Peptidoglycan degradation by specialized lytic transglycosylases associated with type III and type IV secretion systems

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Specialized lytic transglycosylases are muramidases capable of locally degrading the peptidoglycan meshwork of Gram-negative bacteria. Specialized lytic transglycosylase genes are present in clusters encoding diverse macromolecular transport systems. This paper reports the analysis of selected members of the specialized lytic transglycosylase family from type III and type IV secretion systems. These proteins were analysed *in vivo* by assaying their ability to complement the DNA transfer defect of the conjugative F-like plasmid R1-16 lacking a functional P19 protein, the specialized lytic transglycosylase of this type IV secretion system. Heterologous complementation was accomplished using IpgF from the plasmid-encoded type III secretion system of *Shigella sonnei* and TrbN from the type IV secretion system of the conjugative plasmid RP4. In contrast, neither VirB1 proteins (*Agrobacterium tumefaciens*, *Brucella suis*) nor lagB (*Salmonella enterica*) could functionally replace P19. *In vitro*, IpgF, lagB, both VirB1 proteins, HP0523 (*Helicobacter pylori*) and P19 displayed peptidoglycanase activity in zymogram analyses. Using an established test system and a newly developed assay it was shown that IpgF degraded peptidoglycan in solution. IpgF was active only after removal of the chaperonin GroEL, which co-purified with IpgF and inhibited its enzymic activity. A mutant IpgF protein in which the predicted catalytic amino acid, Glu42, was replaced by Gln, was completely inactive. IpgF-catalysed peptidoglycan degradation was optimal at pH 6 and was inhibited by the lytic transglycosylase inhibitors hexa-*N*-acetylchitohexaoase and bulgecin A.

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INTRODUCTION

Type III secretion systems (T3SS) and type IV secretion systems (T4SS) of Gram-negative bacteria are composed of supramolecular protein complexes which span the cell envelope and secrete proteins and/or DNA in a contact-dependent manner (for recent reviews on T3SS see Ghosh, 2004; Tampakaki *et al.*, 2004; for a recent review on T4SS see Cascales & Christie, 2003). Due to structural constraints imposed by the peptidoglycan (PG) meshwork and its sieving capacity (Demchick & Koch, 1996; Pink *et al.*, 2000; Vázquez-Laslop *et al.*, 2001; Yao *et al.*, 1999), it has been proposed that for the assembly of a macromolecular secretion system specialized enzymes are required to enlarge holes in the PG by controlled local degradation (Dijkstra & Keck, 1996; Koraimann, 2003). Analyses of bacterial genome

sequences revealed that many, if not all, T3SS and T4SS gene clusters encode their own murein-degrading enzymes, which belong to the lytic transglycosylase (LT) family of proteins. These small proteins (usually between 150 and 250 aa residues) have been termed 'specialized' LTs because of their defined role in macromolecular transport (Koraimann, 2003) and contain the characteristic SLT sequence motif (see the PFAM database, accession code PF01464; <http://www.sanger.ac.uk/Software/Pfam/>). Originally LTs were characterized as enzymes involved in growth and division of the stress-bearing murein sacculus (Höltje & Heidrich, 2001). LTs are widely distributed among Gram-negative bacteria and cleave the β -1,4 glycosidic bond between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc). Despite their observed redundancy, LTs have been proposed to represent potential targets for novel antibacterial agents (Korsak *et al.*, 2005). Known inhibitors of LTs are hexa-*N*-acetylchitohexaoase (Leung *et al.*, 2001; Song *et al.*, 1994) and bulgecin A (Templin *et al.*, 1992); very recently a novel LT inhibitor, *N*-acetylglucosamine thiazoline, has been identified (Reid *et al.*, 2004a, b).

Abbreviations: GlcNAc, *N*-acetylglucosamine; IL-8, interleukin-8; LT, lytic transglycosylase; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; MurNAc, *N*-acetylmuramic acid; PAI, pathogenicity island; PG, peptidoglycan; SPI, *Salmonella* pathogenicity island; T3SS, type III secretion system(s); T4SS, type IV secretion system(s).

In contrast to structurally and functionally related lysozymes, the reaction catalysed by LTs is not a hydrolysis but rather a transglycosylase reaction which results in formation of an internal 1,6 anhydro bond in the MurNAc residue (Höltje *et al.*, 1975). Experimental data strongly suggest that the cleavage reaction is triggered by a conserved glutamyl residue in the active site of LTs (van Asselt *et al.*, 2000). A natural breakdown product produced by LTs, the muro-peptide GlcNAc-(1,6 anhydro)MurNAc-tetrapeptide has been shown to induce inflammatory cytokines in human monocytes (Dokter *et al.*, 1994). An intracellular receptor and component of the human innate immune system, Nod1, has been shown to specifically recognize Gram-negative PG and PG breakdown products (Girardin *et al.*, 2003). Recognition of PG and PG breakdown products from Gram-negative and Gram-positive bacteria has been acknowledged as a key part of the innate immune system present in diverse organisms (Girardin & Philpott, 2004), yet the source and structural composition of PG breakdown products that elicit a defence reaction in the host of a pathogen are unknown. A potential link between the innate immune system recognizing PG from Gram-negative bacteria and the activity of specialized LTs could be the finding that the delivery of PG to epithelial cells by the human pathogen *Helicobacter pylori* is mediated by the T4SS, which is an essential virulence determinant of this organism (Viala *et al.*, 2004).

Here we focus on biochemical characterization of specialized LTs from diverse T3SS and T4SS. We cloned and overexpressed specialized LTs from both types of secretion systems. From T3SS: IpgF from *Shigella sonnei*, which is 98 % identical to IpgF, encoded within the entry region of the virulence plasmids pWR100 and pCP301 of *Shigella flexneri* serotypes 5a and 2a, respectively (Allaoui *et al.*, 1993; Buchrieser *et al.*, 2000; Jin *et al.*, 2002); and IagB, which is encoded by the *Salmonella enterica* pathogenicity island SPI-1 (Miras *et al.*, 1995). From T4SS: P19 encoded by the conjugative resistance plasmid R1 (Koraimann *et al.*, 1993), VirB1 encoded by the Ti plasmid of *Agrobacterium tumefaciens* (Thompson *et al.*, 1988), VirB1 encoded by the small chromosome of *Brucella suis* (O'Callaghan *et al.*, 1999), TrbN from the IncP plasmid RP4 (Pansegrau *et al.*, 1994) and HP0523 encoded by the *cag* pathogenicity island (*cag*-PAI) of *H. pylori* (Tomb *et al.*, 1997). Inactivation of the genes encoding these proteins in the respective system mostly resulted in virulence-attenuated phenotypes which could have arisen from the observed redundancy of LTs in bacteria (Koraimann, 2003). Whereas mutations in *ipgF* and *iagB* were reported to have no obvious effects on pathogenicity or needle complex formation (Allaoui *et al.*, 1993; Sukhan *et al.*, 2001), a deletion of the corresponding LT gene in the PAI of *Citrobacter rodentium*, *rorf3*, led to decreased T3SS assembly, effector translocation and virulence (Deng *et al.*, 2004). Mutations inactivating P19 of the derepressed F-like plasmid R1-16 reduced the conjugation frequency five- to tenfold (Bayer *et al.*, 1995, 2001). Mutations inactivating VirB1 encoded by the Ti plasmid of *A. tumefaciens* led to attenuated tumour formation in plants

(Mushegian *et al.*, 1996). Notably, HP0523, the *cag*-PAI-encoded LT from *H. pylori* was found to be essential for both CagA translocation into the human host cells and IL-8 induction (Fischer *et al.*, 2001).

