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Superlytic Hemolysin Mutants of *Serratia marcescens*

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Hemolysis by *Serratia marcescens* is caused by two proteins, ShlA and ShlB. ShlA is the hemolysin proper, and ShlB transports ShlA through the outer membrane, whereby ShlA is converted into a hemolysin. Superhemolytic ShlA derivatives that displayed 7- to 20-fold higher activities than wild-type ShlA were isolated. ShlA80 carried the single amino acid replacement of G to D at position 326 (G326D), ShlA87 carried S386N, and ShlA80III carried G326D and N236D. Superhemolysis was attributed to the greater stability of the mutant ShlA derivatives because they aggregated less than the wild-type hemolysin, which lost activity within 3 min at 20°C. In contrast to the highly hemolytic wild-type ShlA at 0°C, the hyperlytic hemolysins were nonhemolytic at 0°C, suggesting that the hyperlytic derivatives differed from wild-type ShlA in adsorption to and insertion into the erythrocyte membrane. However, the size of the pores formed at 20°C by superhemolytic hemolysins could not be distinguished from that of wild-type ShlA. In addition to the N-terminal sequence up to residue 238, previously identified to be important for activation and secretion, sites 326 and 386 contribute to hemolysin activity since they are contained in regions that participate in hemolysin inactivation through aggregation.

The hemolysin of *Serratia marcescens* is a recently discovered type of hemolysin (4, 22) that is found in nearly all clinical isolates of *S. marcescens* and *Serratia liquefaciens* (24) and in several other *Serratia* species (24). The *Serratia* type of hemolysin is also the most frequently occurring cytotoxin in *Proteus mirabilis* and *Proteus vulgaris* (37). The hemolytic activity resides in the ShlA protein, which is secreted across the outer membrane with the help of the ShlB protein located in the outer membrane (28). In the absence of ShlB, ShlA remains in the periplasm and is inactive. ShlB is required for conversion to a hemolytic protein, which can be achieved in vitro without transport across the outer membrane (16). ShlA and ShlB are synthesized as precursors containing typical signal sequences (22) and are exported across the cytoplasmic membrane via the Sec system (28). The genes are transcribed from *shlB* to *shlA* with no transcriptional termination motif downstream of *shlB* but with a promoter in front of *shlA*. Because *Escherichia coli* K-12 transformed with cloned *shlA* and *shlB* genes secretes active hemolysin, we have been able to characterize the hemolysin with the aid of the advanced *E. coli* genetic systems (5). The region important for activation is localized in the first 150 N-terminal amino acid residues; the region important for secretion consists of 238 N-terminal residues (mature ShlA contains 1,578 residues). A sequence motif reading Ala-Asn-Pro-Asn occurs twice in ShlA and is important for activity and secretion since replacement of the central Asn (residues 69 and 109) by either Ile or Lys inactivates ShlA and prevents secretion (31).

ShlA forms pores of a defined size in erythrocyte membranes. Dextrin 15 (molecular mass, 1.4 kDa) nearly abolishes and dextran 4 (molecular mass, 4 kDa) completely prevents osmotic hemolysis since they do not diffuse through the pores formed by ShlA (5, 30) and they counterbalance the internal osmotic pressure at 30 mM. The size of the pore is somewhat larger at 20°C than at 2°C (30). In artificial lipid bilayer membranes, single-channel conductance is distributed over a larger range (30). ShlA integrated into erythrocyte membranes is resistant to proteases added from the outside but is cleaved from inside the erythrocytes in the C-terminal region, resulting in a reduction of the molecular mass from 160 to 143 kDa (27). The C-terminal region is important for pore formation. The shortest C-terminally truncated ShlA derivative found with a residual hemolytic activity (3%) has a molecular mass of 104 kDa (28).

The hemolysis zones formed by ShlA are very small, which may explain why the hemolytic activity of the clinically important *S. marcescens* and *S. liquefaciens* had been largely overlooked. The small hemolysis zone comes from the low diffusion rate of the large molecule and its instability. ShlA is inactivated by aggregation, which can be prevented by 6 M urea, leading to stable samples suitable for investigation.

In this paper, we describe the isolation of ShlA derivatives with a greatly increased hemolytic activity (superhemolytic variants) used to define sites important for activity and to learn more about factors important for activity, such as the rate of synthesis and secretion, the stability of the secreted hemolysin, the binding to erythrocytes, and the pore formation in the erythrocyte membrane. The superlytic derivatives contained single point mutations that reduced inactivation by aggregation. The superlytic derivatives differed from wild-type ShlA in several additional respects, which may, however, be less important for their high activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. marcescens* hemolysin genes were overexpressed in *E. coli* BL21 (DE3) (35, 36, 38), which is lysogenic for a λ phage carrying the T7 RNA polymerase gene controlled by the *lacUV5* promoter. The strain was transformed with plasmids encoding the genes *shlA* and *shlB* cloned downstream of the T7 promoter. Cells were grown in tryptone-yeast extract (TY) medium at 37°C (5), and *shlA shlB* overexpression was induced at an optical density at 578 nm of 0.5 by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 1 mM). After 2 h of incubation, the cells were sedimented (10,000 × *g* for 10 min), and the supernatant containing the secreted ShlA protein was used either directly or after stabilization with urea or guanidine hydrochloride (6 M final concentration).

Plasmids pRO3 and pES15 have been described previously (22, 28, 31). pMH1 was constructed by ligation of the 2.7-kb *Bgl*II-*Hin*dIII fragment of pES56 (28) into pACYC184 (6) digested with *Bam*HI and *Hin*dIII. Plasmid pMH1 carries

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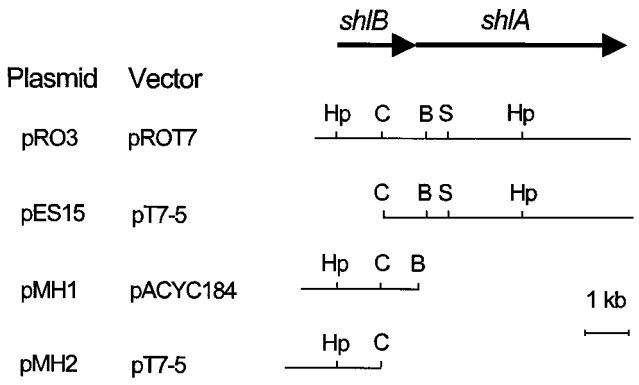

FIG. 1. Plasmids employed in this study. The location of the *shl* genes and the restriction sites used are shown. B, *Bgl*II, C, *Cla*I, Hp, *Hpa*I, S, *Sac*I.

shlB and *shlA′* that encodes only the signal sequence and two amino acids of the mature polypeptide. pMH2 was derived from pES14 (22) by digestion with *Cla*I and religation, resulting in *shlB′* that encoded only the N terminus (amino acids 1 to 327). This plasmid was used to construct pES14 derivatives by inserting the 5.7-kb *Cla*I fragment of mutagenized pES15 plasmids. The mutated *shlA* genes and wild-type *shlB* were together transcribed by the inducible T7 polymerase. pMH80 and pMH87 were constructed by isolating the *Sac*I-*Hpa*I fragment of mutated pES15 plasmids, causing the superlytic phenotype (termed pES15/80 and pES15/87) and ligating the fragment into pRO3 cut with the same endonucleases. Plasmid pSK-INTIII is a pSK-INT derivative (31) with an amino acid replacement in *shlA* (an exchange of N at position 236 to D [N236D]) constructed by oligonucleotide-directed mutagenesis by the method described by Stappert et al. (33). The 0.5-kb *Bgl*II-*Sac*I fragment of *shlA* was excised from pSK-INTIII with *Xho*I and *Sac*I and ligated into pMH80 (*Bgl*II and *Sac*I digested), obtaining plasmid pMH80III.

