JOURNAL OF BACTERIOLOGY, May 1987, p. 2113–2120
0021-9193/87/052113-08$02.00/0

Vol. 169, No. 5

Identification of the *Serratia marcescens* Hemolysin Determinant by Cloning into *Escherichia coli*

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Received 17 November 1986/Accepted 10 February 1987

A cosmid bank of *Serratia marcescens* was established from which DNA fragments were cloned into the plasmid pBR322, which conferred the chromosomally encoded hemolytic activity to *Escherichia coli* K-12. By transposon mutagenesis with Tn*1000* and Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*), the coding region was assigned to a DNA fragment, designated *hly*, comprising approximately 7 kilobases. Two proteins with molecular weights of 61,000 (61K protein) and 160,000 (160K protein) were expressed by the pBR322 derivatives and by a plasmid which contained the *hly* genes under the control of a phage T7 promoter and the T7 RNA polymerase. When strongly overexpressed the 160K protein was released by *E. coli* cells into the extracellular medium concomitant with hemolytic activity. The genes encoding the 61K and the 160K proteins were transcribed in the same direction. Mutants expressing a 160K protein truncated at the carboxy-terminal end were partially hemolytic. Hemolysis was progressively inhibited by saccharides with increasing molecular weights from maltotriose (M_r 504) to maltoheptaose (M_r 1,152) and was totally abolished by dextran 4 (M_r 4,000). This result and the observed influx of [^{14}C]sucrose into erythrocytes in the presence of hemolytic *E. coli* transformants under osmotically protective conditions suggest the formation of defined transmembrane channels by the hemolysin.

We characterized an exoprotease of *Serratia marcescens* and studied its secretion (24). The strains we used were all strongly hemolytic in a liquid assay but caused only very narrow lysis zones on blood agar. Hemolysis was unrelated to the formation of the exoprotease (4). The hemolytic activity resided in the membrane fraction (4). Hemolytic bacteria have been shown to induce the release of the leukotrienes LTC4 and LTB4 from polymorphnuclear leukocytes, the release of histamine from rat mast cells, and chemiluminescence of neutrophils (2, 3). It was concluded that via these inflammatory mediators hemolysin may increase vascular permeability, edema formation, and granulocyte accumulation and thus contribute to the pathogenicity of *Serratia* spp. (18; W. König, Y. Faltin, J. Scheffer, B. König, H. Schöffler, and V. Braun, unpublished data).

Hemolysis required actively metabolizing cells and could be inhibited by various energy poisons (4). Hemolytic activity was not found in the culture supernatant of various *Serratia* strains grown under different conditions, nor could it be released from cells (4). Therefore, we used a genetic approach to characterize the hemolytic determinant of *S. marcescens*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used are listed in Table 1. The rough mutant SN8 was obtained as a partially bacitracin-resistant derivative by using a method developed by H. Rotering (personal communication, this institute). Bacitracin affects O-antigen synthesis by inhibiting recycling of the O-antigen polyisoprenoid carrier (25). Colonies of *S. marcescens* W1436 that grew on 1/8 TY agar plates near a filter paper disk which contained 10 mg of bacitracin were examined by gel electrophoresis to determine whether they expressed smooth or rough lipopolysaccharide (27). Of 20 colonies tested, 4 lacked the ladder of bands characteristic of O-antigen heterogeneity.

Cells were routinely grown in TY medium (0.8% tryptone [Difco Laboratories], 0.5% yeast extract, 0.5% sodium chloride, pH 7). The antibiotic ampicillin (25 or 50 μg/ml) or kanamycin (50 μg/ml) was added to maintain the plasmids.

Cosmid cloning. Chromosomal DNA of *S. marcescens* W225 was isolated from a 600-ml culture grown to a density of 3×10^8 cells per ml at 30°C. DNA was extracted and purified by CsCl density gradient centrifugation, digested with the endonuclease *Sau*3A, and size fractionated by centrifugation through a sodium chloride gradient (9). DNA of the 40-kilobase fraction (25 μg in 70 μl) (7) was ligated with the same molar amount of cosmid pHC79 cleaved with *Bam*HI.

In vitro packaging (8, 15) was performed with the kit of Amersham-Buchler, Braunschweig, Federal Republic of Germany. *Escherichia coli* 5K, grown overnight in 10 ml of TY medium supplemented with 0.2% maltose to induce the formation of the phage λ receptor, was centrifuged and suspended in 5 ml of 10 mM $MgSO_4$. Samples of the packaging reaction (50 μl) were incubated with 0.1 ml of *E. coli* 5K for 15 min at 37°C. Double-strength TY medium (0.15 ml) was added; after incubation for 20 min at 37°C, samples of 0.1 ml were spread on TY agar plates which contained ampicillin (25 μg/ml) and streptomycin (0.1 mg/ml). A gene bank with 7,000 colonies was stored on nitrocellulose filters.