The main aim of the work presented here was to unambiguously demonstrate the muralytic activity of these enzymes, which up to now has not been shown for any of the specialized LTs encoded by T3SS or T4SS. This goal was reached by combined *in vivo* studies, zymogram analyses and in-solution muramidase assays, including a novel test system employing Cy3-labelled PG.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* cells were grown in 2 × TY (16 g tryptone l⁻¹, 10 g yeast extract l⁻¹ and 5 g NaCl l⁻¹). The following antibiotics, when appropriate, were added at the indicated final concentrations: 100 µg ampicillin ml⁻¹, 40 µg kanamycin ml⁻¹.

Enzymes, chemicals and oligonucleotides. Restriction endonucleases and other enzymes used for standard cloning and DNA modification procedures were purchased from TaKaRa Biomedicals, Roche Molecular Biochemicals or New England Biolabs. Oligonucleotides were purchased from MWG Biotech. Rabbit anti-MalE antibody was purchased from Merck; goat anti-rabbit and anti-GroEL antibodies were purchased from Sigma-Aldrich. For DNA manipulations, PG isolation and purification, zymogram analyses, Cy3 labelling of PG, and the Cy3 PG spot assay RNase-free, double-distilled water (Fresenius Kabi Austria) was used.

DNA manipulations and recombinant plasmids. Recombinant DNA techniques were performed as described by Sambrook *et al.* (1989) or Ausubel *et al.* (1987) or according to the manufacturer's protocols. DNA fragments amplified by PCR using the primers given below were cloned into pMAL-p2X (New England Biolabs) for periplasmic and into pMAL-c2X (New England Biolabs) for cytoplasmic expression of fusion proteins; the inserted sequences were verified by DNA sequencing in each case. Signal sequences, when present, were removed during the cloning procedure. All constructs and their relevant features are listed in Table 1. For gene 19 amplification the forward primer BamP19_T20 (5'-ACGCGGATCCACTGATTGCTT-TGATCTTGC-3') was used. Reverse primers were P19end_Hind (5'-ACCCAAGCTTTAATTATTCTGCAGCGTG-3'), 19Hind150G (5'-CATTAAGCTTTTATTAACCTTTACTGCTTTTATATCCC-3'), 19-Hind144G (5'-CCTAAAGCTTTATTACCCGGTATAAATCC-3') and 19Hind140R (5'-CATTAAGCTTTTATTACCCGTAACCTCTG-3'). Primers for site-specific PCR mutagenesis for the creation of the P19_E44A mutant were P19E44A_1 (5'-CATGGAAAGCATCCCCGTTAC-3') and P19E44A_2 (5'-GTAACGGGATGCTTTCCATG-3'), which were used in combination with the primers BamP19_T20 and P19end_Hind. Plasmids pCK217 (Koraimann *et al.*, 1993) and pETBla19E44QHis+ (Bayer *et al.*, 2001) were used as templates for the PCR amplifications. *hp0523* was amplified from a DNA clone containing the *cag*-PAI of *H. pylori* using primers Bam_HP0523 (5'-CTTAGCCGGATTCTGCCAAAAGGTAACCATAGTTTC-3') and HP0523_end_Hind (5'-GGGAAAGCTTACTACTCGTTATATCGC-ACTTGAG-3'). All gene 19 fragments and the *hp0523* fragment were digested with *Bam*HI and *Hind*III and ligated into the pMAL vectors. Oligonucleotides used to amplify *ipgF* from a clinical isolate of *Sh. sonnei* were IpgF-EcoRI (5'-GCTTGAATTCGATTGTTGGGA-TAAGGCTGG-3') and IpgF_HindIII (5'-GAGCAAGCTTTTATTA-TATCCTTCGATTATTCTGCTTGCTC-3'). Site-specific mutagenesis

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i> strains		
XL-1 Blue	F'::Tn10 <i>proAB lacI^qΔM15/endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 lac</i>	Stratagene
SF100	F ⁻ <i>lacX74 galE galK thi rpsL (strA) phoA (PvuII) ompT</i>	Baneyx & Georgiou (1990)
J5	F ⁻ Lac ⁺ λ ⁺ <i>pro met</i>	IMB collection
MC1061	F ⁻ <i>hsdR2 hsdM⁺ hsdS⁺ araD139 Δ(ara-leu)7697 Δ(lac)X74 galE15 galK16 rpsL mcrA mcrB1</i>	Minton (1984)
Plasmids		
R1-16	Derepressed variant of IncFII plasmid R1, Km ^r	IMB collection
R1-16/mut1	R1-16 encoding an inactive N-terminal fragment of P19 (aa 1–46)	Bayer <i>et al.</i> (1995)
pMAL-c2X	Vector for cytoplasmic expression of MalE fusion proteins	New England Biolabs
pMAL-p2X	Vector for periplasmic expression of MalE fusion proteins	New England Biolabs
pMalc-	Expression constructs based on pMAL-c2X	This work
pMalp-	Expression constructs based on pMAL-p2X	This work
-P19	MalE-P19 (P19 aa 20–169)	This work
-19_150G	MalE-P19_150G (P19 aa 20–150)	This work
-P19_144G	MalE-P19_144G (P19 aa 20–144)	This work
-P19_140R	MalE-P19_140R (P19 aa 20–140)	This work
-P19_E44Q	MalE-P19_E44Q (P19 aa 20–169, E44Q)	This work
-P19_E44A	MalE-P19_E44A (P19 aa 20–169, E44A)	This work
-IpgF	MalE-IpgF (IpgF aa 19–152)	This work
-IpgF_E42Q	MalE-IpgF (IpgF aa 19–152, E42Q)	This work
-IagB	MalE-IagB (IpgF aa 20–160)	This work
-VirB1_AT	MalE-VirB1_AT (VirB1 aa 29–239)	This work
-VirB1_BS	MalE- VirB1_BS (VirB1 aa 2–238)	This work
-TrbN	MalE-TrbN (TrbN aa 35–234)	This work
-HP0523	MalE-HP0523 (HP0523 aa 19–169)	This work