Recombinant DNA techniques. Plasmid DNA was isolated with either disposable Qiagen columns (Diagen Co., Düsseldorf, Germany) or by a rapid alkaline lysis method described by Zhou et al. (39). Agarose gel electrophoresis was performed as described by Sambrook et al. (25). DNA fragments were isolated from the agarose gel with the aid of the Qiaex gel extraction kit (Diagen) by following the manufacturer's directions. T4 ligase, alkaline phosphatase, and restriction endonucleases were obtained from Boehringer GmbH (Mannheim, Germany) and used as described in the manufacturer's product instructions. DNA was sequenced by the dideoxy chain termination method, as described previously (22).

SDS-PAGE. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Lugtenberg et al. (14) with 9% acrylamide in the running gel. ShlA obtained from gradient fractions or culture supernatants was concentrated by precipitation in trichloroacetic acid (TCA; 10% [wt/vol]). The proteins were dissolved by heating in sample buffer, loaded onto the gel, and stained after SDS-PAGE with silver (Bio-Rad Plus kit).

Mutagenesis with hydroxylamine. To mutagenize the plasmid pES15, 1 μg of Qiagen-purified DNA was incubated with hydroxylamine (1.2 M hydroxylamine-HCl, 200 mM potassium phosphate [pH 6], 2 mM EDTA) for 30 min at 75°C. The mutagenized DNA was purified with the Qiaex kit (Diagen) and transformed into *E. coli* 5K pMH1 for screening on blood agar plates.

Determination of the hemolytic activity. Hemolytic activities were tested on human blood obtained from the Blood Centre of the University of Tübingen. The assays were performed as described previously (16). Washed erythrocytes were suspended to a final concentration of 8% (vol/vol) in 0.9% NaCl. Hemolytic activities are presented either as the percentage of the total erythrocytes lysed (percent hemolysis) or as hemolytic units per milliliter. The number of hemolytic units was determined by the method described by Bernheimer (1). Dilutions of hemolytic samples (in HU buffer or 0.9% NaCl) were incubated with 1 ml of erythrocyte suspension for 15 min at room temperature and then centrifuged for 1 min in a microcentrifuge. The released hemoglobin was measured spectrophotometrically at 405 nm. One hemolytic unit is described as the amount of hemolysin that causes a release of 50% of the total hemoglobin in an erythrocyte suspension within 15 min. Total hemoglobin (100% hemolysis) was determined by lysing erythrocytes with 1% SDS.

shlA mutants were screened on TY agar plates overlaid with 4 ml of top agar containing 10% human erythrocytes. The blood agar plates were evaluated after incubation for 12 h at 37°C and after further incubation for 12 h at 8°C.

Sucrose density gradient centrifugation. Cell-free culture supernatant (1 ml) was layered on top of a 12-ml 10 to 55% sucrose gradient in TY medium and centrifuged (150,000 × *g*, 22 h, 4°C). Thirteen fractions of 1 ml each were collected, and the hemolytic activity of the fractions was determined in the presence and absence of 6 M guanidine hydrochloride. The remainder of the fractions were precipitated with 10% (wt/vol) TCA and separated by SDS-PAGE (9%).

RESULTS

Mutagenesis of the *shlA* gene. Plasmid pES15 contains the entire *shlA* gene and a small *shlB* 3′ fragment (Fig. 1) (22). It was mutagenized by treatment with 1.2 M hydroxylamine for 1 h at 75°C, yielding mutations that caused a discernible phenotype at a frequency of 1.3%. The phenotypes were tested by use of transformants of *E. coli* 5K carrying, in addition to the mutagenized pES15 *shlA* derivatives, plasmid pMH1 *shlB*, which encodes ShlB, the signal peptide, and two amino acids of mature ShlA (Fig. 1). Single colonies were transferred onto blood agar plates and incubated at 30, 37, and 42°C. Of the 4,500 colonies tested, 56 were nonhemolytic at 37°C, 2 were superhemolytic, and none showed a temperature-sensitive phenotype. However, the *S. marcescens* hemolysin forms only small hemolysis zones, which may have prevented recognition of different degrees of hemolysis at different temperatures. To determine production of the hemolysin protein, the *shlA* of 10 nonhemolytic mutants was cloned into pMH2 (Fig. 1) and expressed in *E. coli* BL21, which chromosomally encodes the phage T7 RNA polymerase that upon IPTG induction transcribes the mutated *shlA* genes cloned downstream of the T7 gene 10 promoter in pMH2. SDS-PAGE of [^{35}S]methionine-labeled whole-cell protein and culture supernatant fractions revealed that all of the mutated ShlA derivatives tested were truncated (data not shown), with molecular masses ranging from 35 to 120 kDa (wild type, 160 kDa). The derivatives were secreted into the culture medium to various extents. Since we have previously described the phenotype of such mutants (22, 28, 31), they were not studied further.

Properties of superhemolytic ShlA derivatives. To identify the mutation(s) in the superlytic ShlA proteins (named ShlA80 and ShlA87), which created a larger zone of hemolysis on blood agar plates than wild-type ShlA did (Fig. 2), restriction fragments were exchanged with wild-type *shlA* fragments in

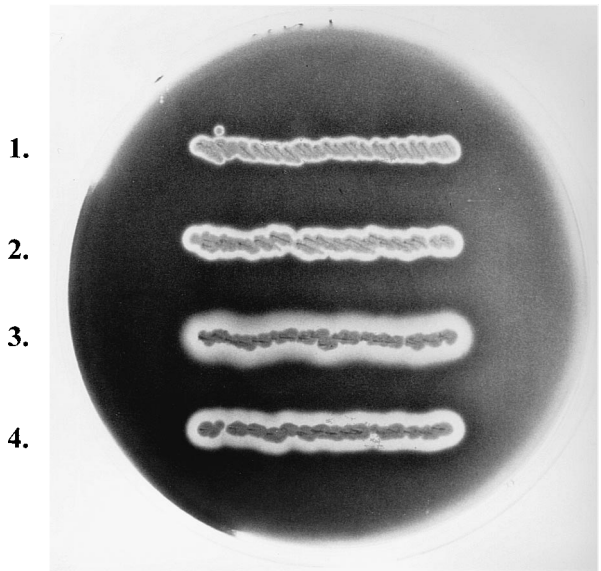

FIG. 2. Hemolytic activity of ShlA proteins secreted by *E. coli* BL21 pRO3 (wild-type ShlA) (streak 1), *E. coli* BL21 pMH80 (ShlA mutant) (streak 2), *E. coli* BL21 pMH80III (ShlA mutant) (streak 3), and *E. coli* BL21 pMH87 (ShlA mutant) (streak 4) on blood agar plates after 12 h of incubation at 37°C and a further 12-h incubation at 8°C.

