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ShlB mutants of *Serratia marcescens* allow uncoupling of activation and secretion of the ShlA hemolysin

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Abstract

The ShlB protein in the outer membrane of *Serratia marcescens* secretes hemolytic ShlA protein into the culture medium. In the absence of ShlB, nonhemolytic ShlA remains in the periplasm. ShlB mutants were isolated in which secretion was uncoupled from activation. Mutants with a tetrapeptide insertion after residues 136 or 224 of mature ShlB and a mutant with an insertion after residue 154 and a deletion secreted inactive ShlA. In vitro, secreted nonhemolytic ShlA was converted into hemolytic ShlA by isolated wild-type ShlB and by complementation with an N-terminal ShlA fragment of 255 residues (ShlA-255). The isolation of secretion-competent, but activation-negative mutants indicates that secretion alone is not sufficient for activation of ShlA. Rather, ShlB is required for activation and secretion, and the mutants define sites in ShlB which are involved in activation. According to a predicted transmembrane model of ShlB, the mutations that retain secretion competence but abolish activation competence are located in the most prominent surface loop and the following transmembrane loop. In one tetrapeptide insertion mutant, ShlB-332, most of the ShlA remained cell-associated in an inactive form and low amounts (6 %) were hemolytic. Secreted inactive ShlA° was completely degraded by trypsin, in contrast to hemolytic ShlA, which was cleaved into two fragments of 60 and 100 kDa. This result indicates that the conformational change from a highly trypsin-sensitive to a highly trypsin-resistant protein with only a single cleavage site in a polypeptide of 1578 residues occurs upon activation of ShlA and not during secretion.

Key words: *Serratia marcescens* – hemolysin – activation – secretion – mutants – conformation

Introduction

Serratia marcescens secretes a hemolysin that also acts as a cytotoxin (Braun et al., 1985, 1992, 1993; Braun and Hertle, 1999; Hertle et al., 1999; König et al., 1987; Marre et al., 1989; Schönherr et al., 1994). The hemolytic activity is determined by the *shlA* and *shlB* genes (Braun et al., 1987; Poole et al., 1988). The ShlA and ShlB proteins are synthesized with signal peptides which are no longer present in the mature proteins. Only ShlB is required for the secretion of ShlA across the outer membrane (Schiebel et al., 1989; Sieben et al., 1998), which distinguishes this type of secretion system from all other known secretion systems (Braun and Hertle, 1999; Pugsley, 1993). Secretion of a hemolysin by a single protein is not confined to ShlA since the hemolysins of *Proteus mirabilis* (Uphoff and Welch, 1990; Welch, 1991), *Haemophilus ducrey* (Palmer and Munson, 1995), and *Edwardsiella tarda* (Chen et al., 1996; Hirono et al., 1997, 1998) are also

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determined by two proteins, which have strong sequence similarities to ShlA and ShlB. In addition, the FHA filamentous hemagglutinin of *Bordetella pertussis* (Guedin et al., 1998; Jacob-Dubuisson et al., 1996; Willems et al., 1994) and the HMWP1 and HMWP2 adhesins of nontypable *Haemophilus influenzae* (St. Geme and Grass, 1998) require a B-component for secretion to the cell surface. These latter proteins share sequence similarities with a region of ShlA in which point mutations lead to an inactive hemolysin (Schönherr et al., 1993); point mutations in the corresponding region of FHA (Jacob-Dubuisson et al., 1997) and HMW1 (Grass and St. Geme, 2000) also lead to an inactive protein. The most advanced studies have been carried out with the *S. marcescens* hemolysin and serve as a paradigm for the secretion and activation of the related hemolysins and may have relevance for the secretion of FHA and HMWPs.

In addition to the secretion function, ShlB converts the ShlA protein into a hemolysin. In the absence of ShlB, nonhemolytic ShlA (termed ShlA*) remains in the periplasm and displays at most 0.2 % of the ShlB-dependent and secreted hemolytic activity (Schiebel et al., 1989).

In in vitro experiments, activation of ShlA* can be uncoupled from secretion of ShlA. Crude extracts of cells that synthesize only ShlB activate ShlA* contained in crude extracts of cells that synthesize only ShlA* (Hertle et al., 1997). Highly purified ShlB does not activate highly purified ShlA* unless phosphatidylethanolamine (PE) is added. Of the outer membrane phospholipids, only PE serves as a co-substrate of ShlA* activation. Phospholipase 2, which releases the fatty acid at the C2 position of the glycerol moiety, inactivates ShlA. ShlA* can also be converted to a hemolysin by addition of an N-terminal fragment of ShlA containing 255 residues (ShlA-255). In order to complement ShlA*, ShlA-255 has to be activated and secreted by ShlB. ShlA becomes inactive upon removal of ShlA-255 (Ondraczek et al., 1992). ShlA-255 itself is nonhemolytic because it lacks the C-terminal sequence of complete ShlA (1576 residues), which is required for pore formation in erythrocyte membranes (Schiebel and Braun, 1989; Schönherr et al., 1994). ShlA-255 also restores the hemolytic activity of the missense proteins ShlA68.69(AN→GI) and ShlA109-(N→I), and of the deletion proteins ShlAΔ4–69, ShlAΔ68–97, and ShlAΔ99–117 (Schönherr et al., 1993).