To score hemolytic colonies, replica plates on TY-ampicillin medium were overlaid with 3.8 ml of blood top agar. Blood top agar was prepared from erythrocytes of outdated human blood which were collected by centrifugation for 10 min at 1,100 × *g* at 4°C. Serum and the white layer of leukocytes were removed before the erythrocytes were diluted into the same volume of 0.9% NaCl. The erythrocyte suspension (0.3 ml) was mixed with 3.5 ml of 0.75% TY agar at 44°C. Hemolytic colonies were scored after 5 h of incu-

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TABLE 1. Bacterial strains used

Strain	Genotype or description	Source
Serratia marcescens		
W225	Wild type	4
W1436	Exoenzyme-deficient strain	4
SN8	Rough derivative of W1436	This study
Escherichia coli		
5K	*hsdR hsdM lac rpsL thi thr*	This institute
CR63	F^+	This institute
MC4100	F^- *araD139 ΔlacU169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR*	M. Casadaban
H1443	*aroB rpsL lac araD*	K. Hantke
CC118	*araD139 Δ(ara,leu)7697 ΔlacX74 ΔphoA20 galE galU thi rpsE rpoB argE*(Am) *recA1*	26
CC202	As CC118, but F42 *lacI3 zzf-2*::Tn*phoA*	22
DS410	*minA minB rpsL lacY xyl thi mtl*	10
YR68	pES2 transformant of DS410	This study
YR71, 77 to 79	Tn*1000* insertion mutants in the *Ava*I A fragment of pES2 of strain YR68	This study
YR69, 70, 76, 80	Tn*1000* insertion mutants in the *Ava*I B fragment of pES2 of strain YR68	This study
YR72, 73	Tn*1000* insertion mutants in the *Ava*I C fragment of pES2 of strain YR68	This study
YR82	DS410 (pBR322::Tn*1000*)	This study
BN110	pUC191 transformant of MC4100	This study
WM1576	HfrC lambda (pGP1-2)	26
BN650	WM1576(pNE1)	This study
BN660	WM1576(pNE2)	This study

bation at 28°C. The cosmids were isolated from hemolytic colonies and used to transform different strains of *E. coli*. Transformants of strain MC4100 showed the strongest activities. Cosmid pHS33 was used for further studies.

Recombinant DNA techniques. Subcloning of the *hly* locus from the cosmid pHS33 into plasmids pBR322 (pES2) and pUC19 (pUC191) was performed by standard procedures (21), which involved cleavage with restriction enzymes, ligation, transformation, selection for inserts, and determination of fragments and their sizes by agarose gel electrophoresis. Plasmids were isolated by the method of Birnboim (1). Newly available endonucleases were used as recommended by the supplier (Boehringer, Mannheim, Federal Republic of Germany).

Transposon mutagenesis. (i) Tn*1000*. The method of Guyer was used (13). *E. coli* CR63 F^+ was transformed with pES2. Cointegrates formed between the F plasmid and pES2 were detected by the ability to transfer the ampicillin resistance marker of pES2 into *E. coli* H1443 by conjugation. Exconjugants were scored on TY plates containing ampicillin and streptomycin. Colonies were purified by restreaking twice.

The hemolytic activity of the colonies was tested by carefully dropping blood top agar, in this case a more concentrated suspension (0.2 ml of erythrocytes in 2.5 ml of agar) than described above, onto the colonies. Nonhemolytic derivatives were tested in a liquid assay which consisted of 0.4 ml of an overnight culture in 2 ml of washed erythrocytes (8%, vol/vol) in 0.9% NaCl (4). Samples of 0.2 ml were withdrawn at time intervals of 30 min, diluted 10-fold in 0.9% NaCl, and centrifuged for 10 min at 6,000 × *g*, and the absorbance of the supernatant fluid was measured at 405 nm.

(ii) Tn*phoA*. Plasmids pES2 and pUC191 were mutagenized by the procedure of Manoil and Beckwith (22). The highest yield of mutants was obtained by using the following protocol. *E. coli* CC202 carries Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*) on an F plasmid and *phoA recA* mutations on the chromosome. It was separately transformed with pES2 and pUC191. After transformation, cells (0.1 ml) were shaken in 2 ml of TY medium for 1 h at 37°C. Transformants were selected on TY agar plates containing 50 μg of ampicillin per ml. Colonies were isolated and suspended in TY medium. They were plated on TY agar containing ampicillin and kanamycin (0.5 mg/ml). The high kanamycin concentration was necessary since strain CC202 showed already a rather high kanamycin resistance (50 μg/ml) without transposition of Tn*phoA* into the multicopy plasmids pES2 and pUC191. Plasmids were isolated from the pooled colonies. *E. coli* CC118 was transformed with the pooled plasmids. The transformants were selected on TY plates containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml). The hemolytic activity of the colonies was tested as described above. Alkaline phosphatase was assayed on TY plates which contained 40 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml. Six percent of the nonhemolytic mutants formed blue colonies. The Tn*phoA* insertion sites and their orientation were determined by restriction endonuclease analysis with single and double digests with the enzymes *Ava*I, *Cla*I, *Hpa*I, and *Sal*I.

Determination of plasmid-encoded proteins. Transformants of *E. coli* DS410 with pES2, pUC191, and their transposon insertion derivatives were grown in 300 ml of TY medium with added glucose (1 g/liter) and ampicillin (25 mg/liter). Cells of the overnight cultures were collected by centrifugation. Minicells were separated from normal cells by three cycles of centrifugation through a 10 to 30% sucrose density gradient in M9 salt buffer (10). They were suspended in M9 medium containing 4 mM cycloserine to an absorbance of 1.0 at 578 nm. The culture was shaken for 1 h at 37°C. [^{35}S]methionine (600 kBq in 50 μl of 10% [wt/vol] Difco methionine assay medium in M9 salt solution) was added to a 0.5-ml suspension of the minicells and incubated for 1 h at 37°C. Then the minicells were pelleted and dissolved by heating for 5 min at 100°C in 50 μl of double-strength sample buffer used for polyacrylamide gel electrophoresis (19). The gel was prepared with 11% acrylamide. Human transferrin, bovine serum albumin, ovalbumin, and chymotrypsinogen A were used as unlabeled standard proteins. T5 phage proteins isolated from phage grown on cells in the presence of a ^{14}C-amino acid mixture were employed as radioactive standards (28). Polyacrylamide gels were stained with Coomas-

sie brilliant blue, treated with Amplify (Amersham-Buchler) for 15 min, dried, and autoradiographed.

