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Cloning, Expression, and Mapping of the *Staphylococcus aureus* α-Hemolysin Determinant in *Escherichia coli* K-12

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A fragment of *Staphylococcus aureus* DNA encoding the α-hemolysin determinant was cloned from strain Wood 46 by inserting *Sau*3A-generated genomic DNA fragments between the *Bam*HI sites of the λ replacement vector L47.1. Phages expressing α-hemolysin were detected by overlaying plaques formed from several thousand independent recombinant phage with erythrocytes and looking for zones of hemolysis. One phage expressing α-hemolysin was purified and named λwα3. This was subsequently shown to contain a 10.2-kilobase pair insert of *S. aureus* DNA. A 7.6-kilobase pair *Hin*dIII fragment encoding the α-hemolysin was subcloned from λwα3 into the plasmid vector pACYC184 to form the hybrid plasmid pDU1148. *Escherichia coli* K-12 cells harboring pDU1148 synthesized a low level of α-hemolysin which remained associated with the cells and was not secreted into culture supernatants. When the same strain was stabbed onto blood agar plates, no zones of hemolysis were detected after overnight growth at 37°C but hemolysis developed if the plates were left at room temperature for 48 h. By introducing specific deletions or Tn*5* insertions into plasmid pDU1148, the α-hemolysin gene was mapped to a region within a 3.3-kilobase pair *Eco*RI-*Hin*dIII fragment which was subcloned onto the vector plasmid pBR322. A specific enzyme-linked immunosorbent assay with peroxidase-labeled rabbit anti-α-hemolysin antibodies was used to measure the levels of α-hemolysin antigen expressed in *E. coli* K-12 cells harboring pDU1148 or a variety of pDU1148::Tn*5* and pDU1148 deletion mutants.

The gram-positive organism *Staphylococcus aureus* is a major bacterial pathogen and is the etiological agent of a variety of infectious diseases in humans and animals, including mastitis, nosocomial infections, toxic shock syndrome, and scalded skin syndrome (14). The organism can colonize a number of different sites on body surfaces from which, under certain conditions, it can invade host tissues. Clinical isolates of *S. aureus* normally secrete a variety of extracellular proteins which may play important roles in establishing and maintaining infections (3). Many of these products can be detected during both in vivo growth in the host organism and during in vitro growth on laboratory medium (10). They include several distinct cytolytic toxins, DNases, proteases, lipases, and a leucocydin (1, 3, 13, 21). One of the major virulence determinants of *S. aureus* is α-hemolysin, which is secreted by almost all isolates (13). The protein is an extracellular product with potent cytolytic activity. The mechanism of action of the hemolysin is not fully understood, although it has been shown to penetrate the hydrophobic regions of biological membranes (13). No enzymatic activity has been associated with the purified protein (2, 13).

Results obtained from experimental infections in animals suggest that clinical isolates of *S. aureus* can differ greatly in virulence (18). Attempts have been made to supplement these studies by isolating strains carrying mutations affecting particular extracellular products. The mutants have been compared with the wild-type strain for virulence (19). Such studies have confirmed that the α-hemolysin is a major virulence factor. The studies were hampered, however, by the poorly developed genetic manipulation systems currently available for use with *S. aureus* and other gram-positive pathogens. The introduction of specific lesions into extracellular protein determinants is complicated initially by the danger of selecting mutations in genes controlling the secretion of several extracellular pro-

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TABLE 1. Bacterial strains used

Strain	Genotype	Source or reference
S. aureus		
Wood 46	High-level α-hemolysin producer	J. P. Arbuthnott (13)
E. coli K-12		
C600	*lac thr leu thi tonA hspR hspM*	17
XAc Su⁻	*lac-pro*$_{VIII}$ *ara thi rif nal Su*⁻	8
WL95	*lac thr leu thi tonA hspR hspM* P2 lysogen	20
BHB2688	*imm*^{434} cI1897 *b2 red3 E*am4 *S*am7	16
BHB2690	*imm*^{434} cI857 *b2 red3 D*aml5 *S*am7	16

teins, which lead to pleiotropic effects and also the inability to confidently map lesions to a particular gene.