In this paper, tetrapeptide insertions at 12 sites along the ShlB polypeptide (mature form consists of 539 residues) were analyzed. Three ShlB mutants secreted inactive ShlA protein (designated ShlA°). A fourth mutant displayed a very low secretion activity, but contained some cell-bound hemolytic activity.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The strains and plasmids used are listed in Table 1. Plasmids pSHL3Δ, pES14, pSH7, and pRO2 have been described previously (Könninger, 1998; Ondraczek et al., 1992; Poole et al., 1988). Plasmids pTAB136, pTAB153, pTAB153(Δ154–252), pTAB177, pTAB220, pTAB224, pTAB268, pTAB332, pTAB362, pTAB368, pTAB437, and pTAB448 were constructed by TAB (*t*wo-*a*mino-acids-*B*arany) linker insertion mutagenesis of plasmid pSH7 *shlB shlA*(Δ130–1578) with plasmid pKINN/NarI (Barany, 1988), as described (Könninger et al., 1999). pSH7 was cleaved with NruI and ligated with pUC4KINN, and the kanamycin resistance cassette was excised with BglII.

Plasmid pTB136 was obtained by excision of the AocI-MluI fragment (1.1 kb) of pTAB136 (5.6 kb) and ligation with the AocI-MluI fragment (3.9 kb) of pSHL3Δ *shlB shlA* (Δ4–1578). The other pTM plasmids were constructed in the same way, except for pTB437 and pTB448, where MluI-BsmI fragments were used.

Plasmid pTM136 was obtained by excision of the AocI-EcoRI fragment (1.9 kb) of pTAB136 (5.6 kb) and ligation with the AocI-EcoRI fragment (7.7 kb) of pES14 *shlB shlA*. The other pTM plasmids were constructed in the same way, except for pTM437 and pTM448, where EcoRI-BsmI fragments were used.

Standard genetic procedures such as cleavage with restriction enzymes, ligation, transformation (Pope and Kent, 1996), selection for inserts, and determination of fragments and their sites by agarose gel electrophoresis were performed as described (Sambrook et al., 1989). Plasmids were isolated by the method of TENS (Zhou et al., 1990) or with columns (Qiagen, Hilden, Germany).

E. coli cells were grown aerobically in tryptone yeast extract (TY) medium (0.8 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, pH 7.0) at 37 °C (Miller, 1972). Ampicillin (100 μg/ml) and chloramphenicol (40 μ/ml) were added to the liquid media or nutrient agar plates to maintain the plasmids.

Transcription of the *shlA* and *shlB* genes by the phage T7 RNA polymerase

The *shlA* and *shlB* genes were inserted downstream of the T7 gene 10 promoter in plasmids pT7-5 and pT7-6, which contain polylinkers in opposite orientations (Tabor and Richardson, 1985). *shlA* and *shlB* were transcribed by the T7 RNA polymerase encoded under *lac* control on the lysogenic λ phage of *E. coli* BL21(DE3) (Studier and Moffat, 1986). In addition, *E. coli* BL21(DE3) contained pLysS, which encodes the T7 lysozyme that inactivates small amounts of the T7 RNA polymerase. Cells were grown in TY medium containing ampicillin or chloramphenicol at 37 °C to an OD_{578} of 0.4. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added (1 mM final concentration) to induce synthesis of T7 RNA polymerase, and incubation was continued for 2 h.

Isolation of ShlB and ShlB mutant proteins

Cells of a 150-ml IPTG-induced culture of *E. coli* BL21-(DE3) harboring pES14 *shlA shlB* or pUK0 *shlB* or pTB

Table 1. Strains of *E. coli* and plasmids used.

Strains/ plasmids	Genotype	Source or reference
E. coli		
BL21(DE3)	F$^-$, *hsds gal,* lysogenic for λDE3 pLysS carrying phage T7 RNA polymerase under *lac*UV5 control	Studier and Moffat, 1986
C9	*phoR18 relA fadL pit-10 t onA22 spoT1,* HfrC	Garen and Garen, 1963
Plasmids		
pBluescriptSK$^+$	pColE1 *ori* Amp^R, high copy number	Stratagene
pT7-5	pColE1 *ori*, Amp^R	Tabor and Richardson 1985
pT7-6	Like pT7-5, but polylinker reversed	Tabor and Richardson 1985
pES14	pT7-5 *shlB shlA*	Schiebel et al., 1989
pSH7	pBluescript SK$^+$ *shlB shlA* (Δ139–1578)[a]	Hobbie, 1993
pSHL3Δ	pT7-6 *shlB shlA*(Δ4–1578)	Ondraczek et al., 1992
pRO2	pT7-6 *shlB shlA*(Δ256–1578)	Schönherr et al., 1994
pTM136	pT7-5 *shlB136*(ARSG)[b] *shlA*	this work
pTM153	pT7-5 *shlB153*(PDLA) *shlA*	this work
pTM154	pT7-5 *shlB153* (PDLA, Δ154–252) *shlA*	this work
pTM177	pT7-5 *shlB177*(PDLA) *shlA*	this work
pTM224	pT7-5 *shlB224*(PDLA) *shlA*	this work
pTM268	pT7-5 *shlB268*(ARSG) *shlA*	this work
pTM332	pT7-5 *shlB332*(ARSG) *shlA*	this work
pTM362	pT7-5 *shlB362*(ARSG) *shlA*	this work
pTM368	pT7-5 *shlB368*(ARSG) *shlA*	this work
pTM437	pT7-5 *shlB437*(ARSG) *shlA*	this work
pTM448	pT7-5 *shlB448*(ARSG) *shlA*	this work
pTB136	pT7-6 *shlB136*(ARSG)[b] *shlA* (Δ4–1578)	this work
pTB153	pT7-6 *shlB153*(PDLA) *shlA* (Δ4–1578)	this work
pTB154	pT7-6 *shlB153*(PDLA, Δ154–252) *shlA*(Δ4–1578)	this work
pTB177	pT7-6 *shlB177*(PDLA) *shlA* (Δ4–1578)	this work
pTB224	pT7-6 *shlB224*(PDLA) *shlA* (Δ4–1578)	this work
pTB268	pT7-6 *shlB268*(ARSG) *shlA* (Δ4–1578)	this work
pTB332	pT7-6 *shlB332*(ARSG) *shlA* (Δ4–1578)	this work
pTB362	pT7-6 *shlB362*(ARSG) *shlA* (Δ4–1578)	this work
pTB368	pT7-6 *shlB368*(ARSG) *shlA* (Δ4–1578)	this work
pTB437	pT7-6 *shlB437*(ARSG) *shlA* (Δ4–1578)	this work
pTB448	pT7-6 *shlB448*(ARSG) *shlA* (Δ4–1578)	this work
pTAB136	pSH7 *shlB136*(ARSG)	[c]
pTAB153	pSH7 *shlB153*(PDLA)	
pTAB154	pSH7 *shlB153*(PDLA Δ154–252)	
pTAB177	pSH7 *shlB*177(PDLA)	
pTAB220	pSH7 *shlB220*(ARSG)	
pTAB224	pSH7 *shlB224*(PDLA)	
pTAB268	pSH7 *shlB268*(ARSG)	
pTAB332	pSH7 *shlB332*(ARSG)	
pTAB362	pSH7 *shlB362*(ARSG)	
pTAB368	pSH7 *shlB368*(ARSG)	
pTAB437	pSH7 *shlB437*(ARSG)	
pTAB448	pSH7 *shlB448*(ARSG)	