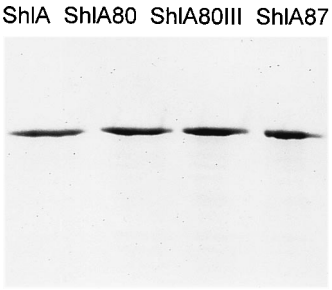

FIG. 3. Electrophoretic mobility of radiolabeled ShlA proteins secreted by *E. coli* BL21 transformed with the plasmids pRO3, pMH80, pMH80III, and pMH87 (lanes left to right, respectively). Cells were labeled in the presence of rifamycin for 5 min with [^{35}S]methionine (370 kBq/ml). The proteins of 1 ml of culture supernatant were precipitated with TCA and then subjected to SDS-PAGE and autoradiography.

pRO3 to see which fragment conferred superhemolysis. The *shlA* gene contains 4,824 nucleotides; therefore, identification of the fragment conferring superhemolysis restricted the DNA sequence to be determined for identification of the mutation. The 1.24-kb *Sac*I-*Hpa*I fragment (Fig. 1) of *shlA80* and *shlA87*, when cloned into wild-type *shlA* (yielding plasmids pMH80 and pMH87), resulted in superhemolytic ShlA derivatives. Sequencing of this fragment revealed in ShlA80 an exchange of glycine at position 326 to aspartate (G326D) and in ShlA87 an exchange of serine at position 386 to asparagine (S386N). Each fragment contained two or three additional nucleotide replacements, respectively, that caused no amino acid substitutions. The mutation in ShlA87 created an INT amino acid sequence, which is contained twice in wild-type ShlA (IN122T and IN236T). To examine whether this motif plays a role in ShlA activity, N was replaced at both sites by S and D in wild-type ShlA. Each of the ShlA derivatives showed a reduced hemolytic activity. However, the combined mutations in ShlA80 (N236D and G326D; termed ShlA80III) increased the superhemolytic activity of ShlA80 (Fig. 2). An increased hemolysis was also observed when ShlA87 was double mutated at sites N122D and N236D (data not shown). The superlytic ShlA derivatives showed the same electrophoretic mobility as that of wild-type ShlA (Fig. 3). ShlA80, ShlA87, and ShlA80III were studied further.

The half-life of the hemolytic activity of ShlA is only 3 min at 37°C (28). It can be preserved in 6 M urea and partially restored by adding 6 M urea to inactive ShlA (22). Aggregation has been suggested to be the major cause of ShlA inactivation (28). Therefore, inactivation of the ShlA derivatives and the effects of 6 M urea and 6 M guanidine hydrochloride were determined and compared with the inactivation of wild-type ShlA. (In this and all other assays using ShlA in 6 M urea or 6 M guanidine hydrochloride, the final concentration of urea and guanidine hydrochloride was 0.2 M maximum after the addition of the erythrocytes, which does not harm the erythrocytes).

Various volumes of the culture supernatant of freshly grown cells were incubated with a suspension of human erythrocytes (8%) to yield lysis of 50% of the cells after a 15-min incubation at 20°C. Under these conditions, ShlA80 was 7-fold more active, ShlA87 was 20-fold more active, and ShlA80III was 18-fold more active than wild-type ShlA (Table 1). In 6 M urea, the activity of wild-type ShlA was fourfold higher and that of ShlA80 was twofold higher, while urea barely affected the activity of the mutant ShlA87. The activity of ShlA80III was reduced. In 6 M guanidine hydrochloride, all activities (except that of ShlA80III, which was not tested) were raised to the maximum level.

TABLE 1. Hemolytic activity of ShlA proteins[a]

Assay conditions[b]	Hemolytic activity (HU)			
	ShlA	ShlA80	ShlA80III	ShlA87
20°C	10	70	175	200
20°C, 6 M urea	40	157	84	198
0°C	15	103	25	78
0°C, 6 M urea	120	0	0	0
20°C, 6 M GnCl	200	200	ND[c]	200
0°C, 6 M GnCl	170	0	0	0

[a] Hemolysis assays were performed with cell-free culture supernatants. Hemolytic activity is given in hemolytic units (HU). The activity was determined after 15 min. ShlA, wild-type hemolysin; ShlA80, ShlA80III, and ShlA87, mutant hemolysins.
[b] GnCl, guanidine hydrochloride.
[c] ND, not determined.

A large difference between wild-type and mutant ShlA was observed at 0°C in the presence of 6 M urea or 6 M guanidine hydrochloride: all three mutant ShlA proteins were inactive, but wild-type ShlA displayed a higher activity at 0°C than at 20°C. In the absence of urea, the hemolysis rate of wild-type ShlA was higher at 0°C than at 20°C, as was observed previously (Table 1). The higher lysis rates at 0°C than at 20°C were taken as evidence that wild-type ShlA does not aggregate in the erythrocyte membrane to form a channel, and it does not do so because the restricted mobility of proteins at 0°C makes such an aggregation impossible (10). In the absence of urea, ShlA80 also caused a faster lysis at 0°C, while ShlA87 and ShlA80III were less active at 0°C than at 20°C (Table 1).

To support further the lack of activity of the mutant ShlA proteins at 0°C as opposed to the high activity of wild-type ShlA, the hemolysins in 6 M urea were incubated with erythrocytes in the presence of 30 mM dextran 4 (molecular mass, 4 kDa) (Table 2). After the removal of dextran 4, hemolysis occurred in salt solution (0.9% salt concentration). Wild-type ShlA incubated with the erythrocytes for 1 min lysed 50% of the cells within 15 min after the removal of dextran 4. The activity of wild-type ShlA was the same regardless of whether the adsorption to erythrocytes was performed at 0 or 20°C and whether incubation in the saline solution was continued at 0 or 20°C. Under assay conditions in which the adsorption in dextran 4 and further incubation in a saline solution were both carried out at 20°C, ShlA80, ShlA80III, and ShlA87 lysed 50% of the erythrocytes, but only 25% of the erythrocytes were

TABLE 2. Nonradioactive erythrocyte binding studies[a]

Adsorption temp (°C)	Incubation temp (°C)	A_{405}			
		ShlA	ShlA80	ShlA80III	ShlA87
20	20	0.500	0.512	0.490	0.530
20	0	0.526	0.236	0.234	0.241
0	0	0.480	0	0	0
0	20	0.490	0	0	0

[a] Wild-type ShlA and mutant ShlA80, ShlA80III, and ShlA87 were expressed in *E. coli* BL21 strains harboring either plasmid pRO3, pMH80, or pMH80III. Cell-free culture supernatants were supplemented with urea (6 M). The amount of supernatant used caused 50% lysis ($A_{405} \approx 0.5$) after 15 min of incubation at 20°C. The hemolysins were incubated with osmotically protected erythrocytes (30 mM dextran 4) for 60 s. Unadsorbed proteins and dextran 4 were removed by centrifugation and washing twice in a saline solution adjusted to the incubation temperature. Lysis was determined in saline after 15 min of incubation at the incubation temperature indicated.

