coli* Shiga toxin-producing (STEC, O26:H) | LEE locus, type III secretion genes | chromosome | *st13* | Q8VLC9 (CAC81851) | 154 (21–141) 121 8.53 |
| *Ralstonia solanacearum (Pseudomonas solanacearum)* | *hrp* gene cluster putative lipoprotein | megaplasmid | *RSP0841* [113] | Q8XRJ0 (CAD17992) | 232 (32–150) 119 7.18 |
| *Salmonella enterica* serovar typhi CT18 | SPI-I type III secretion genes | chromosome | *iagB sty3000* [110, 114] | P43018 (CAD05984) | 160 (20–139) 120 8.89 |
| *Salmonella enterica* serovar typhimurium | SPI-I type III secretion genes | chromosome | *iagB stm2877* [85, 115] | P43017 (AAL21757) | 160 (20–139) 120 8.87 |
| *Shigella flexneri* serotype 2a | entry region type III secretion genes | virulence plasmid pCP301 | *CP0135* [116] | Q07568 (AAL72337) | 152 (19–138) 120 9.02 |
| *Shigella flexneri* serotype 5a | entry region type III secretion genes | virulence plasmid pWR100 | *ipgF* [83, 84] | Q07568 (CAC05810) | 152 (19–138) 120 9.02 |
| *Shigella sonnei* | entry region type III secretion genes | plasmid | *ipgF* | Q55287 (BAA09144) | 152 (19–138) 120 9.02 |
| *Xanthomonas axonopodis* (pv. citri) | *hrp* gene cluster | chromosome | *hpa2* or *XAC0417* [88] | Q8PQB1 | 138 (16–128) 113 8.67 |
| *Xanthomonas campestris* (pv. campestris) | *hrp* gene cluster | chromosome | *hpa2* [88] | Q8PB82 (AAM40539) | 138 (16–134) 119 9.30 |
| *Xanthomonas campestris* (pv. vesicatoria) | *hrp* gene cluster | chromosome | *hpaH* [86] | Q8RJP7 (AAL78295) | 157 (35–147) 113 8.64 |
| *Xanthomonas oryzae* pv. oryzae | *hrp* gene cluster | chromosome | *hpa2* [87] | Q9LBD5 | 162 (17–129) 113 8.64 |
| *Yersinia enterocolitica* | type III secretion | chromosome | *ysaH* [117] | Q9KKJ1 (AAF82325) | 158 (22–139) 118 9.39 |
| **LT genes in type IV secretion systems** | | | | | |
| *Actinobacillus actinomycetemcomitans (Haemophilus actinomycetemcomitans)* | conjugative DNA transfer? | plasmid pVT745 | *magB01* | Q9F243 (AAG24436) | 216 (5–141) 137 6.94 |

Table 1 (continued)