[a] In the *shlA* mutant, the encoded ShlA protein lacks residues 139 to 1578.
[b] The ShlB protein encoded by the mutated *shlB* carries an ARSG insertion after residue 136. The same nomenclature is used for all *shlA* and *shlB* mutant genes and the corresponding mutant proteins.
[c] The pTAB series is from Hobbie, 1993.

plasmids were harvested by centrifugation (6500 × g, 10 min, 4 °C). The membrane fraction was prepared (Könninger et al., 1999) and treated sequentially with *i*) 2 ml 0.2 M Tris-HCl, pH 8.0 at 4 °C, *ii*) 4 ml 0.2 M Tris-HCl, pH 8.0, 1 M sucrose, 400 µl 10 mM EDTA, pH 8.0, 400 µl lysozyme (2 mg/ml), and 12 ml distilled water. After 15 min incubation at 4 °C, 20 ml extraction buffer (2 % Triton X-100, 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$) and 400 µl DNaseI (1 mg/ml) were added. The extraction was followed by centrifugation at 40 000 × *g* for 30 min at 4 °C. The pellet was suspended in 10 ml distilled water and again centrifuged at 43 000 × *g* for 15 min at 4 °C. Triton X-100 was removed by repeating the procedure twice. Then the pellet was suspended in 1 % octylglucoside, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA for 30 min at 4 °C, and the solution was centrifuged at 13 500 × *g* for 1 h at 4 °C.

Isolation of ShlA, ShlA*, and ShlA-255

Cells of a 150-ml IPTG-induced culture of *E. coli* BL21(DE3) (pES14 *shlA shlB*) were sedimented by centrifugation (6500 × *g*, 10 min, 4 °C). Urea was added to a final concentration of 6 M to the supernatant, and ShlA was precipitated with ammonium sulfate (final concentration 55 %). The solution was centrifuged at 15 000 × *g* for 30 min at 4 °C. The pellets were suspended in urea buffer composed of 20 mM HEPES, 6 M urea, pH 6.0. ShlA-255 was precipitated from the culture supernatant of *E. coli* BL21(DE3) (pRO2) without prior addition of urea. For solubilization of ShlA*, the pellet from an IPTG-induced 150-ml culture of *E. coli* BL21(DE3) (pES15) was suspended in 2 ml 20 mM HEPES, 6 M urea, pH 4.0 at 4 °C and sonicated twice for 1 min. The suspension was left at 4 °C overnight and was then centrifuged for 20 min at 60 000 × *g*.

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), and immunoblotting

For SDS-PAGE (Lugtenberg et al., 1975), 9 % or 11 % (w/v) acrylamide was used in the running gel. For Western immunoblotting, the proteins after SDS-PAGE were transferred to a nitrocellulose membrane (0.45 μm) with blotting buffer (3.0 g Tris, 1.0 g SDS, 14.4 g glycine, 200 ml methanol/liter). After transfer of proteins, the nitrocellulose membrane was incubated with 3 % bovine serum albumin in TNT buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.01 % Tween 20) for 1 h, then washed with the same buffer three times for 10 min. Then the membrane was incubated with 1000-fold diluted antibody solution for 1 h and washed as above. Anti-rabbit IgG antibody labeled with alkaline phosphatase was diluted 5000-fold in TNT buffer and incubated for 1 h with the membranes. Nitroblue tetrazolium chloride (80 μl, 50 mg/ml in dimethyl-formamide) and bromo-4-chloro-indolylphosphate (50 μl, 40 mg/ml in water) was used in 10 ml AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) to determine alkaline phosphatase activity.

Hemolysis assays

Outdated human blood stored for approximately four weeks was obtained from the Blood Center of the University of Tübingen. Samples were incubated with an erythrocyte suspension (8 %, v/v) in 0.9 % NaCl for 10 min at room temperature and then centrifuged for 5 s at 13 500 × *g* in an Eppendorf centrifuge (Bernheimer and Rudy, 1986). Released hemoglobin in the supernatant was diluted 100-fold with distilled water and measured spectrophotometrically at 405 nm. The hemolysin concentration causing release of 50 % of total hemoglobin was defined as 1 hemolytic unit (1 HU), from which the hemolytic units in the undiluted samples were calculated. Complete hemolysis of erythrocyte suspensions was achieved by adding 5 % Triton X-100.