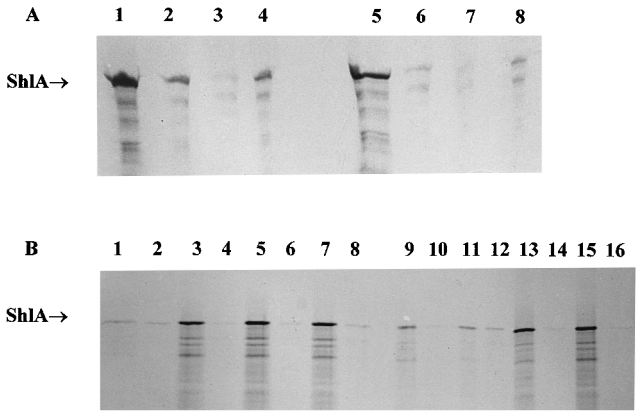

FIG. 4. Binding of radiolabeled ShlA proteins to erythrocytes. Proteins ShlA, ShlA80, ShlA80III, and ShlA87 were expressed in *E. coli* BL21. At an optical density of 0.5 at 578 nm, the cells were induced with IPTG for 2 h and then labeled with [^{35}S]methionine (370 kBq ml^{-1}). One milliliter of radiolabeled supernatant proteins was precipitated with TCA and dissolved in 50 μl of urea buffer (6 M in 20 mM HEPES). For adsorption to erythrocytes, 50 μl of the protein solutions was added to 0.5 ml of osmotically protected erythrocytes (8%; 30 mM dextran 4) kept on ice or at 20°C. After 60 s, the erythrocytes were sedimented and washed once with saline. The proteins in both supernatant fractions were precipitated with 10% TCA. The erythrocyte membranes were then lysed and washed with lysis buffer until hemoglobin was completely removed. Membranes (A) and the TCA precipitates (B) were boiled for 5 min in sample buffer and subjected to SDS-PAGE (9% acrylamide). (A) Lanes of samples incubated at 20°C: 1, ShlA; 2, ShlA80; 3, ShlA80III; 4, ShlA87. Lanes of samples incubated at 0°C: 5, ShlA; 6, ShlA80; 7, ShlA80III; 8, ShlA87. (B) Lanes of samples incubated at 20°C: 1 and 2, ShlA; 3 and 4, ShlA80; 5 and 6, ShlA80III; 7 and 8, ShlA87. Lanes of samples incubated at 0°C: 9 and 10, ShlA; 11 and 12, ShlA80; 13 and 14, ShlA80III; 15 and 16, ShlA87.

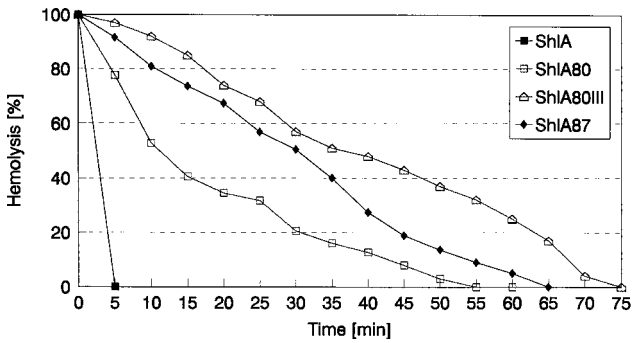

FIG. 5. Decrease in the hemolytic activities of culture supernatants containing ShlA, ShlA80, ShlA80III, and ShlA87 secreted by *E. coli* BL21 carrying pRO3, pMH80, pMH80III, and pMH87, respectively. Culture supernatants containing similar amounts of the proteins to be tested were collected and kept on ice until the hemolytic activity was determined. At time zero, the spent media were shifted to 20°C, and the volume used for hemolysis was adjusted such that the erythrocytes at the onset of the experiment were completely lysed (100% hemolysis). The culture supernatants were incubated, and samples were withdrawn at 5-min intervals to determine the hemolytic activity. The hemolytic activity is presented as percent hemolysis relative to the complete lysis at time zero.

lysed when incubation in saline solution was done at 0°C. When adsorption of the mutant hemolysins was carried out at 0°C, no hemolysis occurred regardless of whether incubation in a saline solution took place at 0 or 20°C.

To examine whether mutant hemolysins adsorbed to erythrocytes and integrated into the erythrocyte membrane at 0°C, radioactively labeled hemolysins were incubated for 1 min with erythrocytes in the presence of dextran 4 (to prevent hemolysis). Erythrocytes were recovered by centrifugation, washed once with a saline solution, and then incubated with lysis buffer until complete lysis occurred. Unadsorbed proteins and proteins in the saline solution of the first washing step were precipitated with 10% TCA. Erythrocytes and the TCA precipitates were dissolved in sample buffer and separated by SDS-PAGE after boiling for 5 min. Most of wild-type ShlA was found associated with the erythrocytes when incubated at 20 and 0°C (Fig. 4A, lanes 1 and 5). Only trace amounts of unadsorbed wild-type ShlA were found (Fig. 4B, lanes 1, 2, 9, and 10). By contrast, much less ShlA80 (lane 2), ShlA80III (lane 3), and ShlA87 (lane 4) were found associated with the erythrocytes after incubation at 20°C, an amount reduced even further after incubation at 0°C (lanes 6 to 8). Most of the mutant proteins remained in solution at both temperatures (Fig. 4, B, lanes 3 to 8 and 11 to 16). However, the different amounts of mutant hemolysins bound to erythrocytes at 0 and 20°C do not explain the complete inactivity of the mutant hemolysins at 0°C.

Inactivation of mutant ShlA proteins. Time-dependent activities of wild-type and mutant ShlA proteins were determined to see whether the much higher activities of the mutant ShlA proteins were a property of the nascent proteins or caused by a slower inactivation of the mutant proteins than of wild-type ShlA. Inactivation kinetics were determined at 20°C in the absence of urea. The half-life of wild-type ShlA (Fig. 5) was much shorter (2.5 min) than those of ShlA80 (10 min), ShlA87 (30 min), and ShlA80III (40 min). The half-lives correlated with the activities measured in samples taken from the spent media (Table 1), meaning that the hemolytic activities measured in the spent media do not reflect the amount produced by the cells but reflect what remains after inactivation. Consequently, no absolute but only relative hemolytic values can be obtained under carefully controlled growth and assay conditions.

Recovery of active from inactive ShlA. To obtain sufficient amounts of ShlA protein for renaturation experiments, wild-type *shlA* and mutant *shlA* were transcribed by the T7 phage polymerase in *E. coli* BL21. Two hours after IPTG induction, cells were removed by centrifugation, and the supernatant fraction was stored for 24 h at 20°C. Only residual activities were left after storage (Table 3). The original values were recovered by the addition of urea to 6 M final concentration. Activity was also substantially restored by heating the samples for 10 min at 80°C (Table 3). Whereas wild-type ShlA was only slightly reactivated, ShlA80 and ShlA87 (ShlA80III was not measured) regained 30 and 50% of their original activities, respectively. Both treatments presumably dissociated the aggregated inactive ShlA protein and thus restored most of the activity, especially of the mutated ShlA proteins.