| Organism | System | Plasmid/ chromosome | Gene name/ reference | Accession numbers[a] | Protein data[b] |
|---|---|---|---|---|---|
| *Actinobacillus actinomycetemcomitans (Haemophilus actinomycetemcomitans)* | type IV secretion | chromosome | *cagE* (C terminus is similar to CagE and VirB4) | Q8KI83 (AAM68957) | 340 (2–143) 142 6.14 |
| *Agrobacterium rhizogenes* | T-DNA and protein transfer to plant cells, *virB* operon | plasmid pRi1724 | *virB1* (Riorf153 protein) [118] | Q9F5A2 (BAB16272) | 240 (28–173) 146 6.45 |
| *Agrobacterium tumefaciens* | T-DNA and protein transfer to plant cells, *virB* operon | plasmid pRiA4 | *virB1* | O66281 (BAA28695) | 245 (30–175) 146 6.45 |
| *Agrobacterium tumefaciens* | T-DNA and protein transfer to plant cells, *virB* operon | plasmid pTiA6; plasmid pTi15955 | *virB1* [119, 120] | P05350 (AAA88645 CAA29972) | 245 (34–175) 142 9.56 |
| *Agrobacterium tumefaciens* | T-DNA and protein transfer to plant cells, *virB* operon | plasmid pTiAB2/73 | *virB1* | Q8VTA2 AAL57009 | 238 (28–173) 146 6.77 |
| *Agrobacterium tumefaciens* (strain C58/ATCC 33970) | T-DNA and protein transfer to plant cells, *virB* operon | plasmid pTiC58 | *virB1* [121] | P17791 (AAL46403) | 245 (30–175) 146 4.70 |
| *Agrobacterium tumefaciens* MAFF301001 | T-DNA and protein transfer to plant cells, *virB* operon | plasmid pTi-SAKURA | *virB1* [122] | Q9R6C3 (BAA87766) | 245 (30–175) 146 5.21 |
| *Brucella abortus* | type IV secretion gene cluster | chromosome | *virB1* [123] | Q9KIT0 (AAF73894) | 238 (4–156) 153 9.23 |
| *Brucella melitensis* | attachment-mediating *virB* genes | chromosome II | *bmeii0025 virB1* [124] | Q8YDZ5 (AAL53266) | 238 (4–156) 153 9.23 |
| *Brucellla suis* | type IV secretion gene cluster | chromosome | *virB1* [125] | Q9RPY4 (AAD56611) | 238 (4–156) 153 9.23 |
| *Enterobacter aerogenes* | conjugative DNA transfer | plasmid R751 | *trbN* | P71189 (AAC64455) | 211 (16–135) 120 9.36 |
| *Escherichia coli Salmonella typhimurium* | conjugative DNA transfer | plasmid pKM101(deletion derivative of plasmid R46, IncN) | *traL* [126] | Q46694 (AAL13382) | 244 (30–177) 148 9.30 |
| *Escherichia coli* | conjugative DNA transfer | plasmid R388 (IncW) | *trwN* | Q8KMV0 (CAD42326) | 198 (6–153) 148 6.31 |
| *Escherichia coli* | conjugative DNA transfer | plasmid R721 | *traB* | Q9F522 (BAB12658) | 194 (8–156) 149 8.81 |

Table 1 (continued)

| Organism | System | Plasmid/ chromosome | Gene name/ reference | Accession numbers[a] | Protein data[b] |
|---|---|---|---|---|---|
| *Escherichia coli* *Salmonella enterica* | conjugative DNA transfer | plasmid R1 (IncFII) | *19* [101] | P17738 (CAA33352) | 169 (21–140) 120 8.89 |
| *Escherichia coli* | conjugative DNA transfer | plasmid F (IncFI) | *orf169* | P47737 (BAA97940) | 169 (21–140) 120 8.89 |
| *Escherichia coli* | conjugative DNA transfer | plasmid p307 (IncFI) | *19* | Q00739 (AAA25521) | 169 (21–140) 120 8.57 |
| *Escherichia coli* | conjugative DNA transfer | plasmid R100 (IncFII) | *X* | P14499 (CAA30250) | 169 (21–140) 120 8.89 |
| *Haemophilus influenzae* biotype *aegyptius* | conjugative DNA transfer? | plasmid pF3031 | *bpl11* | Q8VRD8 (AAL47107) | 227 (9–167) 159 6.18 |
| *Helicobacter pylori* | type IV secretion | chromosome | *hp0523* [106] | O25259 (AAD07590) | 169 (33–165) 133 9.64 |
| *Helicobacter pylori* | type IV secretion | chromosome | *Cag-gamma* | Q9JMY1 (AAF80194) | 169 (33–165) 133 9.57 |
| *Helicobacter pylori* J99 | type IV secretion | chromosome | *jhp0472* | Q9ZLV4 (AAD06055) | 169 (33–165) 133 9.64 |
| *Mesorhizobium loti* strain R7A | symbiosis island type IV secretion genes | chromosome | *virB1* [127] | Q8KJL3 (CAD31462) | 260 (30–175) 146 9.04 |
| Proteobacteria | conjugative DNA transfer | broad-host-range plasmid pIPO2 | *virB1* [128] | Q91UX8 (CAC82747) | 253 (4–153) 150 8.7 |
| *Pseudomonas putida* | conjugative DNA transfer? | plasmid pWW0 (IncP-9) | *mpfJ* | Q8VMF7 (CAC86859) | 315 (41–187) 147 6.94 |
| *Pseudomonas* sp. | conjugative DNA transfer | plasmid RP4 (IncP) | *trbN* | Q03538 (AAA26440) | 234 (46–165) 120 9.12 |
| *Pseudomonas* sp. | conjugative DNA transfer | plasmid pADP-1 | *trbN* | Q9AHG6 (AAK50307) | 211 (16–135) 120 9.36 |
| *Rhizobium etli* | conjugative DNA transfer? | symbiotic plasmid p42d | *virB1* | Q8KIM5 (AAM54907) | 222 (5–148) 144 4.88 |
| *Salmonella enterica* serovar Typhi | conjugative transfer | plasmid R27 (IncHI) | *bfpH* [129] | Q9L5L2 (AAF69968) | 171 (21–142) 122 9.20 |