For the blood-agar plate test, bacteria were incubated overnight at 37 °C on a TY agar plate supplemented with the required antibiotics. Then, a 10 % erythrocyte suspension was poured over the plates, the plates were incubated at 4 °C for 20 h, and the hemolysis zones were inspected around the bacterial colonies.

In vitro activation of ShlA* and ShlA derivatives by ShlB, and complementation by ShlA-255 were carried out as described previously (Ondraczek et al., 1992). Equal volumes of ShlA* (10 μl) and ShlB, purified as described above, were mixed and incubated for 10 min. In the complementation experiments, ShlA* lysates (10 μl) were mixed with ShlA-255 (10 μl). After 3 min of incubation at room temperature, 200 μl of erythrocyte suspension (8 %, v/v) was added and hemolysis was measured after incubation at room temperature for 10 min.

The hemolytic activity in culture supernatants was determined by growing 50-ml cultures of *E. coli* BL21(DE3) transformed with one of the plasmids to be tested to an OD_{578} of 0.4. Then IPTG (1 mM final concentration) was added, and samples of 1 ml were taken at intervals of 30 min. After centrifugation, 6 M urea was added to the supernatants and hemolysis was determined as described above.

For the determination of cell-associated hemolysin, the sedimented cells were suspended in 20 μl lysis mix (1 mg lysozyme/ml, 2 mM EDTA, pH 8), the mixture was vortexed three times for 10 s, 20 μl urea buffer was added, and the mixture was incubated for 1 h at 4 °C. Undissolved material was pelleted at 13 500 × *g* for 30 min. Samples (10 μl) of the supernatant were added to 200 μl of 8 % human erythrocytes to measure hemolysis.

Treatment of inactive and active ShlA proteins with trypsin

The supernatant fraction of 200-ml cultures of *E. coli* BL21(DE3) transformed with one of the plasmids to be tested was adjusted to 55 % ammonium sulfate, and the precipitate was dissolved in 2 ml 6 M urea buffer. Samples of 15 μl were digested with 5 μl of trypsin (1 mg/ml; Serva, Heidelberg, Germany) for 30 min at 37 °C and then treated with 10 μl of trypsin inhibitor (2 mg/ml) for 30 min at 37 °C. Sample buffer (15 μl) (Lugtenberg et al., 1975) was added to 15 μl trypsin-treated probes, the mixture was boiled for 5 min, and 15 μl was analyzed by SDS-PAGE and immunoblotting.

Determination of alkaline phosphatase activity

Bacteria were grown in 50 ml of M9 medium supplemented with 0.3 % vitamin assay casamino acids, 0.1 mg ampicillin/ml at 37 °C to an OD_{578} of 0.2. Samples of 1 ml bacterial culture were centrifuged at 13 500 × *g* for 5 min, the supernatant was incubated with 0.1 ml *p*-nitrophenylphosphate (30 mg/ml, 1 M Tris-HCl, pH 8.8) for 1 h at 37 °C, and the absorbance was read at 405 nm. Cells in the sediment were lysed with 20 μl lysis mix (1 mg lysozyme/ml, 2 mM EDTA, pH 8) and 10 μl 0.1 % SDS and vortexed three times for 10 s. Samples of 1 to 5 μl were incubated with 1 ml M9 medium and 0.1 ml *p*-nitrophenylphosphate for 1 h at 37 °C and centrifuged for 5 min at 13 500 × *g*, and the absorbance of the supernatant was read at 405 nm.

Results

shlB mutants that secrete inactive ShlA (termed ShlA°)

The experiments were performed with *E. coli* BL21 (DE3) transformed with the *shlA* and *shlB* genes; this strain displays hemolytic properties that cannot be distinguished from *S. marcescens* hemolysis (Braun et al., 1987; Poole et al., 1988).Wild-type *shlB* and mutant *shlB* genes were each cloned separately and together with wild-type *shlA* under the control of the phage T7 gene 10 promoter on the medium-copy-number plasmids pT7-5 and pT7-6 and transcribed by the T7 RNA polymerase encoded on the lysogenic λ phage. For the determination of hemolytic activities in liquid cultures, transcription of the *shlA shlB* genes occurred after induction of the RNA polymerase gene transcription with 1 mM IPTG. Secretion of hemolytic ShlA was tested by measuring hemolysis zones around colonies on blood agar plates and by lysis of erythrocytes in liquid culture. To prevent aggregation and sedimentation

of secreted ShlA and ShlA°, 6 M urea was added to the culture supernatants; ShlA is soluble and stable under these conditions (Ondraczek et al., 1992). Secretion of inactive ShlA° into the culture medium and the presence of inactive ShlA* in the periplasm were tested by complementation with isolated ShlA-255 from the culture medium of *E. coli* BL21(DE3) (pRO2 *shlA' shlB*) (Ondraczek et al., 1992).

shlB was mutated by insertion of tetrapeptides using TAB linkers as employed by us previously for *shlB* insertion mutagenesis (Könninger et al., 1999). The mutated *shlB* genes had to be co-expressed with the *shlA* gene on the same medium-copy-number plasmid in the order *shlB shlA* – the same order as on the chromosome in *S. marcescens* – to find mutants that secreted ShlA°. Expression of shlA and *shlB* cloned on high-copy-number plasmids damaged cells to an extent that secretion was strongly reduced. Synthesis of the ShlA and ShlB proteins encoded on distinct plasmids in amounts that differed from the wild-type stoichiometry also reduced secretion. Neither condition was suitable to screen for *shlB* missense mutants that secreted ShlA° or that retained active ShlA in the periplasm.

Twelve *shlB* insertion mutants were studied which showed hemolytic activities in the culture supernatants ranging from 0 to 45 % of that of the wild-type *shlB* transformant (Table 2). More of the residual hemolytic activity remained associated with the cells of all *shlB* mutants than was secreted into the culture medium, whereas more of the hemolysin produced by the *shlB* wild-type transformant was secreted than remained cell-associated (Table 2).