Expression of the *hly* genes under control of the phage T7 polymerase-promoter system. Tabor and Richardson (26) constructed vectors in which inserted genes are transcribed by the T7 RNA polymerase under the control of a T7 promoter. The plasmids pT7-5 and pT7-6 contain polylinkers in opposite orientations preceded by the promoter of the T7 gene 10. In addition, they contain the *bla* gene with a transcription polarity opposite to the gene 10 promoter region (S. Tabor, personal communication). The *Eco*RI-*Hin*dIII fragment of pUC191 was isolated from an agarose gel and ligated into pT7-5 and pT7-6 digested with *Eco*RI and *Hin*dIII. *E. coli* WM1576 containing plasmid pGP1-2, which carries the gene for the T7 RNA polymerase under the control of the λ p_L promoter and the gene for the temperature-sensitive λ repressor c_I857 (26), was transformed with the ligation mixtures. Ampicillin-resistant clones that were hemolytic on blood agar were scored. The resulting strain BN650 and BN660 contained the plasmids pNE1 and pNE2, respectively. In pNE1 the *hyl* DNA fragment had the same transcription polarity as the T7 promoter. pNE2 contained *hly* in the reverse orientation.

To measure the hemolytic activity of *E. coli* BN650 and BN660, the cells were grown overnight in TY medium in the presence of 50 μg of ampicillin per ml and 50 μl of neomycin per ml at 27°C. Half of the cultures were shifted to 42°C for 30 min and then maintained for an additional 30 min at 37°C. The other half was incubated at 27°C. Hemolysis was assayed by incubating a suspension of 8% (vol/vol) human erythrocytes in saline with the *E. coli* samples in a ratio of 30 erythrocytes to 1 bacterial cell as described previously (4). The hemoglobin released was measured at 405 nm in the supernatant fluid after removal of the cells and ghosts by centrifugation at 6,000 × *g*.

To identify the proteins expressed by the plasmids pNE1 and pNE2, strains BN650 and BN660 were grown in TY medium to a density of 5×10^8 cells per ml. Two samples of 0.2 ml each were centrifuged, washed, and suspended in 1 ml of Difco methionine assay medium and incubated for 60 min at 30°C. One sample of each strain was shifted for 15 min to 42°C; then 0.2 mg of rifampin was added, and the cultures were shaken for an additional 10 min at 42°C and then for 20 min at 30°C. Then, the cultures were incubated for 10 min at 37°C with 0.37 MBq of [^{35}S]methionine, after which the cells were collected by centrifugation. They were suspended in 0.12 ml of sample buffer and heated for 5 min. The proteins were separated by polyacrylamide gel electrophoresis (11% polyacrylamide, 0.8% methylenebisacrylamide), and the bands were identified by fluorography.

Determination of sucrose influx into human erythrocytes. Strain BN110 was grown overnight in TY medium at 37°C. Bacteria (10^{10} per ml) were suspended with erythrocytes (6×10^9 per ml) in phosphate-buffered saline medium which contained 30 mM dextran 4 to a final volume of 2 ml. [^{14}C]sucrose (5 kBq in 5 μl) was added. Samples were withdrawn and centrifuged for 30 s in the Eppendorf centrifuge, and 20 μl of the supernatant fraction was measured in a liquid scintillation counter. Controls were run under identical conditions with samples containing the nonhemolytic strain MC4100 instead of BN110. Results were expressed as percent change in marker concentration in the supernatant, which reflected the influx into the erythrocytes. Hemolysis was measured in parallel experiments in the absence of radioactive substances in phosphate-buffered saline buffer that contained no dextran 4.

RESULTS

Cloning of the hemolysin (*hly*) locus. Chromosomal DNA fragments of about 40 kilobases were ligated into the *Bam*HI site of cosmid pHC79. *E. coli* 5K was infected with the suspension of reconstituted λ phage. Hemolysin activity of colonies was tested on blood agar and in liquid culture, and a colony with a high, stable hemolytic activity and containing the cosmid pHS33 was further investigated.

Subcloning of the *hly* locus of the cosmid pHS33. Two strategies were used for subcloning to avoid the formation of artifacts, for example, the assembly of DNA fragments which are not contiguous on the *S. marcescens* chromosome resulting from repeated restriction cleavage and ligation of the DNA. First, cosmid pHS33 was partially digested with *Sau*3A, and the fragments obtained were cloned into the *Bam*HI site of pUC19. A hemolysin-positive transformant of *E. coli* H1443 containing the smallest insert (7.5 kilobases) was designated pUC191. Second, cosmid pHS33 was cleaved with *Hin*dIII, and the fragments obtained were ligated into the *Hin*dIII site of pACYC184. A *Bam*HI-*Eco*RI fragment of a hemolysin-positive transformant of *E. coli* H1443 was excised and cloned into the *Bam*HI-*Eco*RI fragment of pBR322. The insert was further shortened by cleavage with *Bam*HI and partial *Sal*I digestion and cloned into the *Bam*HI-*Sal*I fragment of pBR322, yielding as the final product plasmid pES2 (Fig. 1).