resulting in the IpgF_E42Q mutant was done with the mutagenic primers IpgF_EQ_fw (5'-GCGATTGCGGAAAAACAGTCCGGA-TTTA-3') and IpgF_EQ_rev (5'-ATTAAATCCGGACTGTTTTC-GCAATCG-3') in combination with the two *ipgF*-specific primers above. The *iagB* gene was amplified from *Sal. enterica* using primers *iagB_EcoRI* (5'-GATTGAATTCGATTGCTGGCTTCAGGC-3') and *iagB_HindIII* (5'-CCGCAAGCTTTTATTATTTGTTTACCGCGAT-AGAAAGTC-3'). The *B. suis virB1* gene was amplified using primers *Bruc_EcoRI* (5'-GTACGAATTCGTGCCATTCCTTGTCTCTCGC-3') and *Bruc_HindIII* (5'-CAGTAAGCTTTTATTAGAAAACAACTAC-GCCGTC-3'). *ipgF*, *iagB* and *virB1* fragments were digested with *EcoRI* and *HindIII* and ligated into the pMAL vectors. Primers for the amplification of *virB1* from *A. tumefaciens* were *VirB1_BamHI* (5'-CTAAGGATCCGAGTTCGACCATGTTGCTCG-3') and *VirB1_NotIa* (5'-GATTGCGGCCGCTTATTAGTATAAGTCGAATA-AGAC-3'); finally, *trbN* primers were *TrbN_BamHI* (5'-CTA-AGGATCCCCCGATCTGTTCGGAACAGATGG-3') and *TrbN_NotI* (5'-CTAAGCGGCCGCTCCTTATTATGGCGTGTGTG-3'). The amplified fragments were digested with *BamHI* and *NotI*, cloned into the vector pET-32a(+) (Novagen), and subsequently *BamHI*-*XhoI* fragments were ligated into the *BamHI* and *SalI* restriction sites of the pMAL vectors.

DNA and protein sequence analyses. The Wisconsin Package Version 10.3 (Accelrys) was used for the analysis and *in silico*

manipulations of DNA and protein sequences. The calculation of similarity scores was performed using the BESTFIT program with the BLOSUM 62 matrix (gap weight 6; length weight 2, gaps longer than 5 not penalized).

Expression and affinity chromatography purification of fusion proteins. *E. coli* SF100 cells (*ompT*) harbouring recombinant plasmids for cytoplasmic expression were cultivated at 30 °C to an OD₆₀₀ of 0.4–0.7. Expression of fusion proteins was induced by addition of 0.3 mM IPTG. After 1.5 h cells were harvested, resuspended in 2 ml chromatography buffer (20 mM Tris/HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) and lysed using a French pressure cell. Cell debris was removed by low-speed centrifugation at 500 g; the supernatants were diluted to 10 mg total protein ml⁻¹ and applied to an amylose resin (New England Biolabs) column of 2 ml bed volume. The column was washed first with 20 ml chromatography buffer and subsequently with 10 ml chromatography buffer supplemented with complete protease inhibitor (Roche Molecular Biochemicals). Fusion proteins were eluted with 4 ml elution buffer (20 mM Tris/HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM maltose). Protein concentrations in the samples were determined using the Bio-Rad Protein Assay.

Anion-exchange chromatography. Affinity-purified fractions of MalE-IpgF and the active-site mutant MalE-IpgF_E42Q were

dialysed against 200 vols 20 mM Tris/HCl pH 7.4 at 8 °C overnight. To allow dissociation of GroEL from the fusion protein, the dialysed fractions were incubated with 1 mM ATP, 1 mM MgCl₂ and 0.1 % Triton X-100 for 30 min at 25 °C (Keresztessy *et al.*, 1996; Mattingly *et al.*, 1995). The fractions were centrifuged at 10 000 *g* to remove any insoluble material. The supernatant corresponding to 8 mg fusion proteins was applied to a Mono Q HR 5/5 column (Amersham Pharmacia) using FPLC and eluted with a linear gradient from 0 to 2 M NaCl in 20 mM Tris/HCl pH 7.4 containing 0.1 % Triton X-100 at 8 °C. Fractions were collected and analysed by SDS-PAGE, Western blotting, zymogram analyses, in-solution muramidase assay, and the Cy3-PG spot assay.

MALDI-TOF analysis. Samples (12 µg) of affinity-purified fractions of MalE-P19_{153L} and MalE-IpgF were separated electrophoretically on a 12.5 % SDS polyacrylamide gel. Proteins were Coomassie stained and the bands corresponding to the unknown protein were excised. The gel slices were destained with 500 µl 50 % methanol/50 % acetic acid in water at room temperature overnight. After a second destaining procedure, the gel slices were dehydrated in 200 µl acetonitrile. The samples were then reduced in 50 µl 10 mM DTT for 30 min at room temperature. After removal of DTT, proteins were alkylated with 50 µl 50 mM iodoacetamide for 30 min at room temperature and then washed with 100 µl 100 mM ammonium bicarbonate for 10 min. Subsequently, the gel slices were dehydrated as described above. The gel slices were then rehydrated by swelling in 100 µl 100 mM ammonium bicarbonate for 10 min. Ammonium bicarbonate was removed and the gel dehydrated as above. Acetonitrile was removed and 200 µl acetonitrile was added. Subsequently, the gel slices were dried *in vacuo*. Reswelling of the gel slices was achieved by incubation with 50 µl 20 ng µl⁻¹ trypsin (Promega) in ice-cold 50 mM ammonium bicarbonate for 10 min on ice. Excess trypsin solution was removed and 10 µl ammonium bicarbonate was added. Tryptic digestion of the proteins in the gel slices was performed overnight at 37 °C. Peptides were first extracted with 30 µl 100 mM ammonium bicarbonate. After collection of the supernatant, peptides were extracted twice with 30 µl formic acid in 50 % acetonitrile. The three supernatant fractions were pooled and the sample volume was reduced to 15 µl in a vacuum centrifuge. MALDI-TOF analysis was performed by piChem, Graz. The GroEL chaperone of *E. coli* was identified using the mass fingerprint data obtained by MALDI-TOF and the PeptIdent software (<http://ca.expasy.org/tools/peptident.html>).