Table 1 (continued)

| Organism | System | Plasmid/ chromosome | Gene name/ reference | Accession numbers[a] | Protein data[b] |
|---|---|---|---|---|---|
| *Salmonella enterica* serovar Typhimurium | conjugative DNA transfer | plasmid pSLT | *PSLT072* | Q93GP2 AAL23542 | 156 (21–140) 120 8.59 |
| *Salmonella typhi* | conjugative DNA transfer | plasmid pED208 (IncFV) | *X* [130] | Q8KQN1 (AAL78046) | 152[d] (20–141) 122 8.65 |
| *Sinorhizobium meliloti* | conjugative DNA transfer | plasmid pSB102 | *virB1* [131] | Q91UQ4 (CAC79183) | 259 (4–150) 147 8.39 |
| *Sinorhizobium meliloti* | type IV secretion? | plasmid pSymA (megaplasmid 1) | *virB1* [132] | Q92YZ3 (AAK65377) | 223 (5–149) 145 4.66 |
| Uncultured bacterium | conjugative DNA transfer | plasmid pB4 | *trbN* | Q8RS14 (CAD24380) | 199 (16–135) 120 9.36 |
| *Xanthomonas axonopodis* (pv. citri) | conjugative DNA transfer? | plasmid pXAC64 | *virB1* [88] | Q8PRJ6 (AAM39282) | 292 (24–170) 147 8.93 |
| *Xanthomonas axonopodis* (pv. citri) | type IV secretion? | chromosome | *virB1* [88] | Q8PJB8 (AAM37466) | 280 (4–144) 141 9.23 |
| *Xanthomonas campestris* (pv. campestris) | type IV secretion? | chromosome | *virB1* [88] | Q8P7X8 (AAM41753) | 277 (4–144) 141 9.23 |
| *Xylella fastidiosa* | conjugative DNA transfer | plasmid pXF51 | *XFA0002 traL* [133] | Q9PHK3 (AAF85571) | 191 (3–150) 148 7.96 |
| *Xylella fastidiosa* | conjugative DNA transfer | plasmid pXF51 | *XFA0036* (*trbN*) [133] | Q9PHG9 (AAF85605) | 199 (15–134) 120 9.23 |

| Organism | Closest homologues/ System | Plasmid/ chromosome | Gene name/ reference | Accession numbers[a] | Protein data[b] |
|---|---|---|---|---|---|
| **LT genes not obviously associated with secretion system genes** | | | | | |
| *Aquifex aeolicus* | *ipgF Shigella flexneri*/ type III secretion (Q07568) | chromosome | *iagB aq_1579* | O67519 *(*AAC07484) | 184 (63–183) 121 8.99 |
| *Escherichia coli* K12 (MG1655) | *pilT* of plasmid R721/ type IV pilus (Q9F539) | chromosome | *ORF_o138* [71] | Q46790 (AAA83036) | 158[e] (27–153) 127 9.32 |
| *Escherichia coli* EHEC O157:H7 (EDL 933) | *pilT* of plasmid R721/ type IV pilus (Q9F539) | chromosome | *Z4175* [71] | Q8X6H3 (AAG57967) | 167 (27–153) 127 9.56 |