Restriction map and transposon mutants of pES2 and pUC191. The cleavage sites of the inserts of pES2 and pUC191 were identical for the restriction enzymes tested (Fig. 1). No cleavage sites were found for enzymes *Xho*I, *Bcl*I, *Xba*I, *Kpn*I, *Stu*I, *Eco*RI, *Sma*I, *Dra*I, and *Hin*dIII. The insert of pUC191 is at the left end about 1 kilobase larger and at the right end about 0.1 kilobase shorter than pES2.

Plasmid pES2 was mutagenized by random insertion of the transposon Tn*1000*. The F^+ strain CR63 was transformed with pES2. The intermediary cointegrates formed between the F and pES2 plasmids were transferred by conjugation into *E. coli* H1443 *rpsL*. Colonies resistant to streptomycin and ampicillin were selected. Their hemolytic activities were tested on blood agar.

In the nonhemolytic derivatives the *Ava*I B fragment had disappeared (see Fig. 1 for designations). Insertion mutants in fragment C exhibited a reduced hemolytic activity. Less than half of the erythrocytes was lysed in the liquid assay after 1.5 h by *Ava*I-C insertion mutants. Insertions of Tn*1000* in fragment A yielded hemolytic and nonhemolytic cells. All cells transformed with plasmids carrying insertions in fragment D were hemolytic.

To confirm that the *hly* locus extended beyond the left end of fragment B, the small *Cla*I fragment was excised from pES2. Transformants carrying the shortened pES2 derivative were nonhemolytic. This means that the hemolysin locus extends beyond the left *Ava*I site of fragment B. To test the importance of the *Ava*I C fragment to the right of fragment B for full expression of the hemolytic activity, fragment C was excised by partial digestion with *Ava*I and religation. The products obtained were examined by restriction analysis. Four transformants were tested which contained only the fragments A and B in the correct orientation. Their hemolytic activity was in the same range as that of the Tn*1000* insertion mutants in fragment C. One transformant contained a pES2 derivative composed of fragments A, B, C and was fully hemolytic. These results support the involvement of fragment C in hemolysis.

Transposon insertion mutants were also constructed with

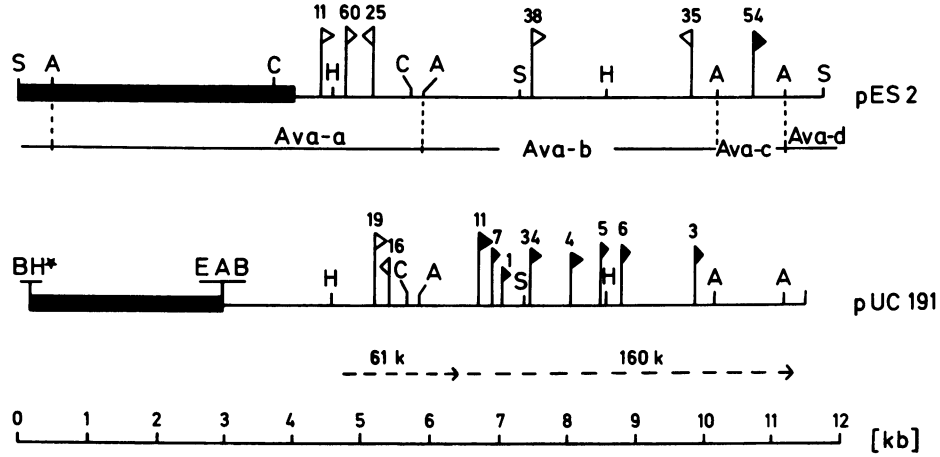

FIG. 1. Physical map and restriction sites of the *S. marcescens hly* chromosomal fragment (—) cloned into the vectors (■) pBR322 (pES2) and pUC19 (pUC191), respectively. The cleavage sites of restriction endonucleases are as follows: *Hpa*I (H), *Cla*I (C), *Ava*I (A), *Hin*dIII (H*), *Sal*I (S), *Eco*RI (E), and *Bam*HI (B). The restriction sites used in the polylinker region of pUC19 are indicated above the horizontal bars. The filled flags indicate insertion sites of the transposon Tn*phoA* where alkaline phosphatase was expressed; the open flags mark positions of Tn*phoA* without enzyme expression. The orientations of the flags correspond with the orientations of Tn*phoA* from $IS50_L$ to $IS50_R$. The arrows indicate the transcription polarity of the 61K and 160K proteins, encoded by the DNA insert. Ava-a, -b, -c, and -d designate *Ava*I fragments A, B, C, and D, respectively of pES2.

Tn*phoA* for two reasons. Tn*phoA* contains suitable restriction sites (C. Manoil, personal communication) for determining the sites of insertion and the orientation of Tn*phoA* in pES2. Moreover, Tn*phoA* may be used as a probe for protein export (26). Thus, Tn*phoA* insertion mutants could reveal the transcription polarity and identify export signals of the *hly* locus.

The site and orientation of Tn*phoA* insertions of mutants of pES2 and pUC191 affected in hemolysis are shown by flags in Fig. 1. The insertion sites confirm the location of the *hly* locus within and to the left and right of the *Ava*I B fragment. The insertions number 11 and 60 and the insertion number 54 of pES2 only reduced the rate of hemolysis. Insertions number 25 of pES2 and number 19 of pUC191 completely abolished hemolysis.

Six percent of the nonhemolytic Tn*phoA* insertion mutants formed blue colonies on 5-bromo-4-chloro-3-indolyl phosphate plates. One pES2 insertion and eight pUC191 insertions were mapped by restriction endonuclease analysis. All showed the same orientation, suggesting that in-frame *phoA* fusions which form an active alkaline phosphatase are all transcribed from left to right (Fig. 1). The fusion products and their subcellular location are being studied.