Our laboratory has been investigating the pathogenic mechanisms employed by *S. aureus* in a number of different infections. To facilitate these studies we needed to construct strains carrying mutations in any of several potential virulence determinants. We decided to employ recombinant DNA techniques to clone and mutagenize *S. aureus* genes in *E. coli* with the intention of reintroducing the mutagenized genes back into *S. aureus*. In this paper we report the isolation and mapping of the α-hemolysin determinant in *E. coli* and describe the expression of α-hemolysin in this gram-negative organism.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and bacteriological media. The bacterial strains employed in this study are listed in Table 1. *S. aureus* Wood 46 is a well-characterized isolate which produces high levels of α-hemolysin in vitro (13). Strain Wood 46 was routinely cultured on tryptic soy broth (BBL Microbiology Systems, United Kingdom). The λ L47.1 bacteriophage replacement vector (*chi*A131, *sr*1 1-2, *sr*1 3, *imm*^{434} cI, *sr*1 4 *nin*5, *shn* 6, *sr*1 5, *sbh*1) was described by Loenen and Brammar (20). λ467(*b*221, *rex*::Tn*5*, *D*am29, *P*am80, *c*I857) was obtained from N. Kleckner (Harvard University, Cambridge, Mass.). λwα3 is a derivative of L47.1 which has the central *Bam*HI fragment replaced by a 10.2-kilobase pair (kbp) *S. aureus* chromosomal DNA fragment which carries the α-hemolysin determinant (this study). Bacteriophage were propagated using λ broth, λ base agar, and λ top agar (9). Plasmid cloning vectors were pBR322 (4), pBR327 (4), and pACYC184 (5). *E. coli* strains were grown in L broth (9) plus any selective agents if required. Blood agar was prepared by adding 2.5 ml of packed washed erythrocytes to 100 ml of tryptic soy agar tempered to 50°C.

***S. aureus* chromosomal DNA preparation.** *S. aureus* Wood 46 was grown with shaking for 18 h at 37°C in 50 ml of tryptic soy broth and the cells were recovered by centrifugation. After washing, the cells were lysed as described previously (23). The lysate was diluted in 2 volumes of 10 mM Tris-hydrochloride, pH 8.0–1 mM EDTA (TE) buffer and extracted several times with phenol equilibrated in TE buffer. The aqueous layer was then extracted once with chloroform to remove residual phenol, and the DNA was precipitated by adding 1/10 volume of 20% (wt/vol) sodium acetate and 2 volumes of ethanol. After standing at −70°C for 60 min, the DNA was recovered by centrifugation at 10,000 × *g* for 10 min and dissolved in 2 ml of TE buffer. The ethanol precipitation was repeated three times, and the DNA was dried under a vacuum and dissolved in 700 μl of TE buffer.

Isolation of plasmid DNA and transformation. Plasmid DNA was isolated as previously described (17). Transformation was carried out by the method of Cohen et al. (6). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverley, Mass.) and were used according to the instructions of the manufacturer.

Purification of phage DNA and in vitro packaging. Phage stocks were prepared routinely by using *E. coli* C600 as a host and indicator (17). Large-scale phage lysates for DNA purification were prepared as follows. A 200-ml volume of minimal medium (9) in 2-liter Erlenmeyer flasks was inoculated with 10^9 *E. coli* C600 cells and 2×10^8 phage particles. The flasks were incubated with shaking (200 rpm) for several hours until debris of lysed bacterial cells accumulated. Chloroform (0.5 ml) was then added and the shaking was continued for 10 min. The lysate was centrifuged at 10,000 × *g* for 10 min and the supernatant was collected. Phage particles were then precipitated by adding NaCl to a final concentration of 0.5 M and polyethylene glycol 6000 to 10% (wt/vol) followed by incubation at 4°C overnight. The precipitated phage were recovered by centrifugation at 500 × *g* for 30 min and suspended in 5 ml of 10 mM Tris-hydrochloride—5 mM $MgCl_2$–0.25 M NaCl, pH 7.5. Phage particles were further purified by sedimentation through two successive CsCl step gradients (9), and phage DNA was released by extraction with formamide (9). The extracted phage DNA was precipitated with ethanol, dried under vacuum, and dissolved in TE buffer.