Table 1 (continued)

| Organism | Closest homologues/ System | Plasmid/ chromosome | Gene name/ reference | Accession numbers[a] | Protein data[b] |
|---|---|---|---|---|---|
| *Ralstonia solanacearum (Pseudomonas solanacearum)* | *orfC Burkholderia pseudomallei*/ type II secretion (Q9ZF87) | chromosome | *RSC2896* [113] | Q8XVD5 (CAD16603) | 170 (46–164) 119 9,07 |
| *Xylella fastidiosa* 9a5c | *trbN* of plasmid pXF51/ conjugation (Q9PHG9) | chromosome | *XF2045* | Q9PBU0 (AAF85605) | 201 (15–134) 120 9.23 |
| *Escherichia coli* | *magB01* of plasmid pVT745/conjugation (Q9F243) | plasmid R6K (IncX) | *pilX1* | Q9EUG1 (CAC20138) | 214 (7–155) 149 8.68 |
| *Pseudomonas syringae* pv. maculicola | seems to be a *virB1-virB4* hybrid like *cagE* of *Actinobacillus actinomycetemcomitans* (Q8KI83) | plasmid pFKN | *orf28* | Q93TD0 (AAK49560) | 868 (7–151) 145 9.01 |

BLAST [134] searches with already identified members of the LT group were performed to identify potential novel LTs belonging to secretion systems. Each list is sorted alphabetically by the name of the organism. n.a., not available.
[a] Both the Swiss-Prot/TrEMBL and the GenBank accession numbers (in parentheses) are given.
[b] The length of the protein, the part of the protein corresponding to the PFAM SLT domain (in parentheses), the length of the SLT domain, and the computed pI of the SLT part are given.
[c] These entries seem to be the same protein. The real size of Q93D61 is very likely 157 amino acids.
[d] This ORF is 24 amino acids longer than given in the database entry (earlier GUG start codon).
[e] This ORF is 20 amino acids longer than given in the database entry (earlier AUG start codon).

genes present on the chromosome of *Burkholderia pseudomallei*, *orfC*, a gene encoding a specialised LT homologue is located next to the type II secretion genes [74]. Transposon mutagenesis revealed that the gene was not required for the secretion of exoenzymes [74]. Intriguingly, a chromosomally located type III secretion gene cluster has been identified in the same organism containing another LT homologue, *bapC* [75].

## Type III secretion

Many plant, animal and human pathogenic Gram-negative bacteria assemble a secretion machinery in the bacterial cell envelope which is coupled to a bacterial translocation complex inserted into the membrane of a eukaryotic cell [19]. Using this machinery, effector proteins like the Yop proteins of Yersiniae are injected into the eukaryotic cell to facilitate host invasion [76]. The core components of the secretion machinery, the TTSS complex, is composed of approximately 20 proteins and is conserved among different bacterial pathogen species [77]. The TTSS is evolutionarily related to the flagellar assembly machinery [78]. The transported effector proteins and the proteins constituting the translocon are not conserved [19]. The proteins of the TTSS of some bacterial pathogens have been isolated and form stable supramolecular structures, 'needle' complexes, that could be visualised by cryo-electron microscopy [79–81]. Clearly, the assembly of such a structure with a diameter of approximately 10 nm in its periplasmic part [80] requires a localised rearrangement of the cell wall. This rearrangement could be achieved by the presence of specialised LTs facilitating the assembly of the supramolecular complexes. Although an essential role for a muramidase (FlgJ) in flagellar assembly has been demonstrated [82], such an enzymatic function has not yet been shown to be of importance in any one of the type III secretion systems. Nevertheless, the genetic elements harbouring TTSS genes generally encode one protein containing the SLT motif (see table 1). For one of these proteins, IpgF, encoded within the entry region of the *Shigella flexneri* virulence plasmid pWR100 [83], we recently obtained definite proof for a peptidoglycan-degrading activity [unpublished observation]. A mutation in *ipgF* was reported to have no effect on pathogenicity [84]. Similarly, a mutation in the closely related *iagB* gene encoded by *Salmonella* SPI-1 did not result in reduced needle complex formation. As the authors concluded, this could either be due to the fact that the type III secretion complex can assemble without the assistance of this putative LT, or an unidentified functionally redundant protein can substitute for IagB [85]. The *hpaH* gene encoded within a *hrp* gene cluster on the chromosome of the plant pathogen *Xanthomonas campestris* pv. vesicatoria contributes to bacterial growth and the induction of plant reactions. However, the effects of a *hpaH* frameshift mutation was