TABLE 3. Hemolytic activity of secreted wild-type and mutant hemolysins dependent on reactivation by incubation and 6 M urea[a]

Assay conditions	Hemolytic activity (HU)		
	ShlA	ShlA80	ShlA87
IPTG induction	12.5	64	205
Incubation for 24 h at 20°C	0	1	20
Incubation for 10 min at 80°C	0.5	20	100
Incubation in 6 M urea	10	66	200

[a] The proteins were expressed in *E. coli* BL21 harboring either the plasmid pRO3 (ShlB and ShlA), pMH80 (ShlB and ShlA80), or pMH87 (ShlB and ShlA87). The hemolytic activity is given in hemolytic units (HU), which were determined with cell-free culture supernatants as described in Materials and Methods.

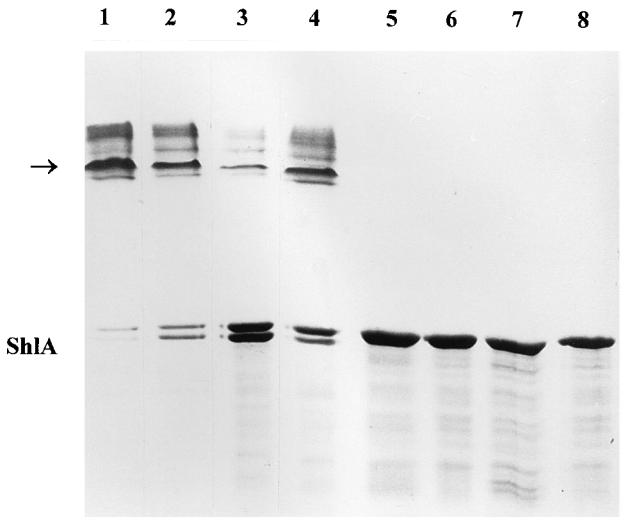

FIG. 6. Autoradiograph after SDS-PAGE of radiolabeled ShlA, ShlA80, ShlA80III, and ShlA87 secreted by *E. coli* BL21 carrying plasmids pRO3, pMH80, pMH80III, and pMH87, respectively. Proteins in 1 ml of cell-free culture supernatant, labeled with [^{35}S]methionine (370 kBq ml^{-1}) for 5 min, were precipitated with 10% TCA, and the sediment was collected by centrifugation and treated with sample buffer lacking mercaptoethanol. Lanes of unheated samples: 1, ShlA; 2, ShlA80; 3, ShlA80III; 4, ShlA87. Lanes of heated samples: 5, ShlA; 6, ShlA80; 7, ShlA80III; 8, ShlA87. Arrow, ShlA dimer.

Demonstration of ShlA aggregation. The results described above suggest that the superlytic activities of the mutated ShlA proteins come from a much lower tendency to aggregate into an inactive form. To demonstrate aggregation, unheated wild-type and mutant ShlA proteins were subjected to SDS-PAGE. The proteins were selectively radiolabeled with T7 RNA polymerase in the presence of rifamycin. To obtain sufficient amounts of protein for SDS-PAGE, culture supernatants of wild-type ShlA and mutant ShlA were precipitated with 10% TCA, and the precipitate was dissolved in 6 M urea. This procedure, surprisingly, yielded ShlA, which, when taken up in the same volume, was as hemolytic as the culture supernatant. Therefore, the physicochemical state of the proteins after treatment with TCA and urea was expected to be similar to the state in the culture supernatant. Wild-type ShlA contained a high proportion of high-molecular-weight material (Fig. 6, lane 1) running in the 9% gel at a position that corresponds approximately to a molecular mass of 320 kDa (marked by an arrow) and larger. Only a small fraction was at the position of the monomeric ShlA (marked ShlA). ShlA80 (lane 2) also aggregated as high-molecular-weight material, but a large proportion displayed the electrophoretic mobility of monomeric ShlA. Most of the ShlA80III was at the ShlA monomeric position, and much less was at the polymer position (lane 3). ShlA87 behaved in a manner similar to ShlA80 (lane 4). The double band at 170 kDa had been described previously (28), and it is observed with other outer membrane proteins. The double band was converted to a single band by heating (Fig. 6, lanes 5 to 8).

Aggregation was studied further by sucrose density gradient centrifugation. The spent medium of cells synthesizing wild-type and mutant ShlA under T7 control was fractionated on a stepwise sucrose gradient (10 layers from 10 to 55%) by centrifugation for 22 h. Wild-type ShlA protein was mainly contained in the 40 to 55% sucrose concentration (Fig. 7), as was horse ferritin (molecular mass, 442 kDa; data not shown). To measure the hemolytic activity of the collected fractions, guanidine hydrochloride had to be added to a concentration of 6 M. The distribution of the hemolytic activity agreed with the protein content. In contrast to wild-type ShlA, ShlA87 (and ShlA80III and ShlA80 [data not shown]) was contained in fractions containing 10 to 30% sucrose (Fig. 6), where alcohol dehydrogenase (molecular mass, 170 kDa) was also found. This shows that most of ShlA87 was present in monomeric form. ShlA87 in the fractions was hemolytic and, unexpectedly, was inhibited by guanidine hydrochloride. The same fractionation pattern and sensitivity to guanidine hydrochloride was obtained with ShlA80 and ShlA80III (data not shown). Since inactivation by guanidine hydrochloride disagreed with the results presented in Tables 1 and 2, where hemolysis by ShlA80 and the ShlA87 was recorded, the effects of guanidine hydrochloride on the activity of the mutant ShlA proteins was studied. Mutant hemolysins in diluted solutions lost most of their activity within 30 to 60 min after the addition of guanidine

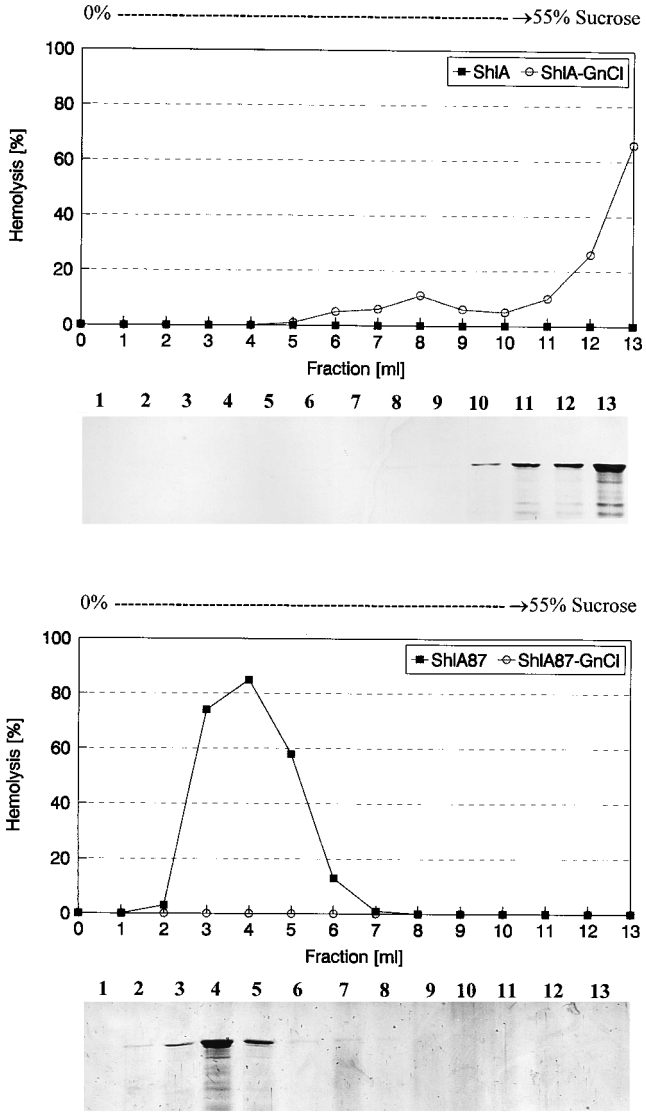

FIG. 7. Sucrose density gradient centrifugation of the hemolysins secreted by *E. coli* BL21(pRO3) and *E. coli* BL21(pMH87) through a 10 to 55% sucrose gradient. The relative hemolytic activity of each fraction was determined prior to (■) and after the addition of 6 M guanidine hydrochloride (○). The TCA-precipitated fractions were subjected to SDS-PAGE and stained with silver.