In vitro packaging extracts were prepared from the *E. coli* strains BHB2688 and BHB2690, and DNA was packaged by the method of Hohn (16).

Isolation of Tn*5* insertion mutations. *E. coli* XAc (Su⁻) harboring pDU1148 was infected with λ467 (*rex*::Tn*5*) and kanamycin-resistant colonies were selected. Approximately 10^5 colonies were pooled, and the plasmid-linked insertions were selected as described previously (8).

Fractionation of *E. coli* cells. Membrane and cytoplasmic fractions of *E. coli* were prepared by passing late-exponential-phase cells through a French pressure cell at 10,000 lb/in^2 as described by Owen et al. (24). Osmotic shock was performed as described by Hazelbauer and Harayama (15).

Measurement of α-hemolysin production. The level of α-hemolysin production by hemolytic *E. coli* was measured by either hemolytic titration or by a specific enzyme-linked immunosorbent assay (ELISA) of extracts of cells prepared as described below. Cultures (100 ml) of the test strain were grown overnight with shaking, and the cells were harvested by centrifugation at 10,000 × *g* for 10 min, suspended in 10 ml of phosphate buffered saline (PBS), and placed on ice. DNase (Sigma London Chemical Co. Ltd., United Kingdom) was added to 100 μg/ml and the cells were lysed by passing them through a French pressure cell as described above. Cell debris was removed by centrifugation at 30,000 × *g* for 10 min and the supernatant was used directly for hemolytic titration or ELISA. Hemolytic titrations were performed in microtiter dishes with doubling dilutions of the hemolysin-containing sample made in PBS. An equal volume of 4% (wt/vol) washed rabbit erythrocytes in PBS was added, and the mixture was incubated at 37°C for 30 min. One hemolytic unit of α-hemolysin was defined as the highest twofold dilution of supernatant in PBS which resulted in visibly detectable lysis of rabbit erythrocytes in 30 min at 37°C in a microtiter assay well containing 25 μl of supernatant and 25 μl of erythrocytes. The ELISA was performed with peroxidase-conjugated specific rabbit anti-α-hemolysin antibody, using the double antibody sandwich technique (25). Unconjugated antibody (100 μl) dissolved in coating buffer (carbonate-bicarbonate, pH 7.6) (25) was added to the wells of a microtiter plate and the plates were incubated for 18 h at 4°C. The wells were then washed six times with PBS containing 0.05% Tween 20, and then antigen (100 μl) consisting of either purified α-hemolysin or lysates was added to the wells in the form of doubling dilutions in PBS-Tween 20 and the plates were incubated for 3 h at room temperature. The plates were then washed as before and dried, and 100 μl of peroxidase-conjugated anti-α-hemolysin was then added to each well; the incubation was continued for a further 3 h. The plates were washed and dried as before and 100 μl of substrate (0.04% [wt/vol] *ortho*-phenylenediamine, 0.05 ml of water, 10 mM phosphate-citrate buffer, pH 5.0) was added and left at room temperature for 30 min. Stopping buffer (50 μl of 2.5 M H_2SO_4) was then added to each well. The plates were then read spectrophotometrically at 405 nm with a MICROELISA MR 590 (Dynatech Instrument Inc., Calif.). The sensitivity was determined as 7.5 ng/ml.

RESULTS

Cloning *S. aureus* α-hemolysin in *E. coli*. A gene bank of *S. aureus* Wood 46, which produces high levels of α-hemolysin, was constructed by cloning *Sau*3A-generated fragments of Wood 46 total cell DNA between the *Bam*HI sites of the *E. coli* λ bacteriophage vector L47.1 (Fig. 1). Total cell DNA of *S. aureus* Wood 46 was partially digested with *Sau*3A under conditions which resulted in a high proportion of the cleaved DNA being in the region of 5 to 20 kbp in size, as determined by agarose gel electrophoresis. The vector L47.1 DNA was digested to completion with *Bam*HI, and without further