only weak [86]. A mutation in the homologous *hpa2* gene of *X. oryzae* pv. oryzae did not result in an altered phenotype when compared to the wild-type strain [87]. Yet, the *hpa2* gene was present in all *Xanthomonas* species that were tested and was detected by Southern blotting [87]. In the two completely sequenced *Xanthomonas* species one additional specialised LT gene (*virB1*) is present on each chromosome within a type IV secretion gene cluster [88]. The function of these type IV secretion genes in *Xanthomonas* species remains to be elucidated. Could there possibly be cross-talk between the type III and type IV secretion systems and a (partial) functional complementation of *hpa2* and *hpaH* by *virB1*? While this question remains to be answered and requires an experimental approach, recently, in *Burkholderia cepacia* genomvar III, a *virB1* homologue has been found to be associated with type III secretion genes [89]. This intriguing observation could mean that specialised LT genes can move from one dedicated secretion system to another without losing their function.

## Type IV secretion

During the past years more and more bacterial pathogens have been found that possess a T4SS as essential virulence determinants. T4SSs deliver proteins to the target eukaryotic host cells and have been adapted from plasmid conjugation systems [for recent reviews see refs 90–92]. One of the best-characterized T4SSs is encoded by the *virB* operon present on Ti plasmids of *Agrobacterium* species. This plant pathogen exports several proteins and a nucleoprotein complex containing tumourigenic DNA into plant cells [93]. The *virB* operon encodes VirB1, a protein identified as a member of the SLT family [9]. Subsequently, deletion of the *virB1* gene in the *Agrobacterium* Ti plasmid was shown to result in a severely reduced tumour formation capacity [94]. Complementation of this defect was possible with a wild-type *virB1* gene but not with a construct expressing a VirB1 variant in which the invariantly conserved Glu residue at position 53 was changed to Gln or Ala [94]. Interestingly, the VirB1 protein possesses a C-terminal extension beyond the SLT motif which is processed to yield VirB1* [95]. This polypeptide contributes to tumourigenesis independently of the LT function [96], but the role of VirB1* in the Ti system remains unclear. The attenuated phenotype observed with *virB1* mutants is also seen in conjunction with the phenomenon that the broad-host-range plasmid RSF1010 is transferred by the Ti-plasmid-encoded T4SS. Whereas all other *virB* mutants were completely defective in transfer, a *virB1* mutation only caused an approximately tenfold reduction in the transfer capacity [97]. The assembly of the *Agrobacterium* T4SS complex in the cell envelope is another interesting area of investigation. Fascinatingly, VirB1 seems to play a key role in the assembly process where the SLT part of VirB1 has been demonstrated to interact with core components of the *Agrobacterium* T4SS complex using a yeast two-hybrid system [98]. Such an interaction of VirB1 with other components of the VirB T4SS could not be identified in another study. In that case, protein-protein interactions in the membranes were analysed after extraction with the mild detergent dodecyl-$\beta$-D-maltoside followed by electrophoretic separation under native conditions [99]. A reason for this discrepancy could be that VirB1 interactions with the core component are only transient and/or weak, such that the interactions cannot be detected when cell extraction methods are used. In another DNA transfer system, a 'classical' F-like conjugation system, the inactivation of gene *19* encoded by the enterobacterial conjugative resistance plasmid R1 led to a tenfold decrease in the conjugation frequency [9]. Protein P19 contains the SLT motif, is transported to the periplasm via the Sec pathway [100] and causes localised lysis in cells overexpressing the protein [101]. Furthermore, the results of in vivo complementation assays demonstrated that the invariantly conserved Glu at position 44 is absolutely required for the biological function of P19 [101]. Another of the identified SLT genes in T4SS is *traL* encoded by the conjugative IncN plasmid pKM101. A disruption of *traL* reduced the conjugation frequency 10- to 100-fold [102]. In the case of the human pathogen *Helicobacter pylori*, the T4SS encoded by the *cag* pathogenicity island (*cag*-PAI) is required for the transport of CagA, a bacterial protein that is translocated into the epithelial host cell and phosphorylated [103–105]. For both CagA translocation/phosphorylation and interleukin-8 induction in the host cells, some of the genes present in the *cag*-PAI were found to be essential, among them *hp0523* [106]. Although the HP0523 protein has not been recognised as a member in the family of specialised LTs, the presence of a weak albeit characteristic similarity has been observed [C. Baron, personal communication]. In our laboratory, we overexpressed and partially purified HP0523 and could demonstrate a peptidoglycan-binding and/or -degrading activity using zymograms [unpublished results]. Because of these results, the presence of the invariantly conserved Glu at position 56 and of the two other characteristic sequence motifs (fig. 1), HP0523 very likely represents a specialised LT which is essential for the function of the *H. pylori* T4SS.