Complementation assays. Complementation of the gene 19 defect was performed with donor strains carrying either the resistance plasmid R1-16 or R1-16/mut1 together with a complementation plasmid. The latter plasmids, pMalpP19 wt, pMalpP19_{150G}, pMalpP19_{144G}, pMalpP19_{E44Q}, pMalpP19_{E44A}, pMalpIpgF, pMalpIpgF_{E42Q}, pMalpIpgB, pMalpVirB1_{AT}, pMalpVirB1_{BS}, pMalpHP0523 and pMalpTrbN, are described in Table 1. Induction of transcription from the *tac* promoter with IPTG was not necessary for complementation. Overnight cultures from single colonies grown in 2 ml 2 × TY, supplemented with kanamycin and ampicillin in case of the donors, were used for mating. Forty microlitres of the donor cells, i.e. *E. coli* MC1061 harbouring R1-16 or R1-16/mut1 and the different complementation plasmids, were pipetted into 0.9 ml prewarmed 2 × TY medium and incubated for 60 min at 37 °C. Then 100 µl of an overnight culture of recipient cells, i.e. *E. coli* J5, was added, and the mixture was incubated for 60 min at 37 °C without shaking. Conjugation was interrupted by vigorously mixing for 1 min and placing the tubes on ice. Dilutions of 10⁻³ to 10⁻⁷ in 0.9 % NaCl were plated on lactose MacConkey agar plates containing kanamycin. The conjugation frequency was determined by counting white donor and red transconjugant colonies and is expressed as the number of transconjugants per 100 donor cells.

PG isolation and purification. PG was isolated from 1 litre of *E. coli* J5 cells in stationary phase according to Rosenthal & Dziarski (1994). Cells were harvested and washed with 40 ml 10 mM Tris/HCl pH 6.8. After washing, cells were resuspended in 30 ml 10 mM Tris/HCl pH 6.8. This suspension was added dropwise to 300 ml boiling 4 % SDS. After an additional 45 min of boiling, PG sacculi were collected by ultracentrifugation at 200 000 *g* for 20 min at 20 °C. The pellet was resuspended in 150 ml 2 M NaCl and incubated overnight at room temperature. After ultracentrifugation as described above, sacculi were washed with 60 ml water, collected by ultracentrifugation and resuspended in 20 ml water containing 0.1 mM MgCl₂. The suspension was incubated first with 50 µg ml⁻¹ DNase I, 50 µg ml⁻¹ RNase A and 200 µg ml⁻¹ α-amylase (Roche Molecular Biochemicals) for 90 min at 37 °C and second, after addition of 200 µg ml⁻¹ Pronase (Roche Molecular Biochemicals) at 60 °C for 60 min. Enzymes were inactivated by addition of SDS to a final concentration of 8 % and 15 min boiling. PG was collected by ultracentrifugation and washed twice with 20 ml water. The final pellet was resuspended in 5 ml water and stored at -20 °C. The amount of PG was estimated by evaporation and weighing an aliquot of the dried material.

Zymogram analyses. Zymogram analyses for the determination of PG-degrading activity was performed according to Bernadsky *et al.* (1994). Purified PG was incorporated into 12.5 % and 16 % polyacrylamide gels to a final concentration of 0.05 % (w/v). Protein samples were separated electrophoretically on polyacrylamide gels containing 0.02 % SDS. After electrophoresis, the gels were incubated with water for 60 min at 4 °C, then transferred to 25 mM potassium phosphate buffer pH 5.2 containing 0.1 % Triton X-100 (renaturation buffer) and incubated for 60 min at 4 °C. After this equilibration step, the gels were incubated with fresh renaturation buffer for 72 h. Peptidoglycan degradation was visualized by staining of gels with 0.1 % methylene blue/0.01 % KOH for 60 min at 4 °C. Gels were destained with water for 45–60 min.

Muramidase assay. Muramidase activity was determined by measuring solubilization of radiolabelled murein polymer (Engel *et al.*, 1991). [³H]A₂pm-labelled murein sacculi (2.5 µg; 5000 c.p.m.) were incubated in the presence of enzyme samples (3–6 µM) in a total volume of 100 µl 20 mM Bistris buffer pH 5.3 for 30 min at 37 °C. To precipitate the insoluble substrate, 100 µl of 1 % cetyltrimethyl ammonium bromide solution was added. The samples were kept on ice for 30 min. After centrifugation for 2 min at 17 000 *g* in an Eppendorf centrifuge at 4 °C, 100 µl of the supernatant was added to 1.5 ml scintillation cocktail, and radioactivity was measured in a Beckman LS 6500 scintillation counter. Inhibition of muramidase activity was assayed by addition of 1 µM GroEL (20 µl) to the reaction mixture prior to incubation at 37 °C.

Cy3 labelling of PG. A 500 µl sample (approx. 1 mg) of PG purified as described above was incubated with 50 µl 1 M borate buffer pH 9.6 and 5 µl Cy3 NHS ester (1 mg ml⁻¹ in dimethylformamide, Amersham Biosciences) for 60 min at room temperature. Excess Cy3 was removed by dialysis against 500 vols water using Slide-A-Lyser dialysis cassettes (MWCO 10 000, Pierce). Cy3-labelled PG was stored at -20 °C.

Cy3-PG spot assay. Eight-well glass slides (ICN) were coated with poly-L-lysine (Sigma-Aldrich) for 30 min at room temperature. After removal of unbound poly-L-lysine by washing with water, the slides were dried. Then 10 µl water and 1 µl Cy3-labelled PG were spotted onto the coated wells and incubated at room temperature for 45 min in the dark. To remove residual SDS and low-molecular-mass material the slides were rinsed with deionized water for 2 min and then dried at room temperature. Ten microlitres of buffer (10 mM or 25 mM sodium phosphate or ammonium acetate, pH 6) was pipetted on each of the Cy3-PG spots. Before and after addition

of 1 µl enzyme, fluorescence microscopy images were taken using a Zeiss Axioskop and Zeiss filter set #15 (BP 546/12, FT 580, LP 590). Fluorescence microscopy images were taken using a cooled CCD camera and analysed with the Metamorph 5.0 software package (Visitron Systems). To determine the pH optimum of MalE-IpgF the Cy3-PG spot assay was performed as described above using different buffers for incubation (pH 4 and 5, 10 mM sodium citrate buffer; pH 6 and 7, 10 mM sodium phosphate buffer; pH 6.8 and 8, 10 mM Tris/HCl). After addition of 1 µl MalE-IpgF the slides were incubated at room temperature for 15 min. To remove the digested material, the slides were rinsed with deionized water for 2 min and dried at room temperature. To quantitate the fluorescence signals, dry slides were scanned before and after incubation with the enzyme using an array scanner (Axon GenePix 4000B, PMT setting 320, scan power 33 %) and images were obtained with the GenePix Pro 4.1 program; the 16 bit tiff files were subsequently analysed using ImageQuant 5.1 software. PG-degrading activity of IpgF was calculated as the ratio of fluorescent PG spot values before and after treatment minus one. To test the effects of bulgecin A – a gift of A. J. Dijkstra (Hoffmann-La Roche, Basel, Switzerland) – and hexa-*N*-acetylchitohexaose (Seikagaku, Japan), various amounts of the compounds were added to the reaction mixture prior to the addition of the enzyme. The stocks of the various enzyme preparations had the following concentrations: MalE-IpgF, 6 µg µl⁻¹; MalE-IpgF_E44Q, 4 µg µl⁻¹; lysozyme from chicken egg white (Sigma), 20 µg µl⁻¹; GroEL, 1.6 µg µl⁻¹.