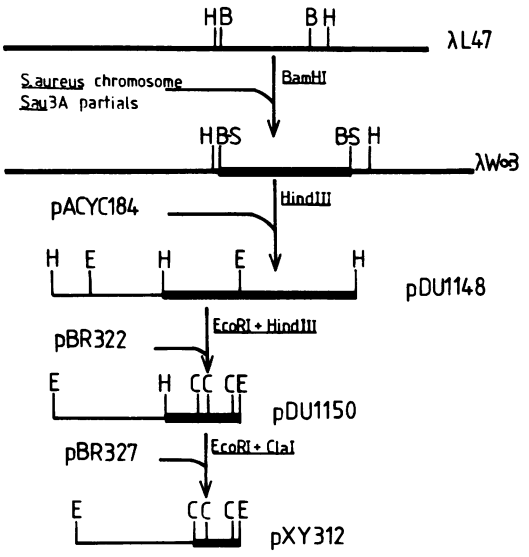

FIG. 1. Scheme for cloning and subcloning α-hemolysin determinant in *E. coli* K-12. The thick horizontal line represents cloned *S. aureus* DNA. L47.1 DNA is represented by the medium horizontal line, and plasmid DNA is represented by the thin horizontal line. Restriction endonuclease sites are: H, *Hin*dIII; B, *Bam*HI; S, *Sau*3A; E, *Eco*RI; C, *Cla*I. The phage and plasmids are drawn to different scales. For simplicity, only the vector encoded *Hin*dIII sites are shown on λwα3.

fractionation of the *Sau*3A-cleaved *S. aureus* DNA or purification of the λ *Bam*HI fragments, equal amounts (0.3 μg) of these digested DNA samples were mixed and ligated. The ligated mixture was packaged in vitro, and the resulting phage were plated onto the P2 lysogenic *E. coli* WL95. This strain selects directly for recombinant phage, since *gam* λ cannot grow on a P2 lysogen and the *gam* genes of L47.1 are located within the *Bam*HI fragment which was replaced in recombinant phage (20). In control experiments where L47.1 was plated at a high multiplicity onto *E. coli* WL95, no phage grew to form plaques. A total of 5,000 plaques, assumed to represent 5,000 independent recombinants, were obtained on plating the packaged ligation mixture on *E. coli* WL95. These were pooled, titrated, and without further amplification were screened for production of the *S. aureus* α-hemolysin.

Recombinant phage were plated on *E. coli* C600 to yield 300 to 500 plaques per plate. These plates were overlaid after 18 h of incubation with 2 ml of 1% (wt/vol) agarose in PBS containing 5% (vol/vol) washed rabbit erythrocytes. After 6 to 8 h of incubation at 37°C, zones of hemolysis approximately 3 to 5 mm in diameter were observed above a number of the plaques. These

plaques, which occurred at a frequency of 1 per 1,000, were picked and purified. The purified phage were plated on *E. coli* C600 to yield 10 to 20 plaques per plate and were overlaid with agarose containing either rabbit, human, or sheep erythrocytes. The largest and most rapidly developing hemolytic zones were observed on the rabbit erythrocyte plates, with much smaller and more slowly developing zones on the sheep erythrocyte overlays. No hemolysis was detectable on the human erythrocyte overlays. Incorporation of specific anti-α-hemolysin serum into the top agar immediately before pouring onto the base agar plate resulted in inhibition of hemolysis in all cases. Hemolysis was not inhibited by anti-β-hemolysin or by preimmune serum. Thus, the hemolytic activity associated with these recombinant phage was apparently due to *S. aureus* α-hemolysin. One of these phage, termed λwα3, was chosen for further study (Fig. 1).

Phage λwα3 DNA was not cleaved by *Bam*HI, indicating that both of the vector *Bam*HI sites were lost upon insertion of the *Sau*3A *S. aureus* DNA fragments. Cleavage of λwα3 with *Hin*dIII yielded five fragments. Two fragments of 23.0 and 8.1 kbp corresponded to the left and right L47.1 *Hin*dIII arms, respectively. The other three *Hin*dIII fragments were 7.6, 3.7, and 1.9 kbp in size. Two of these must be vector-cloned DNA junction fragments containing 0.5 kbp (left junction fragment) and 2.3 kbp (right junction fragment) of vector DNA sequences. Thus, 10.2 kbp of *S. aureus* DNA had been cloned in λwα3.