Hemolytic activity of the clones. *S. marcescens* W225 used for cloning exhibited a low hemolytic activity both in the liquid assay and on blood agar. This strain was purposely chosen to avoid the possible killing of *E. coli* transformed with plasmids bearing a strongly expressed *Serratia* hemolysin. Compared with the highly hemolytic strains W1128 and W1436 described previously (4), the rate of hemolysis by W225 was threefold lower. *E. coli* 5K and *E. coli* H1443 transformed with the cosmid pHS33, or with the plasmids pUC191, or pES2, lysed erythrocytes with about the same rapid rate as *S. marcescens* W1128 and W1436.

Lysis only occurred when the transformed *E. coli* cells were incubated with erythrocytes. No hemolytic activity could be detected in the culture supernatant of the *E. coli* transformants grown in different media for various lengths of time. Hemolysis was not enhanced by the addition of Ca^{2+} to the assay. These properties reflect those of hemolytic *Serratia* strains and demonstrate that synthesis and export of the hemolysin seems to be similar in the two genera.

Proteins encoded by pES2. The minicell-producing strain *E. coli* DS410 was transformed with pES2 (strain YR68) and with pES2 derivatives which contained Tn*1000* insertions in the *Ava*I B fragment (strains YR69, YR70, YR76, YR80), in fragment A (YR71, YR77, YR78, YR79), and in fragment C (YR72, YR73). A Tn*1000* insertion derivative in pBR322, the vector of pES2, served as a control (strain YR82). The strains with mutations in fragments A and B used were all nonhemolytic; those with mutations in fragment C showed a reduced hemolytic activity.

Minicells were prepared and labeled with [^{35}S]methionine. Minicells containing pES2 (Fig. 2, lane 14) expressed a protein with a high molecular weight of about 160,000 (160K protein, marked by an arrow in Fig. 2), which was not found in minicells carrying pBR322::Tn*1000* (lane 8). The same protein band was present in all minicells programmed by Tn*1000* inserts in fragment A (Fig. 2, lanes 4 to 6 and 11; the original fluorograph clearly discerns the 160K protein in lane 6). The 160K protein band was lacking in strains with mutations of fragment B (Fig. 2, lanes 7, 9, and 10) and of fragment C (lanes 12, 13). In lane 12 a shorter peptide appeared below the 160K protein.

An additional protein band related to the presence of the insert had an apparent molecular weight of about 61,000 (61K protein). A protein of this size was also expressed by minicells containing pBR322::Tn*1000*, but minicells with pES2 and its derivatives, with the exception of all Tn*1000* inserts in fragment A, expressed a protein of this size much more strongly. In addition, one strain with an insertion mutation in fragment B (Fig. 2, lane 3) expressed no 61K protein but instead expressed the 160K protein. It appears that the 61K protein is encoded by the fragment A and part of fragment B, and the 160K protein is encoded by fragments B and C (Fig. 1). Almost the entire length of the insert of pES2 is required to encode these two proteins.

A 160K protein was also observed after staining outer membrane proteins of *S. marcescens* SN8 with Coomassie brilliant blue after their separation by polyacrylamide gel

electrophoresis (data not shown). To avoid wavy, poorly resolved bands in the high-molecular-weight region caused by long O antigens, a rough mutant was used. In *S. marcescens* cells we could not assign unambiguously the hemolytic activity to this protein because we failed to obtain hemolytically inactive mutants after chemical mutagenesis. In contrast to the single protein in the high-molecular-weight region several proteins appeared in the 61K region.

Proteins encoded by pNE1 and pNE2. Plasmids pNE1 and pNE2 contained *hly* inserts in pT7-5 and pT7-6, respectively, with two transcription polarities relative to the orientation of the T7 promoter. The *E. coli* strains BN650(pNE1) and BN660(pNE2) carried in addition plasmid pGP1-2 bearing the gene for the T7 RNA polymerase downstream of the λ p_L promoter whose transcription is controlled by a temperature-sensitive λ repressor, c_I857. The T7 polymerase was synthesized at an elevated temperature, which in turn initiated transcription from the T7 gene 10 promoter. The *E. coli* RNA polymerase was inhibited by rifampin. Two protein bands with apparent molecular weights of approximately 61,000 and one band of 160,000 were strongly labeled in strain BN650 after derepression (Fig. 3, lane 1; protein bands marked by arrows). The same proteins were only weakly labeled in repressed cells (too weak to be seen in lane 2). All three protein bands were not apparent in repressed and derepressed cells of strain BN660.

In addition to the radioactively labeled cells applied to lanes 1 to 4 in Fig. 3, the ethanol precipitates of the supernatant fractions of the four cultures were separated on gels to examine whether the strongly overproducing cells released hemolysin proteins. The spent medium of the derepressed strain BN650 only contained detectable amounts of the 160K protein (Fig. 3, lane 6). Moreover, only this supernatant was hemolytically active (data not shown). The lanes showing no protein bands were not included in Fig. 3.