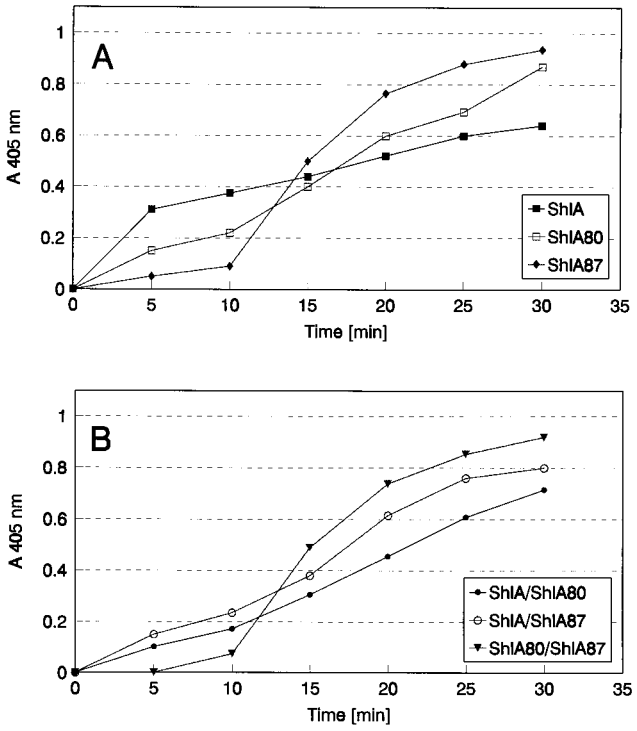

FIG. 8. Lysis kinetics of ShlA, ShlA80, and ShlA87. The hemolysins in the culture supernatants of *E. coli* BL21(pRO3), *E. coli* BL21(pMH80), and *E. coli* BL21(pMH87) were stabilized with urea (6 M). The amount of each hemolysin sample used caused about 45% (A_{405} = 0.45) lysis of an 8% erythrocyte suspension (1 ml). Hemolysis was determined by testing 75 μl of the toxin-treated suspension (1 ml) at 5-min intervals at 20°C. (A) Individual ShlA proteins; the amount caused about 45% hemolysis after 15 min of incubation. (B) Mixtures of ShlA proteins (each protein had half the concentration used in panel A).

hydrochloride. Activity was not stabilized by added serum albumin. More-concentrated mutant hemolysins in culture supernatants were more stable than less-concentrated hemolysins and gave the results presented in Table 1.

Rate of hemolysis by wild-type and mutant ShlA. To obtain additional data on the difference in hemolysis between wild-type and mutant ShlA proteins, the time-dependent rate of hemolysis was determined in the presence of 6 M urea. The concentrations of the proteins were adjusted so that they would release the same amounts of hemoglobin after 15 min. The initial hemolysis rate of the wild-type ShlA was higher than that of ShlA80 and ShlA87 (Fig. 8A). After 15 min, ShlA80 and ShlA87 lysed erythrocytes faster than wild-type ShlA did. The sigmoidal shapes of the curves suggest a cooperative effect of several polypeptides.

To determine whether interaction between ShlA monomers played a role in the different kinetics, wild-type and mutant ShlA were mixed at concentrations of each protein at half of that used for the experiment shown in Fig. 8A. The kinetics obtained after 15 min (Fig. 8B) were dominated by the mutant proteins. Hemolysis by ShlA87 combined with ShlA80 (ShlA87-ShlA80) was faster than hemolysis of ShlA-ShlA87, which was faster than that of ShlA-ShlA80.

Within the first 15 min, the hemolysis rates of all mixtures were lower than the rate of hemolysis obtained with each single protein (used at the same concentration as that of the protein mixture). Moreover, the mutant protein mixture lysed the erythrocytes with a delay of 5 min, which could be an indication of a possible interaction between protein monomers. This interaction was disturbed by the mutations in ShlA80 and ShlA87, whereas wild-type ShlA interacted better with the mutant proteins than they interacted with each other. Therefore, the hemolysis rates obtained suggest an additive effect of the ShlA derivatives or a synergistic effect of heterodimers.

Pore formation in erythrocytes. Since the superhemolytic ShlA derivatives were inactive at 0°C, the sizes of the pores they formed in erythrocyte membranes at 20°C were compared with those formed by wild-type ShlA. The rate of hemolysis was measured over 4 h in the presence of maltotriose (molecular mass, 504 Da), dextrin 20 (molecular mass, 900 Da), dextrin 15 (molecular mass, 1,400 Da), and dextran 4 (molecular mass, 4,000 Da). No difference was found between the wild-type ShlA, ShlA80, and ShlA87 in the reduction of hemolysis by the smaller oligosaccharides and the prevention of hemolysis by dextran 4 (data not shown), indicating that the size of the pores did not contribute to superhemolysis of mutant ShlA.

DISCUSSION

Single amino acid replacements increased ShlA activity 7- to 20-fold. The strongest increase was caused by the rather conservative replacement of serine by asparagine, which in itself is remarkable but more so considering the huge size of mature ShlA (1,578 amino acid residues). The mutations were localized in the N-terminal portion of ShlA, which was shown previously to be important for the hemolytic activity and the secretion of ShlA across the outer membrane catalyzed by ShlB (28, 31). The much higher activities of the mutated ShlA proteins were related to their slower inactivation in the culture supernatants. While wild-type ShlA lost half of its activity in 2.5 min, the mutated ShlA proteins showed half-lives of 10, 30, and 40 min. Loss of activity was caused by aggregation, as demonstrated by SDS-PAGE and sucrose density gradient centrifugation. Wild-type ShlA showed an electrophoretic mobility corresponding mainly to an apparent molecular mass of 320 kDa, while the mutated ShlA proteins displayed electrophoretic mobilities corresponding predominantly to a molecular mass of 170 kDa. A similar result was obtained by sucrose density gradient centrifugation, in which wild-type ShlA accumulated in the fractions containing 40 to 50% sucrose, where horse ferritin (442 kDa) was located, while ShlA87 was localized in the 10 to 30% sucrose fraction in which alcohol dehydrogenase (170 kDa) was found.