Subcloning of α-hemolysin determinant onto pACYC184. The *Hin*dIII fragments of λwα3 were cloned into the vector plasmid pACYC184. Chloramphenicol-resistant, tetracycline-susceptible transformants were stabbed onto rabbit blood agar plates to detect hemolysin production. After overnight incubation at 37°C, no hemolysis could be detected around any of the transformants. However after standing the plates for 2 days at room temperature, distinct zones of hemolysis began to develop around some of the colonies. All of the hemolytic transformants harbored the 7.6-kbp *Hin*dIII fragment of λwα3 cloned in pACYC184. One of these, termed pDU1148, was picked for further study (Fig. 1).

Cell-free supernatants prepared from cultures of *E. coli* C600 harboring pDU1148 possessed no detectable hemolytic activity even if the supernatant proteins were concentrated 200-fold by ammonium sulfate precipitation. However, when cells were harvested from late-exponential-phase cultures (ca. 4×10^8 cells per ml), concentrated 20-fold in PBS, and lysed in a French pressure cell, between 32 and 64 hemolytic units per ml of lysate could be detected. Fresh transformants of *E. coli* C600 grown to late-logarithmic phase and treated by the cold shock procedure of Hazelbauer and Harayama (15) yielded up to 160 hemolytic units per ml in the shock fluid. If the cells were subcultured for several passages on nutrient medium and the shock treatment was repeated, no hemolytic activity was released; however, hemolytic activity could still be detected within the cells if they were lysed as described above. At present, the reason for these changes are unknown. Hemolytic activity detected in cell fractions was completely neutralized by anti-α-hemolysin serum. Normal serum and anti-β-hemolysin serum had no neutralizing effect.

Mapping the α-hemolysin genes in pDU1148. A restriction map of pDU1148 was constructed by a series of double and triple enzyme digestions (Fig. 2). The cloned DNA fragment did not carry sites for the restriction enzymes *Bam*HI, *Xho*I, *Xma*I, *Ava*I, *Sal*I, *Sma*I, *Acc*I, and *Pvu*II. The map was used to construct a number of in vitro-generated deletion mutants of pDU1148, which were used to further localize the α-hemolysin determinant. Deletion of the 2.3 kbp of DNA between the *Pst*I sites of pDU1148 yielded the hemolytic plasmid pDU1194 and subcloning of the 2.1-kbp *Eco*RI-*Pst*I fragment and 4.6-kbp *Eco*RI-*Hin*dIII fragment of pDU1148 into pBR322 yielded the nonhemolytic plasmids pDU1192 and pDU1193, respectively. These plasmids show that the α-hemolysin determinant was either located within the 3.3-kbp *Hin*dIII-*Eco*RI fragment of pDU1148 or spanned the *Eco*RI site. Cloning the 3.3-kbp *Hin*dIII-*Eco*RI fragment into pBR322 yielded the hemolytic plasmid pDU1150 and showed that the α-hemolysin determinant was completely contained within this fragment.

Nine Tn*5* insertion mutants of pDU1148 which abolished the ability to express α-hemolysin were isolated and physically mapped within an approximately 500-base pair (bp) region of pDU1148 located within the 3.3-kbp *Eco*RI-*Hin*dIII fragment. All of the insertions mapped in a 550-bp *Kpn*I fragment. A deletion mutant of pDU1148 lacking the 550-bp *Kpn*I fragment was constructed and named pDU1191. This plasmid no longer directed the expression of α-hemolysin. Thus, part of the hemolysin determinant appeared to be located within the 550-bp *Kpn*I fragment.