## DNA secretion in *N. Gonorrhoeae*

*N. gonorrhoeae* not only displays natural competence but also secretes DNA during the stationary phase, thus providing DNA that can be taken up by recipients. Recently, AtlA [107], an autolysin with a high degree of similarity to the LAL encoded by the $\lambda$R gene, was discovered on

a variable genetic island specific for *N. gonorrhoeae* [108]. Additionally, all versions of the genetic island encoded homologues of F factor conjugation proteins, suggesting that, like some other pathogenicity islands, this region encodes a conjugation-like secretion system. Consistent with this hypothesis, a wild-type strain released large amounts of DNA into the medium during exponential growth without cell lysis, whereas an isogenic strain mutated in *atlA* was drastically reduced in its ability to donate DNA for transformation [108].

## Concluding remarks

During the past few years, remarkable progress has been made in understanding the molecular mechanisms that contribute to bacterial pathogenicity. It has become increasingly obvious that dedicated secretion and translocation systems provide bacteria with the equipment that facilitates host invasion. Not only these systems but also other macromolecular transport systems rely on the presence of complex supramolecular structures composed of protein complexes inserted into the cell envelope of bacteria. Additionally, the single-layered bacterial cell wall has convincingly been shown to constitute a permeability barrier and the efficient assembly and/or function of several transport systems depends on the presence of transport-complex-associated specialised muralytic enzymes that facilitate penetration of the peptidoglycan network. These enzymes, commonly belonging to the LT family, are termed specialised LTs because of their committed function in macromolecular transport systems. In most of the cases where the role of the specialised LT has been investigated, the LT was found to enhance the efficiency of the system but the LT was not absolutely essential in vivo. At certain sites within the cell wall and during defined physiological conditions, the holes in the peptidoglycan meshwork may be large enough for the assembly of the transporter, and, therefore, the specialised LT is not needed. Another, very striking possibility is that genes encoding specialised LTs are redundant and can complement each other. Such a redundancy can be observed in different pathogenic bacteria as has been detailed in this review. In favour of this hypothesis is that genes encoding specialised LTs have been kept during evolution with conjugative plasmids, bacteriophages and pathogenicity islands, or have been added by horizontal gene transfer events. The well-established contribution of specialised LTs to the efficiency of several systems that have been investigated, the remarkable redundancy of LT genes and the evolutionary conservation in diverse systems implies that these enzymes do play an important role. Clearly, additional work in the laboratory is required to shed light on this important aspect. An experiment to assess the effects of a knock-out of one given LT gene probably needs to be performed in a 'clean' background where no other specialised LT genes are present that could functionally replace the deleted or mutated gene. Such experiments are currently being carried out in our laboratory.

In this review, the main objective was to focus on the cell wall as a barrier to trans-envelope transport in bacteria and how this barrier can be overcome. However, I would like to emphasise that the peptidoglycan network in these systems may not only be a hindrance but may also provide a scaffold for the assembly and an anchor for the macromolecular transport complex. Such a scaffold function of the peptidoglycan meshwork might be as important as the function of the murein network in protecting the cell from mechanical stress factors.

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