The hemolytic activity of wild-type ShlA and of mutated ShlA derivatives, stored for 24 h to allow aggregation, was recovered by the addition of 6 M urea or 6 M guanidine hydrochloride. These agents interfere with hydrogen bond formation and usually denature proteins. In the case of ShlA, they dissolve aggregates, resulting in activation rather than inactivation of ShlA monomers (22, 28, 30). Heating to 80°C reactivated the mutated ShlA proteins to a much higher degree than it did the wild-type ShlA protein. Presumably, the mutated forms were more easily disaggregated because of their lower aggregation tendency. The mutated proteins also differed in some other respects from wild-type ShlA. They were inactive at 0°C in 6 M urea and 6 M guanidine hydrochloride, which was caused mainly by an inefficient adsorption to erythrocytes at 0°C. Incubation of the mutant ShlA proteins at 0°C with erythrocytes protected from lysis by dextran 4 resulted in no lysis at 0 and 20°C. Experiments with radiolabeled proteins demonstrated that most of wild-type ShlA associated with the erythrocytes and most of the mutated ShlA remained unadsorbed in solution. The adsorbed fraction of the mutant hemolysins was apparently not able to insert properly into the

erythrocyte membrane to form pores. Once the pores were formed at 20°C, mutant hemolysin pores were not distinguished from wild-type hemolysin pores by osmotic protection experiments with oligosaccharides. Since wild-type ShlA is more active at 0°C than at 20°C (27, 30), it is unlikely that aggregation within the erythrocyte membrane is required for pore formation. However, the aggregation in solution shortly prior to binding or after binding at the erythrocyte surface could take place. Restoration of inactive ShlA derivatives with point mutations or deletions in the N-terminal portion by an N-terminal wild-type fragment (16) suggests that aggregation is a step in pore formation. Since aggregation also inactivates ShlA, it is likely that the aggregates formed in solution differ from the aggregates formed at the erythrocyte membrane. This model is reminiscent of the initial steps of pore formation by aerolysin of *Aeromonas hydrophila*, which forms dimers in solution and then oligomerizes at the erythrocyte surface prior to insertion as heptamers into the erythrocyte membrane. The aerolysin heptamers are very stable and can be isolated from the membrane (18, 19). We have no indication of stable oligomers formed by the *S. marcescens* hemolysin in the erythrocyte membrane. Lysis kinetics suggests that pore formation is a complex multistate process. Hemolysis induced by wild-type hemolysin was initially faster than that of the mutant hemolysins, which after 20 min lysed erythrocytes faster than wild-type hemolysin did. None of the ShlA forms displayed a linear time-dependent lysis rate. At lower hemolysin concentrations than those used in the experiment illustrated in Fig. 8, there was also a delay of up to 30 min in the onset of hemolysis elicited by wild-type ShlA, suggesting a concentration-dependent hemolysin oligomerization as a necessary step for pore formation (5, 30). Pore formation in artificial lipid bilayers indicates that aqueous multistate channels are smaller at 0°C than at 20°C (30). The concentration-dependent conductance elicited by ShlA in lipid-bilayer membranes suggests an association-dissociation equilibrium between hemolysin monomers and oligomers. The inner diameter of the ShlA channels deduced from the conductance measurements in artificial bilayers and the temperature-dependent rate of hemolysis varies between 1 and 3 nm, which supports the conclusion that ShlA can form water-filled channels of complex structure. The large molecule inserts into the erythrocyte membrane in such a way that it is no longer degraded by proteases at the cell surface (27). From the inside of erythrocytes, trypsin releases 16% of the polypeptide from the C-terminal end, resulting in a defined fragment that is probably protected from further digestion by the erythrocyte membrane. In ShlA80 and ShlA80III, the sequence RGE of wild-type ShlA is converted to RDE. RGE is similar to RGD, which has been implicated in the binding of prokaryotes to eukaryotic cells, e.g., *Bordetella pertussis* via the filamentous hemagglutinin (23) and *Haemophilus influenzae* via the high-molecular-weight surface protein (34). Originally, RGD was found in fibronectin (21) and pertactin (12). It is possible that the RGE motif plays a direct role in binding of ShlA to erythrocytes and leukocytes (11, 26). In this case, it would also be involved in aggregation of ShlA. The IST sequence in ShlA87 may play a similar role. Both mutations likely alter the conformation of ShlA so that fewer sites are involved in aggregation or are no longer exposed, thus increasing hemolytic activity by preventing or delaying aggregation. It is reasonable that a protein such as ShlA, although water soluble and lacking any extended hydrophobic region, which spontaneously integrates into the erythrocyte membrane, must have permanently exposed hydrophobic regions or the tendency to expose such regions to lead to aggregation in an aqueous environment. This is a typical feature of many bacterial toxins, and with colicins A (20), E1 (7, 8), and Ia (32), it has been shown that major alterations in secondary and tertiary structures occur upon insertion of these water-soluble proteins into artificial membranes. So, proteins like ShlA that have to be soluble in an aqueous environment but at the same time have to insert spontaneously into the hydrophobic environment of a membrane must keep a delicate balance between their hydrophilic and hydrophobic properties. They lose their activity too rapidly by aggregation when they are too hydrophobic or by inserting too slowly into membranes when they are too hydrophilic. Wild-type hemolysin may be the optimal compromise between the two requirements. The HpmA hemolysin of *P. mirabilis* that shows 47% sequence identity to the *S. marcescens* ShlA hemolysin (37) is less prone to aggregation (29a). The HpmA sequence differs from that of ShlA at the mutation site of ShlA80 (MGD instead of RGE in ShlA) and ShlA87 (TATK instead of ISTQ), which may contribute to its better solubility.

S. marcescens forms such small hemolysis zones on standard blood agar plates that they are hard to recognize. We use erythrocytes suspended in soft agar (0.75%), which facilitates diffusion of the large molecule so that the hemolysis zones become larger (2, 3, 5). In the spent medium of *S. marcescens*, only trace amounts of hemolytic activity were found. *S. marcescens* secretes a number of proteases with a broad specificity (13, 15, 29) that degrade hemolysin and thus add to the low activity of secreted hemolysin. This led us to suggest that ShlA is a contact cytolysin that is transferred from *S. marcescens* to erythrocytes and leukocytes (2, 5). This conclusion may still be valid in insects, animals, and humans, where *S. marcescens* has been found and where it may cause various kinds of diseases (9, 17). Our previous suggestion that aggregation is the major cause of inactivation, based on the preservation of hemolytic activity in 6 M urea, the recovery of hemolytic activity from hemolysin precipitates, and indications that inactive hemolysin has a high molecular weight (30), is strongly supported by the results presented in this paper that show that the delay in aggregation of the mutant hemolysins results in superhemolysis.