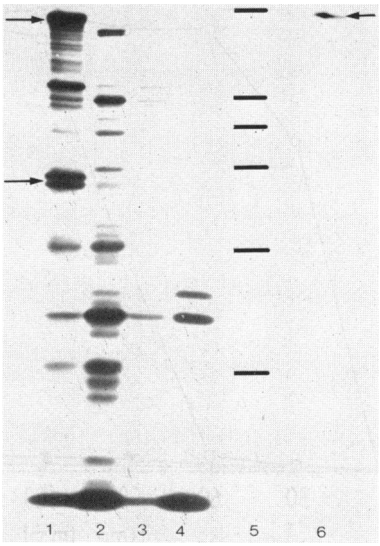

FIG. 3. Fluorograph of [^{35}S]methionine-labeled cells of *E. coli* BN650(pNE1) (lanes 1 and 2) and BN660(pNE2) (lanes 3 and 4) that were temperature induced (lanes 1 and 3) and uninduced (lane 2 and 4). The ethanol precipitate of the culture supernatant of strain BN650 (induced) was applied to lane 6. The positions of the standard proteins identified by staining is given in lane 5 from top to bottom as follows: α-macroglobulin (molecular weight 170,000), phosphorylase *a* (93,000), human transferrin (80,000), bovine serum albumin (67,000), ovalbumin (45,000), and chymotrypsinogen (25,000).

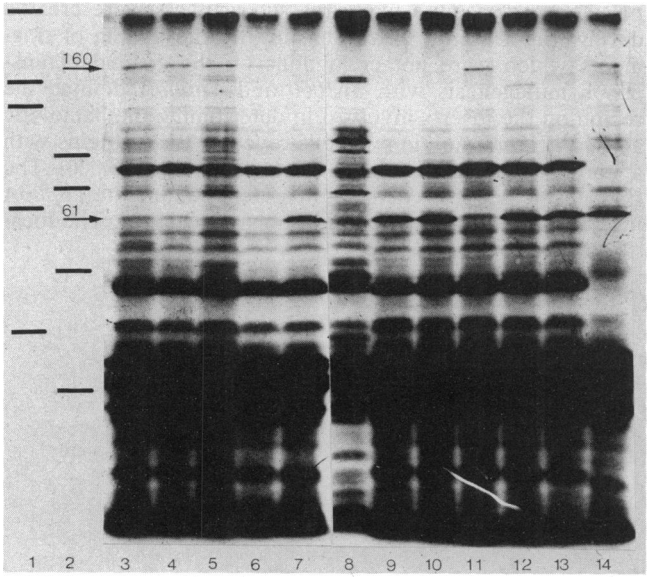

FIG. 2. Fluorograph of [^{35}S]methionine-labeled proteins in minicells derived from *E. coli* DS410 after separation on sodium dodecyl sulfate-polyacrylamide gels. The minicells contained plasmid pES2 (strain YR68; lane 14), pBR322::Tn*1000* (strain YR82, lane 8), Tn*1000* transposon insertions in the *Ava* A fragment of strain YR68 (strains YR77, 78, 79, 71; lanes 4 to 6 and 11), the *Ava* B fragment (strains YR76, 80, 69, 70; lanes 3, 7, 9, and 10), and the *Ava* C fragment (strains YR72, 73; lanes 12 and 13). In lane 1 the position of the structural proteins of phage T5 having molecular weights of 220,000, 125,000, 103,000, 75,000, 67,000, 58,000, and 32,000, respectively (28), and in lane 2 the standard proteins transferrin (molecular weight 80,000), bovine serum albumin (67,000), ovalbumin (45,000), and chymotrypsinogen A (24,000) stained with Coomassie brilliant blue are indicated. The positions of the 61K and 160K proteins are marked by arrows.

Hemolysis by strains BN650 and BN660 under conditions of repressed and derepressed T7 RNA polymerase was tested (Fig. 4). Strain BN650 immediately lysed erythrocytes after temperature induction, whereas lysis induction was delayed by 60 min with uninduced cells which were maintained at 27°C. Strain BN660 exhibited no hemolytic activity regardless whether the cells were preincubated at 42 or 27°C.

Formation of pores by the hemolysin. To counterbalance the intracellular pressure, we used dextran 4, an oligosaccharide with a mean molecular weight of 4,000, which was previously demonstrated to prevent hemolysis by the *E. coli* α-hemolysin (2). Indeed, erythrocytes were not lysed by *S. marcescens* cells in the presence of 30 mM dextran 4. At a ratio of 3 erythrocytes per *S. marcescens* cell, it took 30 min until the onset of lysis in the absence of dextran 4. In the presence of dextran 4 the erythrocytes were stable for hours. However, when *S. marcescens*-treated erythrocytes were precipitated by centrifugation (10 min, 4,500 × *g*) and suspended again in 0.9% NaCl they lysed completely within 5 min. This showed that damage of the erythrocytes had taken place during incubation in the presence of dextran 4.

To obtain an estimate of the size of the pore suggested by the above experiments, the ability of sugars of different sizes to prevent hemolysis was examined. Sorbitol (M_r 182) did not affect the onset of hemolysis (Fig. 5). In contrast, sucrose (M_r 342) and maltotriose (M_r 504) delayed the onset

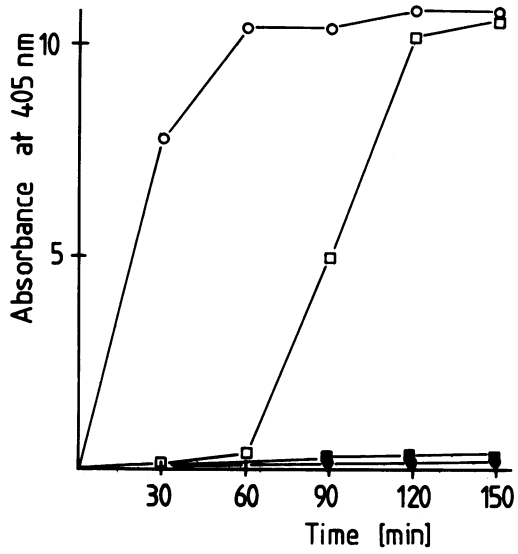

FIG. 4. Lysis of human erythrocytes by temperature induced (○), and uninduced (□) *E. coli* BN650 and by induced (●) and uninduced (■) *E. coli* BN660.

of hemolysis by 30 min, stachyose (M_r 666) delayed hemolysis by 60 min, and the larger maltodextrins maltopentaose (M_r 828), maltohexaose (M_r 990), and maltoheptaose (M_r 1,152) progressively retarded hemolysis (Fig. 5).