E. coli BL21(DE3) (pTM136) encodes an ShlB protein that contains the peptide ARSG inserted after residue 136 of the mature form (Table 1). No hemolytic activity was found in the culture supernatant. An octylglucoside-EDTA extract from the outer membrane of *E. coli* BL21(DE3) harboring pUKO *shlB* was added to the culture supernatant of *E. coli* BL21(DE3) (pTM136) to see whether *E. coli* BL21(DE3) (pTM136) secreted inactive ShlA that could be activated by wild-type ShlB. Hemolytic activity was obtained that amounted to 37 % of the activity obtained when isolated periplasmic ShlA* synthesized by *shlA* transformants lacking *shlB* was treated under identical conditions with isolated wild-type ShlB (Table 3). This experiment showed that the pTM136 transformant secreted inactive ShlA which was termed ShlA°. This conclusion was supported by complementation with ShlA-255, which resulted in a much higher hemolytic activity than activation with wild-type ShlB (Table 3). Similar results were obtained by complementation of ShlA* with ShlA-255 (Table 3). The lower activities obtained with ShlA° as compared to ShlA* may be caused by the lower amounts of secreted ShlA° in the culture supernatant of *E. coli* BL21(DE3) (pTM136) (see later), as compared to *E. coli* BL21(DE3) (pES14), which secretes active ShlA. Similar results were obtained (Table 3) with *E. coli* BL21 (DE3) transformed with plasmid pTM224, which encodes an ShlB protein with a tetrapeptide insertion (Table 1), and with plasmid pTM154 (Table 3), which determines an ShlB derivative with an insertion and a deletion (Table 1). The mutated ShlB proteins of the three strains secreted nonhemolytic ShlA°, which could be activated in vitro by wild-type ShlB and complemented by ShlA-255. Secretion kinetics of ShlA° by the three transformants with no hemolytic activity in the culture supernatant is shown in Fig. 1.

Table 2. Hemolytic activities of *E. coli* BL21(DE3) transformed with plasmids carrying *shlA* and mutant *shlB* genes.

Plasmids	Secreted hemolytic activity[a]	Cell-associated hemolytic activity[b]
pES14 (wild-type)	201	171
pTM136	0	0
pTM153	41	63
pTM154	0	0
pTM177	2	7
pTM220	3	10
pTM224	0	0
pTM268	3	43
pTM332	0	5
pTM362	4	23
pTM368	39	74
pTM437	90	124
pTM448	37	67

[a] The values represent hemolytic units in 1 ml culture supernatant. Urea (6 M) was added to the conditioned media prior to the determination of the hemolytic activity.

[b] The values represent hemolytic units in the cell pellet of a 1-ml culture. Urea (6 M) was used for solubilization of the cell-associated hemolytic activities prior to the determination of the hemolytic activity.

Table 3. In vitro activation of nonhemolytic ShlA° secreted by ShlB insertion mutants.

ShlA° secreted by	Hemolytic units after activation with wild-type ShlB	Hemolytic units after complementation with ShlA-255
ShlB136	0.75	53
ShlB154	0.85	64
ShlB224	0.80	70
(ShlA*)[a]	2.05	160

ShlB was extracted from isolated outer membranes with 1 % octylglucoside, ShlA° and ShlA-255 were precipitated with ammonium sulfate from the conditioned medium.

[a] Experiment performed with periplasmic ShlA* isolated from cells that did not synthesize ShlB.

In addition to wild-type ShlA we also determined secretion and activation of three *shlA* missense mutants that synthesized ShlA proteins with 7- to 20-fold higher hemolytic activities in the culture supernatant than wild-type ShlA (Hilger and Braun, 1995). The superhemolytic ShlA derivatives were less prone to aggregation and for this reason showed a higher hemolytic activity. Under the conditions used in this paper the mutant ShlA proteins displayed 2- to 6-fold higher activities when cosynthesized with wild-type ShlB but no activity when cosynthesized with ShlB136 (data not shown).

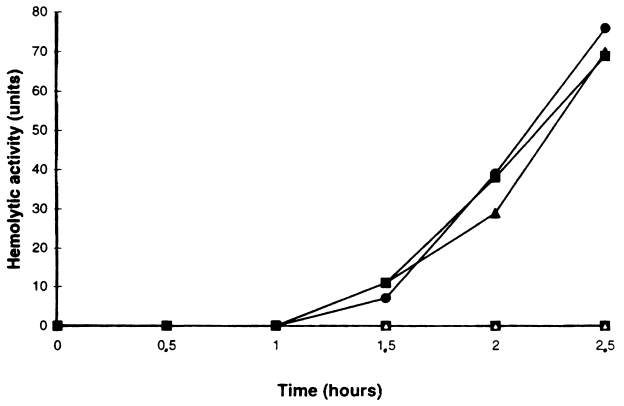

Fig. 1. Time course of the secretion of nonhemolytic ShlA° by *E. coli* BL21(DE3) transformed with plasmids pTM136 (■,□), pTM154 (▲,△), and pTM224 (●,○). The filled symbols represent the hemolytic values after complementation with ShlA-255, the open symbols without complementation. Transcription of the mutant *shlb* genes and wild-type *shlA* was induced by addition of 1 mM IPTG at time zero. Samples of 1 ml were taken at 30-min intervals, cells were precipitated by centrifugation, and urea was added to the supernatant fractions to a concentration of 6 M. Samples of 10 μl were mixed with 10 μl of ShlA-255 and 0.2 ml of 8% human erythrocytes, and the released hemoglobin was measured at 405 nm after 10 min incubation at 20°C.