RESULTS

Overexpression and purification of MalE-LT fusion proteins

Earlier attempts to overexpress specialized LTs in the cytoplasm of *E. coli* resulted in formation of inclusion bodies which could only be solubilized in 8 M urea. Renaturation and recovery of active proteins was not possible. This was observed with native proteins as well as with different fusion constructs that were tested (His-tag, Trx-tag, S-tag; data not shown). The proportion of the soluble target protein was increased significantly when fused to the maltose-binding protein (MBP or MalE) from *E. coli*. Therefore, we constructed plasmids for the expression of N-terminal MalE

fusions of P19 wild-type, C-terminally truncated versions of P19 (P19_153L, P19_150G, P19_144G, P19_140R – the C-terminal amino acid residue in the deletion variant is indicated), and mutated versions of P19 (P19_E44Q and P19_E44A). The C-terminal deletions of P19 were included in the analysis to determine the minimal P19 core that is sufficient to confer *in vivo* and *in vitro* activity. We also constructed plasmids for the expression of MalE fusions with IpgF (*Sh. sonnei*, virulence plasmid T3SS) and the assumed active site mutant IpgF_E42Q, IagB (*Sal. enterica*, chromosomal T3SS), VirB1_AT (VirB1 from *A. tumefaciens*, Ti plasmid T4SS), VirB1_BS (VirB1 from *B. suis*, chromosomal T4SS), TrbN (*Pseudomonas* sp., conjugative resistance plasmid RP4 T4SS) and HP0523 (*H. pylori*, *cag*-PAI T4SS). The relevant protein data and accession numbers for the protein sequences in the databases are listed in Table 2. Both periplasmic and cytoplasmic expression constructs were made (see Table 1). Whereas the periplasmic constructs were used for the *in vivo* complementation assays, overexpression and affinity-purification steps were performed with fusion proteins expressed in the cytoplasm. In the latter case protein yield and stability were considerably higher. Signal sequences, when present, were removed during the cloning procedure. Despite the sequence similarity of the LTs chosen for expression and purification (see Table 3), the corresponding fusion proteins behaved quite differently during affinity chromatography. In the case of MalE-VirB1_AT and MalE-HP0523 at least one-third of the fusion proteins present in the crude extract did not bind to the amylose affinity resin. Best results in the first purification step were obtained with MalE-IpgF and MalE-P19; the average yield from 500 ml cultures was 8–15 mg of approximately 50 % pure fusion protein.

In vivo activity of MalE-LT fusion proteins

To determine the *in vivo* activity of the MalE-P19 fusion protein we used conjugation assays to assess the effects of

Table 2. Relevant protein data and sequence accession numbers

Protein name	Protein data*				Organism	System	Plasmid/chromosome	Accession numbers†
IpgF	152	(19–138)	120	9.02	<i>Sh. sonnei</i>	Entry region, T3SS	Plasmid	Q55287 , BAA09144
IagB	160	(20–139)	120	8.89	<i>Sal. enterica</i>	SPI-I, T3SS	Chromosome	P43018 , CAD05984
VirB1	245	(34–175)	142	9.56	<i>A. tumefaciens</i>	T-DNA and protein transfer to plant cells, T4SS	Plasmid pTiA6	P05350 , AAA88645, CAA29972
VirB1	238	(4–156)	153	9.23	<i>B. suis</i>	T4SS	Chromosome	Q9RPY4 , AAD56611
TrbN	234	(46–165)	120	9.12	<i>Pseudomonas</i> sp.	Conjugative DNA transfer, T4SS	Plasmid RP4	Q03538 , AAA26440
P19	169	(21–140)	120	8.89	<i>E. coli</i> , <i>Sal. enterica</i>	Conjugative DNA transfer, T4SS	Plasmid R1	P17738 , CAA33352
HP0523	169	(33–165)	133	9.64	<i>H. pylori</i>	T4SS	Chromosome	O25259 , AAD07590

*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.

†Both SWISS-PROT/TrEMBL (**bold**) and GenBank accession numbers are given.

Table 3. Similarity scores

*The BESTFIT algorithm with the BLOSUM 62 matrix (gap weight 6; length weight 2, gaps longer than 5 not penalized) of the Wisconsin Package, Version 10.3 was used. The numbers indicate similarity/identity values.

	IagB ^{20–139}	VirB1 ^{34–175} AT	VirB1 ^{4–156} BS	TrbN ^{46–165}	P19 ^{21–140}	HP0523 ^{33–165}
IpgF ^{19–138}	62/52†	49/40	47/35	39/34	53/43	43/26
IagB ^{20–139}		54/45	42/32	40/32	50/39	47/36
VirB1 ^{34–175} AT			46/42	42/35	44/36	49/38
VirB1 ^{4–156} BS				37/29	42/30	45/35
TrbN ^{46–165}					48/38	37/29
P19 ^{21–140} *						39/28

*The sequences of P19 encoded by the F-plasmid and plasmid R1 are 97 % identical.

mutations in protein P19 (Bayer *et al.*, 2001). In addition to investigating the biological activity of P19 and P19 mutants, we were interested in cross-complementation phenotypes of specialized LTs encoded by heterologous T3SS and T4SS. Complementation of the gene *19* defect was taken as an indication that the MalE-LT fusion protein tested degraded *E. coli* PG *in vivo*, and that it was able to appropriately interact with the DNA transport apparatus (i.e. the T4SS) encoded by plasmid R1. Cells harbouring either plasmid R1-16 (a derepressed variant of plasmid R1) or R1-16 and pMAL-p2X (the vector plasmid) served as positive controls. Cells harbouring R1-16/mut1 (R1-16 defective in gene *19*) and pMAL-p2X served as negative control. Table 4 shows that MalE-P19 wild-type fully complemented the conjugation deficiency of R1-16/mut1. We concluded that the MalE portion of the fusion protein did not interfere with the *in vivo* activity of P19 or other LTs. Complementation analysis revealed that amino acid residues between positions 144 and 150 of P19 wild-type are critical for its activity: MalE-P19_150G complemented the conjugation defect, whereas in the case of MalE-P19_144G and MalE-P19_140R no complementation was observed. As expected, the glutamyl residue at position 44 of P19 wild-type, which is invariantly conserved in the SLT family of proteins, was essential for its *in vivo* activity. Neither MalE-P19_E44Q nor MalE-P19_E44A could complement the conjugation defect. Finally, the heterologous complementation experiments showed that MalE-IpgF and MalE-TrbN restore conjugation to wild-type levels. As for MalE-P19_E44Q and MalE-P19_E44A, the exchange of the conserved glutamyl residue in MalE-IpgF_E42Q resulted in loss of the ability to complement the conjugation defect. MalE-IagB, MalE-VirB1_AT and MalE-VirB1_BS did not complement the DNA transfer defect; MalE-HP0523 showed an intermediate phenotype (Table 4).