To measure entry of a low-molecular-weight substance into erythrocytes, [^{14}C]sucrose was added to a suspension of bacterial cells and erythrocytes in the presence of 30 mM dextran 4. The concentration of [^{14}C]sucrose in the medium should decrease upon gaining access to the volume of the erythrocytes through channels formed by the hemolysin. Indeed, the radioactivity in the culture supernatant began to decrease after 6 min of incubation to 80% after 10 min (Fig. 6). As a control, the untransformed nonhemolytic *E. coli* MC4100 parent strain of BN110 was incubated with erythrocytes in the presence of dextran 4 and [^{14}C]sucrose. The result shows that [^{14}C]sucrose was not taken up by erythrocytes or by the bacterial cells (Fig. 6). Hemolysis of the erythrocytes by BN110 in the absence of dextran 4 began after 15 min at the high cell concentrations used in these experiments (Fig. 6). Influx of sucrose in this and other experiments under different conditions (data not shown) always preceded hemolysis.

DISCUSSION

S. marcescens, in contrast to *E. coli*, secretes exoenzymes into the culture medium. However, the hemolysin could not be found in the extracellular fluid but was found only in the membrane fraction (4). Cells of *E. coli* transformed with plasmids carrying *hly* of *S. marcescens* lysed erythrocytes, although no hemolytic activity was found in the spent medium. The activity was roughly proportional to the gene dose and was not enhanced by Ca^{2+} ions. These data indicate that the *S. marcescens* hemolysin exhibited the same properties in *E. coli* as in *S. marcescens* (4). Moreover, the size of the cloned hemolysin found in *E. coli* apparently corresponded to the size observed in *S. marcescens*. Other toxins, for example, those of *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Bacillus cereus*, *Aeromonas hydrophila*, are neither processed nor excreted efficiently when cloned into *E. coli* K-12 (6).

The *hly*-containing DNA inserts in pES2 and pUC191 showed the same restriction enzyme cleavage sites. They were subcloned by different procedures from the cosmid pHS33, so it is quite likely that no artifacts were created during fragmentation of the cosmid by the joining of fragments which were not contiguous on the cosmid. Transposon mutagenesis with Tn*1000* and Tn*phoA* defined the region on the inserts involved in determining the hemolytic activity. Expression in minicells revealed two proteins with apparent molecular weights of 61,000 and 160,000. The transposon insertions defined their location, and the Tn*phoA* inserts defined the transcription polarity (Fig. 1). The loca-

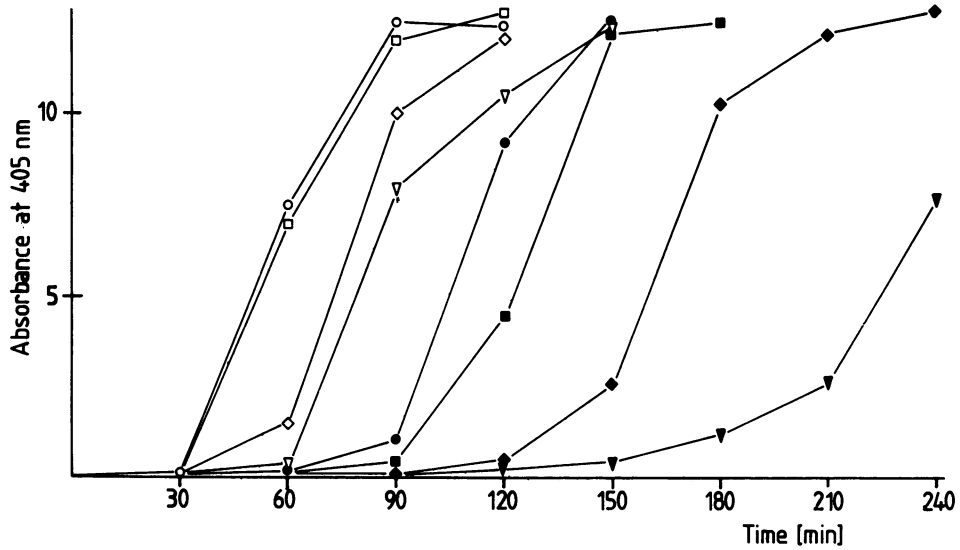

FIG. 5. Inhibition of hemolysis induced by *S. marcescens* W1436 by 30 mM saccharides. Bacteria (3×10^8 per ml) grown overnight in TY medium were incubated with 10^9 human erythrocytes per ml in a buffer consisting of 12.5 mM potassium phosphate, 90 mM NaCl, and 40 mM KCl (pH 7.2) in the absence of saccharides (○), and in the presence of 30 mM sorbitol (□), sucrose (◇), maltotriose (▽), stachyose (●), maltopentaose (■), maltohexaose (◆), or maltoheptaose (▼). The control supernatant without added bacteria had an absorbance below 0.3 read at 405 nm after 240 min of incubation.