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REFERENCES

1. **Bernheimer, A. W.** 1988. Assay of hemolytic toxins. Methods Enzymol. **165:** 213–217.
2. **Braun, V.** 1985. Iron supply as a virulence factor, p. 168–176. *In* G. G. Jackson and H. Thomas (ed.), Bayer-Symposium VIII. The pathogenesis of bacterial infections. Springer-Verlag, Berlin.
3. **Braun, V., H. Günther, B. Neuss, and C. Tautz.** 1985. Hemolytic activity of *Serratia marcescens*. Arch. Microbiol. **141:**371–376.
4. **Braun, V., S. Hobbie, and R. Ondraczek.** 1992. *Serratia marcescens* forms a new type of cytolysin. FEMS Microbiol. Lett. **100:**299–306.
5. **Braun, V., B. Neuss, Y. Ruan, E. Schiebel, H. Schöffler, and G. Jander.** 1987. Identification of the *Serratia marcescens* hemolysin determinant by cloning into *Escherichia coli*. J. Bacteriol. **169:**2113–2120.
6. **Chang, A. C. Y., and S. N. Cohen.** 1979. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
7. **Cramer, W. A., F. S. Cohen, A. R. Merrill, and H. Y. Song.** 1990. Structure and dynamics of colicin E1 channel. Mol. Microbiol. **4:**519–526.
8. **Cramer, W. A., J. B. Heymann, S. L. Schendel, B. N. Deriy, F. S. Cohen, P. A. Elkins, and C. V. Stauffacher.** 1995. The structure-function of the channel-forming colicins. Annu. Rev. Biophys. Biomol. Struct. **24:**611–641.
9. **Flyg, C., K. Kenne, and H. G. Boman.** 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to

Cecropia immunity and a decreased virulence to *Drosophila*. J. Gen. Microbiol. **120:**173–181.
10. **Fowler, V., and D. Branton.** 1977. Lateral mobility of human erythrocyte integral membrane proteins. Nature (London) **268:**23–26.
11. **König, W., Y. Faltin, J. Scheffer, H. Schöffler, and V. Braun.** 1987. Role of cell-bound hemolysin as a pathogenicity factor for *Serratia* infections. Infect. Immun. **55:**2554–2561.
12. **Leininger, E., M. Roberts, J. G. Kenimer, I. G. Charles, N. Fairweather, P. Novotny, and J. Brennan.** 1991. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. Proc. Natl. Acad. Sci. USA **88:**345–349.
13. **Letoffe, S., P. Delepelaire, and C. Wandersman.** 1991. Cloning and expression in *Escherichia coli* of the *Serratia marcescens* metalloprotease gene: secretion of the protease from *E. coli* in the presence of the *Erwinia chrysanthemi* protease secretion functions. J. Bacteriol. **173:**2160–2166.
14. **Lugtenberg, B., M. F. J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. FEBS Lett. **58:**254–258.
15. **Miyazaki, H., N. Yanagida, S. Hirinouchi, and T. Bepu.** 1989. Characterization of the precursor of *Serratia marcescens* serine protease and COOH-terminal processing of the precursor during its excretion through the outer membrane of *Escherichia coli*. J. Bacteriol. **171:**6566–6572.
16. **Ondraczek, R., S. Hobbie, and V. Braun.** 1992. In vitro activation of the *Serratia marcescens* hemolysin through modification and complementation. J. Bacteriol. **174:**5086–5094.
17. **Pagani, L., F. Luzzaro, P. Ronza, A. Rossi, P. Micheletti, F. Porta, and E. Romero.** 1994. Outbreak of extended-spectrum β-lactamase producing *Serratia marcescens* in an intensive care unit. FEMS Immunol. Med. Microbiol. **10:**39–46.
18. **Parker, M. W., J. T. Buckley, J. P. M. Postma, A. D. Tucker, K. Leonard, F. Pattus, and D. Tsernoglou.** 1994. Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. Nature (London) **367:**292–295.
19. **Parker, M. W., J. P. Postma, F. Pattus, A. D. Tucker, and D. Tsernoglou.** 1992. Refined structure of the pore-forming domain of colicin A at 2.4 A resolution. J. Mol. Biol. **224:**639–657.
20. **Pattus, F., D. Carvard, R. Verger, C. Rosenbusch, and H. Schindler.** 1983. Formation of voltage-dependent pores in planar bilayers by colicin A, p. 407–413. *In* G. Spach (ed.), Physical chemistry of transmembrane ion motion. Elsevier, Amsterdam.
21. **Pierschbacher, M. D., and E. Ruoslahti.** 1984. Cell attachment activity can be duplicated by small synthetic fragments of the molecule. Proc. Natl. Acad. Sci. USA **88:**345–349.
22. **Poole, K., E. Schiebel, and V. Braun.** 1988. Molecular characterization of the hemolysin determinant of *Serratia marcescens*. J. Bacteriol. **170:**3177–3188.
23. **Relman, D. A., M. Domenighini, E. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA **86:**2637–2641.
24. **Ruan, Y., and V. Braun.** 1990. Hemolysin as a marker for *Serratia*. Arch. Microbiol **154:**221–225.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Scheffer, J., W. König, V. Braun, and W. Goebel.** 1988. Comparison of four hemolysin-producing organisms (*Escherichia coli*, *Serratia marcescens*, *Aeromonas hydrophila*, and *Listeria monocytogenes*) for release of inflammatory mediators from various cells. J. Clin. Microbiol. **26:**544–551.
27. **Schiebel, E., and V. Braun.** 1989. Integration of the *Serratia marcescens* hemolysin into human erythrocyte membranes. Mol. Microbiol. **3:**445–453.
28. **Schiebel, E., H. Schwarz, and V. Braun.** 1989. Subcellular location and unique secretion of the hemolysin of *Serratia marcescens*. J. Biol. Chem. **264:**16311–16320.
29. **Schmitz, G., and V. Braun.** 1985. Cell-bound and secreted proteases of *Serratia marcescens*. J. Bacteriol. **161:**1002–1009.
29a. **Schönherr, R., and V. Braun.** Unpublished results.
30. **Schönherr, R., M. Hilger, S. Broer, R. Benz, and V. Braun.** 1994. Interaction of *Serratia marcescens* hemolysin (ShlA) with artificial and erythrocyte membranes. Eur. J. Biochem. **223:**655–663.
31. **Schönherr, R., R. Tsolis, T. Focareta, and V. Braun.** 1993. Amino acid replacements in *Serratia marcescens* haemolysin ShlA define sites involved in activation and secretion. Mol. Microbiol. **9:**1229–1237.
32. **Slatin, S. L., X.-Q. Qui, K. S. Jakes, and A. Finkelstein.** 1994. Identification of a translocated protein segment in a voltage-dependent channel. Nature (London) **371:**158–161.
33. **Stappert, J., J. Wirsching, and R. Kemler.** 1992. A PCR method for introducing mutations into cloned DNA by joining an internal primer to a tagged flanking primer. Nucleic Acids Res. **20:**264.
34. **St. Geme, J. W., III, S. Falkow, and S. J. Barenkamp.** 1993. High-molecular-weight proteins of nontypable *Haemophilus influenzae* mediate attachment to human epithelial cells. Proc. Natl. Acad. Sci. USA **90:**2875–2879.
35. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
36. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and W. J. Dubendorff.** 1990. Use of T7 polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–83.
37. **Swihart, K. G., and R. A. Welch.** 1990. The HpmA hemolysin is more common than HlyA among *Proteus* isolates. Infect. Immun. **58:**1853–1860.
38. **Tabor, S., and C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
39. **Zhou, C., Y. Yang, and A. Y. Jong.** 1990. Mini-prep in ten minutes. BioTechniques **8:**172–173.