PG degradation by MalE-LT fusion proteins: zymogram analyses

A method which has been widely used in the past for the detection of PG degradation by muramidases is the analysis

of proteins in zymograms (Bernadsky *et al.*, 1994). In zymograms, polymeric PG is incorporated into a polyacrylamide gel matrix. After electrophoresis under denaturing conditions and removal of SDS, the proteins are allowed to refold (for details see Methods). After methylene blue staining and subsequent destaining, clear, non-stained zones indicate degradation of the PG substrate by the refolded muramidase. In our zymogram analyses we first tested MalE-P19 (Figs 1a and 2c). As expected, clear zones

Table 4. *In vivo* complementation experiments

Mean values of at least three independently carried out experiments are given.

Conjugative plasmid in donor	Coresident plasmid	Conjugation frequency*	Complementation†
R1-16	–	36	–
	pMAL-p2X	32	–
R1-16/mut1	pMAL-p2X	7	23
	pMalp-P19wt	35	111
	pMalp-P19_150G	26	81
	pMalp-P19_144G	7	22
	pMalp-P19_E44Q	7	22
	pMalp-P19_E44A	2	6
	pMalp-IpgF	29	91
	pMalp-IpgF_E42Q	3	7
	pMalp-IagB	10	33
	pMalp-VirB1_AT	7	20
	pMalp-VirB1_BS	5	15
	pMalp-TrbN	27	86
	pMalp-HP0523	21	67

*The conjugation frequencies are represented as number of transconjugants per 100 donor cells.

†Based on the conjugation frequency of cells harbouring wild-type plasmid R1-16 and the vector control pMAL-p2X, which was set to 100 %.

Fig. 1. Zymogram analyses of MalE-P19 and MalE-IpgF proteins. Affinity-purified fusion proteins as indicated were separated by modified SDS-PAGE and renatured in the PG-containing gel as described in Methods. For all proteins, images showing the relevant details of the same gel, produced by staining with either methylene blue (Z) or Coomassie (C), are represented. Arrowheads point to clear, non-stained zones, indicating PG degradation by MalE-P19, MalE-P19_153L, MalE-P19_150G and MalE-IpgF. In contrast, MalE-P19_144G and MalE-IpgF_E42Q are inactive.

appeared in the case of MalE-P19, suggesting a PG-degrading activity which was virtually indistinguishable from the activity seen with the positive control protein MltB (Fig. 2a), the well-characterized membrane-bound LT of *E. coli* (van Asselt *et al.*, 2000). MalE did not produce clear zones (Fig. 2b), unambiguously demonstrating that the activity seen with MalE-P19 was due to the LT part of the fusion protein. Investigation of C-terminally truncated versions of MalE-P19 revealed that both MalE-P19_153L and MalE-P19_150G were able to degrade PG, indicating that amino acid residues between positions 150 and 169 of P19 are not essential for protein folding, substrate recognition and degradation (Fig. 1b, c). In contrast, zymogram analysis of MalE-P19_144G (Fig. 1d) and MalE-P19_140R (data not shown) clearly demonstrated that these C-terminal deletions of the P19 fusion proteins are inactive. These results suggested that these truncated proteins either did not fold correctly or lost the ability to bind to and/or degrade the substrate.

MalE-IpgF also produced cleared zones in the zymogram gels and can therefore be regarded as a PG-degrading

enzyme (Fig. 1e). The catalytic activity of this specialized LT was lost when the conserved glutamyl residue in the presumed active site of this enzyme was changed to glutamine (Fig. 1f). The analysis of the PG-degrading activities of MalE-LT fusion proteins was not only carried out with full-length fusion proteins but also with fusion proteins treated with Factor Xa. This serine protease causes the release of the respective LT from MalE via a specific cleavage site. The tested wild-type forms of the MalE-LT fusion proteins (MalE-P19, MalE-IpgF, MalE-IagB, MalE-VirB1_AT, MalE-VirB1_BS and MalE-HP0523), as shown in Fig. 2(c–h), produced cleared zones in zymogram gels, indicating degradation of polymeric PG. After cleavage with Factor Xa only the released LTs produced cleared zones, whereas the MalE part, as expected, did not show a positive signal.

Interaction of MalE-LT fusion proteins with GroEL

SDS-PAGE of affinity-purified MalE-LT fusion proteins revealed that in all cases except MalE-P19_140R (data not shown), a protein with a molecular mass of approximately 60 kDa co-purified with the MalE-LT fusion protein (arrowhead in Fig. 3b). The protein corresponding to that band was identified by MALDI-TOF analysis of peptide fragments produced by an in-gel tryptic digest. Six prominent peptide fragments were detected which gave an 86.6% match to the *E. coli* chaperonin GroEL (data not shown). The results obtained using peptide mass fingerprinting were confirmed by Western blotting and detection using a commercially available anti-GroEL antibody (Fig. 3c). GroEL did not co-purify with a preparation of MalE alone; thus, GroEL specifically interacted with the LT part of the fusion protein. Several attempts to remove GroEL from MalE-LTs originating from T4SS failed. MalE-P19 turned out to be unstable and precipitated as soon as GroEL was removed. Removal of GroEL was only accomplished in the case of affinity-purified MalE-IpgF (Fig. 3) and MalE-IpgF_E42Q (data not shown) after addition of ATP and Mg^{2+} and subsequent anion-exchange chromatography. In this way, MalE-IpgF and MalE-IpgF_E42Q proteins with an estimated purity of >95% suitable for further enzymic characterizations were obtained.

MalE-IpgF degrades PG in solution

MalE-IpgF, purified as described above, was subjected to an in-solution muramidase assay to demonstrate PG-degrading activity. In this assay purified, radioactively labelled polymeric PG from *E. coli* was used as a substrate. An active muramidase generates soluble fragments that remain in the supernatant after a precipitation step (for details see Methods). As shown in Fig. 4, only purified, GroEL-free, MalE-IpgF degraded PG in a manner comparable to lysozyme, which was used in the same experiment as a positive control. The negative controls MalE and thyroglobulin did not show any PG-degrading activity. When GroEL (1 μ M) was added to the reaction mixture, the activity of MalE-IpgF (3 μ M) decreased by 80%, demonstrating that

Fig. 2. Zymogram analyses of MalE-LT fusion proteins from different secretion systems. The indicated untreated (–) and Factor Xa treated (+) affinity-purified fusion proteins as indicated were separated by modified SDS-PAGE and renatured in the PG-containing gel as described in Methods. For all proteins, images showing the relevant details of the same gel, produced by staining with either methylene blue (Z) or Coomassie (C), are represented. Black and white arrowheads indicate active fusion proteins and active LTs after protease cleavage, respectively. MltB served as a positive control, MalE was used as a negative control.