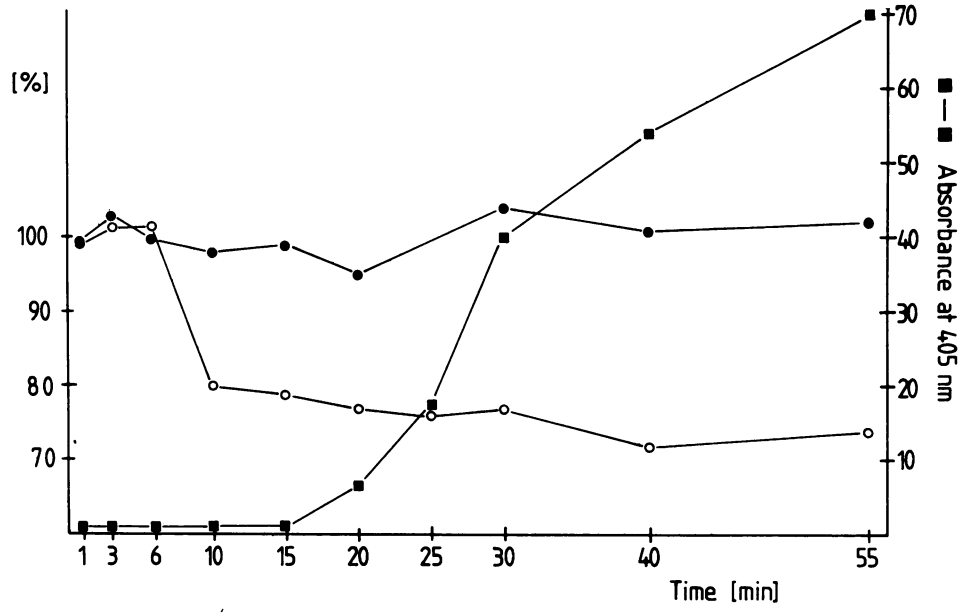

FIG. 6. Influx of [^{14}C]sucrose (○) into human erythrocytes (6 × 10^9 cells per ml) induced by *E. coli* BN110 (1 × 10^{10} cells per ml) and *E. coli* MC4100 (●) in the presence of 30 mM dextran 4. The change in the concentration of the radioactive compounds is given as the percentage of the original concentration. Hemoglobin released from erythrocytes by BN110 (■) into the medium in the absence of dextran 4 was measured at 405 nm after centrifugation of the cells.

tion of these genes was confirmed by cloning the insert of pUC191 behind the T7 promoter in both orientations. The two proteins and strong hemolytic activity were only expressed when transcription proceeded as drawn in Fig. 1. Expression of the hemolysin by pNE1 was so strong that some hemolytic activity and some 160K protein were found in the culture supernatant. This finding suggests that the 160K protein is the hemolysin. The results of the Tn*phoA* insertion mutagenesis support this conclusion, since only hybrids of the alkaline phosphatase with the 160K protein but not with the 61K protein were secreted (blue colonies on 5-bromo-4-chloro-3-indolyl phosphate plates). This 160K protein was also observed in the membrane fraction (data not shown), whereas the 61K has not yet been localized. The 61K protein formed a double band in Fig. 3, lane 1, which may indicate synthesis as a precursor that was not completely processed upon strong overexpression or that two translational start sites are used under these conditions.

The truncated form of the 160K protein synthesized by the Tn*1000* insertion mutant YR72 in the *Ava*I C fragment (Fig. 2, lane 12) confirmed the transcription polarity. A truncated 160K protein was also expressed by the *Ava*I C Tn*1000* mutant YR73 which was located between the 160K and the truncated 160K protein of YR72 (barely to be seen in Fig. 2, lane 13). The isolates with mutations in fragment C exhibit an interesting phenotype in that they show a reduced hemolytic activity. Apparently, the hemolysin can be shortened at the carboxy-terminal end and still retain activity. It remains to be determined whether the specific activity of the truncated hemolysin is reduced, whether it is less stable (the band in Fig. 2, lane 12, is weak and can barely be seen in lane 13), or whether secretion is affected.

Preliminary experiments indicate that the hemolysin forms channels of a defined size. Lysis could be prevented by dextran 4 and initiated immediately upon removal of the oligosaccharide, which apparently counterbalanced the internal turgor pressure of the erythrocytes. Saccharides of increasing molecular weights yielded progressively better protection of erythrocytes from lysis. Maltoheptaose (M_r 1,152) was already highly protective, suggesting that the diameter of the channel was too small to permit diffusion of this sugar. Influx of [^{14}C]sucrose was observed in the presence of 30 mM dextran 4, indicating that lysis protection observed with the larger saccharides was due to osmotic counterbalance and not to the inhibition of channel formation.

The number of genes which determine the hemolytic activity of *S. marcescens*, their sizes, and their arrangement appear to differ from those of the *E. coli* α-hemolysin (3, 5, 11, 12, 14, 17, 20). The latter is synthesized and secreted as a polypeptide with a molecular weight of 107,000, it is activated by the *hlyC* gene product, and the secretion process requires the proteins encoded by the *hlyB* and *hlyD* genes. The α-hemolysin apparently forms a hydrophilic transmembrane pore (16) with an effective diameter of about 3 nm (2). The *S. marcescens* hemolysin seems to exhibit similar pore-forming properties. With expression under the control of phage T7 promoter we expect to obtain sufficient amounts of the *S. marcescens* hemolysin to raise antibodies which will be used to study secretion and channel formation.

ACKNOWLEDGMENTS

We thank Helga Wolff for excellent technical assistance and Keith Poole for reading the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (Br 330/8-2).

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