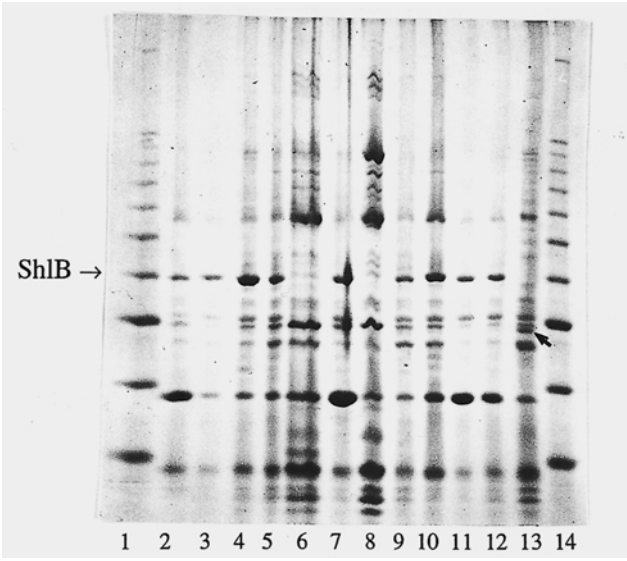

Fig. 2. SDS-PAGE of mutant ShlB proteins ShlB136 (lane 2), ShlB153 (lane 3), ShlB177 (lane 4), ShlB220 (lane 5), ShlB224 (lane 6), ShlB268 (lane 7), ShlB332 (lane 8), ShlB362 (lane 9), ShlB368 (lane 10), ShlB437 (lane 11), ShlB448 (lane 12), and ShlB154 (lane 13) isolated from the outer membrane fractions of *E. coli* BL21(DE3) transformed with the plasmids of the pTB series listed in Table 1. Lanes 1 and 14 contain 10-kDa molecular mass markers starting at the bottom with 30 kDa. The gel was stained with Serva blue.

Amounts of mutant ShlB proteins in the outer membrane

Since very low amounts of ShlB could be sufficient to secrete ShlA but insufficient to activate ShlA, outer membranes containing the mutant ShlB proteins were subjected to SDS-PAGE. The outer membranes were prepared from cells grown under the same conditions as used for the hemolysis assays. Although the amounts of mutant ShlB proteins varied they did not quantitatively correlate with the secreted hemolytic activities and the hemolytic activities obtained after activation with wild-type ShlB or complementation with ShlA-255. For example, the pTM136 and pTM154 transformants with no hemolytic activities in the conditioned medium contained similar amounts of ShlB protein (Fig. 2, lanes 2, 3) as the pTM437 transformant (lane 11) with a high hemolytic activity. The pTM177 transformant with very low residual hemolytic activity (Table 2) contained high amounts of ShlB (lane 4). Despite the only tiny ShlB band derived from the outer membrane of *E. coli* BL21 (DE3) pTM224 (Fig. 2, lane 6) the amount of secreted ShlA° in the conditioned medium was rather high as evidenced by activation with wild-type ShlB and complementation by ShlA-255 (Table 3). The same result was obtained with *E. coli* BL21 (DE3) pTM332 (Fig. 2, lane 8) which contained residual amounts of cell-associated hemolysin (Table 2) and gave rise to 36 hemolytic units after complementation of ShlA° with ShlA-255 (not listed in Table 3). In *E. coli* BL21 (DE3) pTM154 that synthesized the ShlB154 deletion protein SDS-PAGE identified a ShlB protein with a higher electrophoretic mobility than the ShlB insertion mutants (Fig. 2, lane 13, marked by an arrow). The position of the band corresponding to 49 kDa was close to the calculated molecular weight of 50 kDa. ShlB154 secreted ShlA° despite of the large deletion of 99 residues and the insertion of 4 heterologous residues (Table 3).

Identification of secreted inactive ShlA°

To examine the ShlA° proteins secreted by the nonhemolytic *shlB* mutants and to compare them with the ShlA proteins of the hemolytic *shlB* mutants we performed an analysis by Western blotting. The strongest

bands were detected with transformants containing pTM368 and pTM437 (Fig. 3, lanes 13 and 14) which synthesized rather high amounts of secreted and cell-associated hemolysin (Table 2). These amounts were similar in strength to that of the wild-type *shlB* transformant (lane 1). Weaker signals were detected with cells carrying plasmids pTM136 (lane 3), pTM154 (lane 4), pTM224 (lane 8), and pTM332 (lane 10). In the latter transformant, no secreted hemolysin but some cell-associated hemolysin was observed (Table 2). For this reason, we also present the immunoblot of the cell-associated ShlA332 protein which is much stronger than that of the released ShlA protein (lane 11). The immunoblot confirms the secretion of ShlA° in the cases where no hemolytic activity is observed in the conditioned medium.

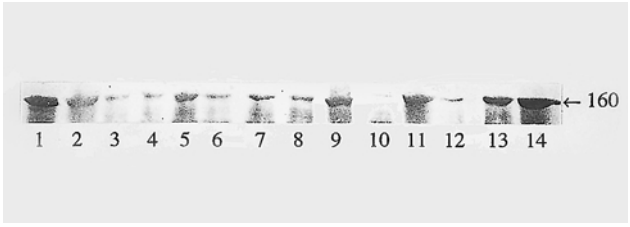

Fig. 3. Immunoblot of the 160-kDa area showing ShlA, ShlA°, or ShlA* after SDS-PAGE of culture supernatants of *E. coli* BL21(DE3) transformed with plasmid pES14 *shlA shlB* (lane 1), pES15 *shlA* (lane 2, solubilized from cells), TM136 (lane 3), pTM154 (lane 4), pTM153 (lane 5), pTM177 (lane 6), pTM220 (lane 7), pTM224 (lane 8), pTM268 (lane 9), pTM332 (lane 10), pTM332 (lane 11, cell-associated ShlA*), pTM362 (lane 12), pTM368 (lane 13), or pTM437 (lane 14). After SDS-PAGE (9% polyacrylamide), the proteins were blotted onto a nitrocellulose membrane and incubated with polyclonal rabbit anti-ShlA antibodies and anti-rabbit IgG alkaline phosphatase conjugate. The proteins in the supernatant were precipitated with ammonium sulfate, dissolved in sample buffer, and applied onto the gel.