GroEL not only interacted with IpgF but also inhibited its muralytic activity. In contrast, the enzymic activity of lysozyme was not adversely affected by addition of GroEL (Fig. 4).

A new test system for PG-degrading enzymes: the Cy3-PG spot assay

The in-solution muramidase assay using radiolabelled PG sacculi has been a useful tool over the last 20 years to investigate the activity of murein-degrading enzymes. Nevertheless, it is a time-consuming method, and PG sacculi need to be radioactively labelled *in vivo*. To permit rapid testing for peptidoglycanase activity we developed a new assay which is easier to perform, reliable, and suitable for screening for inhibitors of murein-degrading enzymes. We used the fluorescent dye Cy3 to label purified PG isolated from the Gram-negative bacterium *E. coli*. Labelling of large amounts of sacculi can be performed within 12 h, and storage of the labelled (insoluble) substrate is possible for at least 6 months at -20°C . The labelling protocol is very efficient, so that small amounts of the substrate are sufficient for the assay (for details see Methods). Fluorescence microscopy showed that Cy3-labelled PG

sacculi appear as intensely fluorescing aggregates (Fig. 5a, no enzyme); at higher magnifications single labelled PG sacculi were visible (Fig. 5c). Addition of MalE-IpgF to the buffer resulted in rapid dissolution of the aggregates and diffusion of the breakdown products through the liquid (Fig. 5a); the fluorescing sacculi collapsed and net-like, less well defined irregular structures were observed (Fig. 5c). After 10 min the Cy3-labelled PG aggregates in the area where MalE-IpgF had been pipetted were completely dissolved (Fig. 5b). Incubation of Cy3-labelled PG with buffer alone or with the mutant MalE-IpgF_E42Q did not lead to any visible changes in the appearance of the aggregates (Fig. 5b). The newly developed test system was verified using lysozyme from chicken egg white. As can be seen in Fig. 5(c), lysozyme degraded Cy3-labelled PG in a manner comparable to IpgF. To verify that not only were aggregates dissolved by the muralytic enzymes but specific breakdown products were formed we performed a modified version of FACE (fluorophore-assisted carbohydrate electrophoresis), a method that has been introduced as an alternative to HPLC analysis of PG degradation products (Li *et al.*, 2004). Specific, low-molecular-mass degradation products were seen only when MalE-IpgF was used and not in the case of MalE-IpgF_E42Q (our unpublished data).

Fig. 3. Purification of MalE-IpgF and removal of GroEL. (a) Elution profile of proteins separated by anion-exchange chromatography. Numbers indicate peaks that were analysed by SDS-PAGE (b) and Western blotting (c, d). (b) SDS-PAGE (12.5% polyacrylamide) of selected fractions. AC, 10 μ g affinity-chromatography-purified MalE-IpgF; fraction 2 contains >95% pure MalE-IpgF; fractions 3–5 contain MalE-IpgF and degradation products. (c) Western blot analysis of the indicated fractions using anti-GroEL antibody. A GroEL signal was detected after affinity purification (AC) and in fractions 6–8. No GroEL signal was detected in fractions 1–5, indicating successful removal of GroEL from MalE-IpgF. (d) After detection of the GroEL signal, the membrane was stripped and analysed with anti-MalE antibody. The most intense MalE signals were detected in fractions 2–5. Double bands in fractions AC, 6, 7 and 8 result from incomplete removal of the anti-GroEL antibody during the stripping procedure.

pH optimum and inhibition of MalE-IpgF by known LT inhibitors

In further experiments using a variation of the Cy3-PG spot assay in combination with an array scanner (see Methods) the optimal pH for IpgF and the effects of LT inhibitors were determined (Fig. 6). As shown in Fig. 6(a), optimal activity of IpgF was observed at pH 6, and less activity at pH 5 and pH 7; no activity could be measured at pH 4 and very little at pH 8. We conclude that the pH optimum of IpgF lies at pH 6. The same assay system was used to determine the activities of IpgF in comparison to IpgF_E42Q and lysozyme. As expected, IpgF and lysozyme were active, whereas IpgF_E42Q was not. As observed earlier, GroEL, when added to the substrate together with the enzyme, inhibited IpgF but not lysozyme (data not shown). A similar observation was made with hexa-*N*-acetylchitohexaose,

Fig. 4. Muramidase activity of MalE-IpgF. Degradation of murein was determined by measuring the amount of soluble radiolabelled fragments released after incubation without enzyme (blank), and with MalE, lysozyme, MalE-IpgF or thyroglobin (grey bars). Radioactivity increased at least fivefold in the supernatants derived from lysozyme- and MalE-IpgF-treated murein sacculi compared to the negative controls (blank, MalE- and thyroglobin-treated sacculi), demonstrating muramidase activity of MalE-IpgF. To test the effect of GroEL on the activity of MalE-IpgF, GroEL was added to the reaction mixture (white bars). Addition of GroEL inhibited the activity of MalE-IpgF whereas no inhibitory effect on lysozyme was detectable.

which completely inhibited IpgF at a concentration of 4 mM (Fig. 6b). In contrast, lysozyme was not affected by the presence of *N*-acetylchitohexaose (data not shown), which is in accordance with the observation that this substance is a substrate for lysozyme and solely inhibits LTs (Leung *et al.*, 2001; Song *et al.*, 1994). We also tested bulgecin A, which has been described as an inhibitor of LTs (Templin *et al.*, 1992) and found that a concentration of 20 mM was sufficient to inhibit IpgF (Fig. 6c).