ShlB mutant that retains cell-associated active ShlA

E. coli BL21(DE3) pTM332, which encodes ShlB with an insertion of ARSG after residue 332, secreted no hemolytic ShlA into the culture medium (Table 2). In contrast to the three *shlB* mutants described above, the pTM332 transformant released after 1 h induction with IPTG only small amounts of ShlA° (2 units after complementation with ShlA-255) and contained a low cell-associated hemolytic activity (5 units). Most of the ShlA protein was periplasmic ShlA* (80 units after complementation with ShlA-255). Thus ShlB332 differs from ShlB136, ShlB154, and ShlB224 in that it shows residual activation capacity for the ShlA protein that seems to be higher than the secretion capacity.

Alkaline phosphatase is not released by the ShlB mutants

Incorporation of mutated ShlB proteins could damage the outer membrane, resulting in release of ShlA° by leakage as opposed to secretion by the ShlB derivatives. To examine whether ShlA° is released by leakage, we determined the activity of periplasmic alkaline phosphatase in the culture supernatant of *E. coli* C9 transformed with plasmid pTM136, pTM154, or pTM224. Untransformed *E. coli* C9 and *E. coli* C9 transformed with the vector pT7-5 served as controls. *E. coli* C9 is mutated in the repressor gene *phoR* and synthesizes alkaline phosphatase constitutively. During a 7-h period, the enzyme activity was determined every hour and remained constant. The activity released into the conditioned medium amounted to 10–15% of the total activity (sum of cell-bound and released activity). The control samples contained between 5 and 10% of the alkaline phosphatase in the supernatant. Although the ShlB mutant transformants released on average 5% more alkaline phosphatase than the control cells, leakage cannot account for the high amounts of ShlA° in the conditioned media of the *shlB* mutants.

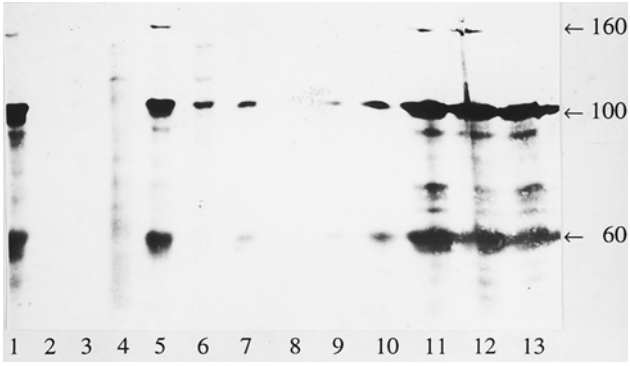

Fig. 4. Anti-ShlA immunoblot after trypsin digestion of the proteins in the culture supernatants of *E. coli* BL21(DE3) transformed with plasmid pES14 *shlA shlB* (lane 1), pES15 *shlA* (lane 2, solubilized from cells), TM136 (lane 3), pTM154 (lane 4), pTM153 (lane 5), pTM 177 (lane 6), pTM220 (lane 7), pTM224 (lane 8), pTM268 (lane 9), pTM362 (lane 10), pTM368 (lane 11), pTM437 (lane 12), or pTM448 (lane 13). The numbers on the right indicate the molecular masses of standard proteins in kDa.

ShlA° is completely degraded by trypsin

To examine whether secreted nonhemolytic ShlA° differs structurally from secreted hemolytic ShlA, these proteins were precipitated from the culture supernatant of *E. coli* BL21(DE3) transformed with one of a number of plasmids that encoded *shlA* and a mutated *shlB* gene. ShlA° secreted by ShlB136 (Fig. 4, lane 3), ShlB154 (lane 4), and ShlB224 (lane 8) was completely degraded by trypsin, in contrast to ShlA (160 kDa), which was cleaved into two fragments of 60 and 100 kDa (lane 1). All other ShlB insertion mutants which secreted different amounts of hemolytic ShlA yielded

60- and 100-kDa ShlA trypsin degradation products (Fig. 4, lanes 5–7, 9–13). Inactive periplasmic ShlA* was completely degraded (lane 2). In the absence of trypsin none of the proteins was cleaved (data not shown).

Discussion

Release of hemolytic ShlA into the culture medium is unique since *i*) it is catalyzed by a single protein (ShlB) which resides in the outer membrane, *ii*) ShlA is converted from a nonhemolytic periplasmic form into a hemolytic secreted form, and *iii*) activation plus the maintenance of the activity specifically require phosphatidylethanolamine. In this paper, we demonstrate that secretion of ShlA in vivo can be uncoupled from activation by insertion of heterologous tetrapeptides into ShlB and by deletion of 99 amino acids of ShlB. The four mutants studied in detail – ShlB136, ShlB154, ShlB177, and ShlB224 – released virtually no hemolytic ShlA into the culture medium and contained no or very little cell-associated hemolytic activity. However, immunoblotting with polyclonal anti-ShlA serum revealed ShlA° in the culture medium, and the complementation assay with ShlA-255 resulted in hemolytic ShlA with an average 39 % of the complementation activity of ShlA* remaining in the periplasm of an *shlB* deletion mutant. The results obtained with these mutants demonstrate that secretion is less affected by ShlB mutations than activation. In fact, nearly all of the 12 ShlB insertion mutants secreted the ShlA protein, although to different extents. This conclusion is further corroborated by results obtained with ShlB mutants that contained 13 amino acids after residue 104 or 350 (Könninger et al., 1999) and secreted ShlA° (data not shown). Furthermore, the deletion mutant ShlB154 (PDLA, Δ154–252) and the previously isolated deletion mutants ShlBΔ65–186, ShlBΔ137–186, and ShlBΔ126–200 (Könninger et al., 1999) released ShlA° into the culture medium. These mutants contained virtually no hemolytic activity in the conditioned medium.

Lack of activation of the secreted ShlA derivatives is probably not caused by a failure to bind phosphatidylethanolamine (PE) since periplasmic ShlA* strongly binds PE in the absence of ShlB without converting ShlA* to hemolytic ShlA (Hertle et al., 1997). We have not tested whether ShlA° contained PE.

The insertions in mutants ShlB136 and ShlB177 are located in the most prominent surface loop in the predicted transmembrane model of ShlB which encompasses residues 125 to 216 (Könninger et al., 1999). The insertion of ShlB154 is in the same region, but the deletion extends to residue 252, which results in the excision of two transmembrane regions. An M2 epitope inserted after residue 251 clearly localized residue 251 at the cell surface. An M2 epitope after residue 224 was localized within the transmembrane region. The deletions in mutants ShlBΔ65–186, ShlBΔ137–186, and ShlBΔ126–200, which secrete ShlA°, are located in the same region. These data suggest that the most prominent surface loop and the following transmembrane loop are important for the activation function of ShlB, but play no essential role in ShlA secretion.

A total of 12 ShlB mutants contained much less hemolytic activity in the culture supernatants than cells synthesizing wild-type ShlB. Addition of ShlA-255 restored activity to nearly the wild-type level, depending on the mutant (data not shown). Wild-type ShlB secretes not only hemolytic ShlA, but also nonhemolytic ShlA°, as revealed by the enhancement of hemolytic activity by addition of ShlA-255 to samples of culture supernatants of *E. coli* BL21(DE3) transformed with pES14 *shlA shlB*. The hemolytic activity was measured during 2 h after addition of IPTG to induce transcription and increased on average 1.5-fold upon addition of ShlA-255.

ShlB332 differed from the other ShlB mutants since it did not secrete ShlA and ShlA°. Lack of secretion was not caused by lack of ShlB332 protein, as shown in this work by immunoblotting of the outer membrane proteins and by the previously shown reaction of a monoclonal antibody with the foreign M2 epitope DYKDDDDK inserted after residue 332 of ShlB (Könninger et al., 1999). Both methods yielded strong reactions. However, the M2 epitope is not exposed at the cell surface since it reacted with the anti-M2 antibody only in isolated outer membranes. Although these data do not reveal proper insertion of ShlB332 into the outer membrane, it is likely that ShlB332 is in the outer membrane because it converted some ShlA* into hemolytic ShlA.

Although ShlB tolerates many mutations without losing its secretion activity, mutants lacking the *shlB* gene do not secrete ShlA and ShlA°. Bacterial outer membrane proteins tolerate many insertions of foreign epitopes and duplications in surface loops and periplasmic turns without losing their properties of export through the cytoplasmic membrane, insertion into the outer membrane, and transport and receptor activities (for examples, see (Agterberg and Tommassen, 1991; Armstrong and McIntosh, 1995; Koebnik and Braun, 1993; Köster et al., 1991; Merck et al., 1997; Moeck et al., 1994; Newton et al., 1996; Sukhan and Hancock, 1995)).

In artificial lipid bilayer membranes, ShlB does not form a permanently open pore (Könninger et al. 1999) through which, if it exists in vivo, unfolded ShlA could be secreted. Only very small pores with a conductance of 0.2 nS and rarely of 1.2 nS and with a very short lifetime of a few hundred milliseconds have been observed.

The deletion derivatives ShlBΔ65–186, ShlBΔ87–153, and ShlBΔ126–200 display a stepwise increase in conductance of 1.2 nS and most of the pores stayed open for 1 min. These results suggest that ShlB has the potential to form open pores, and the results of this work indicate that this property is not strongly affected by quite a number of ShlB insertion and deletion mutations.

Secreted inactive ShlA° was completely degraded by trypsin, as is periplasmic inactive ShlA* (Schiebel et al., 1989). In contrast, hemolytic ShlA was cleaved into two fragments of 60 and 100 kDa, as has been shown previously (Schiebel et al., 1989). This result indicates that the conformational change from a highly trypsin-sensitive to a highly trypsin-resistant protein with only a single cleavage site in a polypeptide of 1578 residues occurs upon activation of ShlA and not during secretion. The high sensitivity to trypsin also suggests that the secretion-competent conformation is not tightly folded.

The results obtained with ShlA and ShlB are of relevance for the understanding of the activation and secretion of the homologous hemolysins synthesized by *Proteus mirabilis*, *Haemophilus ducreyi,* and *Edwardsiella tarda*, and they may have some bearing on the secretion mechanism of the *B. pertussis* filamentous hemagglutinin FHA and the *H. influenzae* adhesins HMW1 and HMW2. FhaC is necessary for secretion of FHA and displays single channel conductance in planar lipid bilayers which fluctuates very fast (Jacob-Dubuisson et al., 1999), comparable to the ShlB channels (Könninger et al., 1999). Amino acids important for translocation to the cell surface and secretion into the culture medium were localized by site-directed mutagenesis to the N-proximal region of HMW1 (residues 150 and 166) (Grass and St. Geme III, 2000), which agrees with the secretion of N-terminal ShlA-255 (Ondraczek et al., 1992).

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