DISCUSSION

In this paper we show for the first time that several selected members of the LT family of proteins, termed specialized LTs (Koraimann, 2003), are indeed capable of degrading PG *in vitro* as suggested by the results of our zymogram analyses. Furthermore, the IpgF protein from *Sh. sonnei*, purified to homogeneity, degraded polymeric radioactively labelled PG in solution, and Cy3-labelled PG in a newly developed Cy3-PG spot assay. We could show by complementation analyses and *in vitro* assays that the conserved glutamyl residue corresponding to the catalytic residue of LTs – position 42 in IpgF – is absolutely necessary for its activity. Since IpgF was inhibited by both bulgecin A and *N*-acetylchitohexaose we conclude that IpgF cleaves the β -1,4 glycosidic bond between the MurNAc and GlcNAc moieties in PG isolated from *E. coli* in a manner characteristic for other well-characterized LTs like Slt70, Slt35 or bacteriophage λ lysozyme (Leung *et al.*, 2001; Thunnissen *et al.*, 1995; van Asselt *et al.*, 2000). In addition to the demonstrated *in vitro* PG-degrading activity of IpgF, this T3SS-encoded LT also cross-complemented a defect in P19, the LT in the conjugation system of the IncF plasmid R1 (a T4SS). We

Fig. 5. Muramidase activity of MalE-IpgF detected by Cy3-PG spot assay. Cy3-labelled PG was bound to poly-L-lysine-covered glass slides as described in Methods. Images were taken using a fluorescence microscope. The PG-degrading activity of MalE-IpgF results in dissolution of PG aggregates and diffusion of breakdown products over the time of incubation. (a) Cy3-PG spot before (no enzyme) and after 2, 4 and 6 min incubation with MalE-IpgF. (b) Degradation of Cy3-PG by wild-type IpgF in comparison to IpgF_E42Q. Images were taken before (no enzyme) and after 10 min incubation with the respective enzyme preparation. (c) Higher magnifications show oval-shaped fluorescing sacculi corresponding to the size of *E. coli* cells. After treatment with IpgF or lysozyme the sacculi collapse to net-like structures indicating degradation of PG. Hexa-*N*-acetylchitohexaose (4 mM) inhibits the muralytic activity of IpgF. Bars represent 1 mm in (a) and (b), and 10 µm in (c).

have proposed earlier that P19 of plasmid R1 interacts with the T4SS apparatus encoded by the conjugative plasmid and that the protein facilitates the efficient assembly of the transport complex in the cellular envelope by local degradation of the cell wall (Bayer *et al.*, 2000, 2001). Our results are not only fully consistent with this view but also suggest that LTs from different secretion systems can fully or partially substitute each other. A similar observation has been made in the case of the VirB1 proteins of *A. tumefaciens* and *B. suis* (Höppner *et al.*, 2004). This intriguingly low level of specificity and the observed genetic redundancy of LT genes could explain the sometimes weak effects of disruptions of a single LT gene in a given secretion system (Koraimann, 2003). IpgF is one of the closest sequence relatives of P19, although it is encoded by a T3SS of *Sh. sonnei*. The sequence identity (43 % in the SLT domain) was sufficient for the restoration of the wild-type conjugation frequency. In contrast to IpgF, IagB did not complement the gene 19 defect *in vivo* (39 % sequence identity in the SLT

domain, Table 3). The differences between the IpgF and IagB proteins could be sufficient to disable complementation by IagB and may hint at important residues necessary for a specific interaction of P19 with its postulated but as-yet-unidentified interaction partner. The fact that VirB1_AT and VirB1_BS did not complement the gene 19 deficiency *in vivo* is probably due to major differences between the VirB1 proteins and P19. VirB1_AT and VirB1_BS both have a C-terminal extension which is only present in the VirB1 class of specialized LTs. In T-DNA transfer of *A. tumefaciens* VirB1_AT is processed and a peptide, VirB1*, representing this C-terminal extension, is secreted into medium or loosely interacts with the bacterial cell surface (Baron *et al.*, 1997). VirB1_AT was shown to interact with several VirB proteins, among them VirB8, which has been proposed to recruit VirB1 to the assembly site for the VirB transporter complex (Ward *et al.*, 2002). Similarly, VirB1_BS has been shown to interact with core components VirB8, VirB9 and VirB11 (Höppner *et al.*, 2005). In complementation assays it was

Fig. 6. pH optimum and inhibition of MalE-IpgF. Quantitative analysis of the Cy3-PG spot assay as described in Methods demonstrated that the pH optimum of the MalE-IpgF fusion protein is at pH 6 (a). The same assay was used to determine the inhibition by known LT inhibitors hexa-*N*-acetylchitohexaose (b) and bulgecin A (c). The enzyme's muralytic activity was calculated (see Methods) and is expressed as arbitrary units. ND, Not detectable. The results of one representative experiment are shown in each panel.

shown that VirB1_BS can complement a VirB1_AT deletion, whereas P19 encoded by the F plasmid (termed Orf169) and HP0523 did not restore wild-type levels of tumour formation and IncQ plasmid transfer (Höppner *et al.*, 2004). Thus, the cross-complementation observed by us and by the Baron laboratory (Höppner *et al.*, 2004) are fully consistent and suggest that specialized LTs are probably late additions to secretion systems and function to replace existing inefficient cellular enzymes designed for PG turnover.

One intriguing observation during our efforts to purify MalE-LT fusion proteins was that they strongly interacted with the chaperone GroEL in the cytoplasm. Since LTs

normally possess signal sequences and are transported into the periplasm like P19 (Bayer *et al.*, 2000), further investigations are currently being performed in this laboratory to elucidate whether the binding of GroEL to the LT is solely an artifact created by overexpression of the fusion proteins with concomitant accumulation of misfolded protein in the cytoplasm or serves a physiological role. Interestingly, the interaction of GroEL with the MalE-IpgF fusion protein also inhibited the PG-degrading activity of this enzyme. Presumably, the LT proteins are unstable or misfolded in the cytoplasm and are recognized by the GroEL/ES complex unless they are transported to the periplasm by the Sec machinery. At least in the case of the 169 aa P19 protein, residues between R140 and G144 (RIYTG) are mainly responsible for the strong interaction with GroEL. Substrate recognition by the GroEL/ES complex is not well defined; the molecular basis of substrate recognition and binding by GroEL is supposed to be the presentation of hydrophobic amino acids on the surface of misfolded proteins (Wang *et al.*, 1999).

It has been recently recognized that PG fragments are specifically recognized by NOD proteins, which have been identified as key constituents of the innate immune system of animals and humans (for reviews see Dziarski, 2003; Girardin & Philpott, 2004; Royet & Reichhart, 2003). Human Nod1 is a cytosolic receptor in epithelial cells and detects a unique diaminopimelate-containing GlcNAc-MurNAc tripeptide found in Gram-negative bacterial PG, resulting in activation of the transcription factor NF- κ B pathway (Girardin *et al.*, 2003). Only very recently has it been found that non-invasive *H. pylori* elicits this innate immune response by delivering bacterial PG fragments into epithelial cells via the *H. pylori* *cag*-PAI (Viala *et al.*, 2004). It is tempting to speculate that in this case HP0523, the specialized LT encoded by the *H. pylori* *cag*-PAI, could produce the PG fragments that are then translocated via the T4SS into epithelial host cells. HP0523 from *H. pylori* was shown here to be capable of degrading PG *in vitro* and it was found earlier to be essential for both CagA translocation and IL-8 induction (Fischer *et al.*, 2001). Future studies will be required to address the question whether HP0523 or other LTs like IpgF can produce PG fragments that are recognized by Nod1 and thus can trigger an innate immune response.

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