To further localize the α-hemolysin determinant plasmid pDU1150, DNA was cleaved to completion with *Eco*RI and partially with *Cla*I, and the digestion products were separated by agarose gel electrophoresis. Two partial digestion products of approximately 1,070 and 1,620 bp were purified together from the gel, and these were ligated to the vector plasmid pBR327, which had been cleaved to completion with

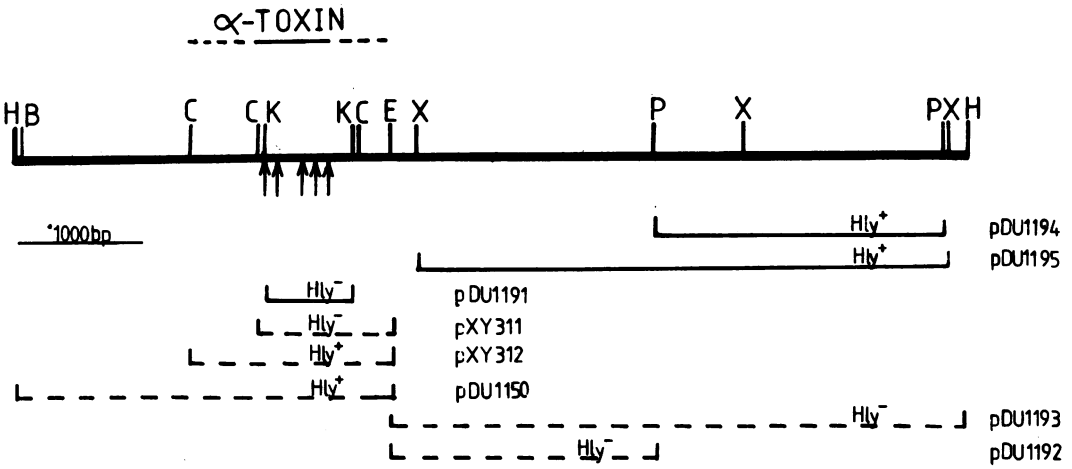

FIG. 2. Restriction map of the cloned DNA in pDU1148. Symbols: ———, sequences of pDNA deleted to form the corresponding plasmids; ------, sequence of DNA subcloned into pBR322 or pBR327 to form the corresponding plasmids (see text for details). Hly$^+$ designates hemolytic plasmids; Hly$^-$ designates nonhemolytic plasmids. Restriction endonuclease sites are: H, *Hin*dIII; B, *Bam*HI; C, *Cla*I; K, *Kpn*I; E, *Eco*RI; X, *Xho*I; P, *Pst*I. The vertical arrows show the position of the Tn*5* inserts in the pDU1148::Tn*5* plasmids.

*Eco*RI and *Cla*I. After transformation of *E. coli* C600 with selection for ampicillin resistance, two differently structured plasmids named pXY311 and pXY312, respectively (Fig. 2), were identified by screening plasmid DNA in the transformants. If *E. coli* C600 cells harboring pXY311 or pXY312 were stabbed onto blood agar plates, zones of hemolysis were only detected around cells harboring pXY312 and not around those harboring pXY311. Thus, the α-hemolysin determinant must be encoded within the 1,620-bp fragment of cloned DNA on pXY312.

Expression of α-hemolysin in *E. coli* K-12. The levels of α-hemolysin expressed in cells harboring pDU1148, pDU1148::Tn*5* plasmids, and other deletion or subcloned derivatives of pDU1148 were measured by a hemolytic titration of cell lysates or by ELISA (Table 2). Lysates prepared from cells harboring plasmids which produced a detectable zone of α-hemolysis after 3 days growth on rabbit blood agar plates all contained low levels of α-hemolysin of approximately 0.01 μg of α-hemolysin per mg of cytoplasmic protein extract as determined by the ELISA. No hemolysin or cross-reactive material was detected in cells harboring plasmids unable to produce zones of hemolysis on rabbit blood agar. Although strains harboring α-hemolytic plasmids synthesized hemolysin in a stable manner when propagated routinely on blood agar plates, some strains of *E. coli* K-12 were difficult or impossible to transform with plasmid DNA encoding active hemolysin synthesis. The reason for this is unknown.

DISCUSSION

α-Hemolysin is one of a number of extracellular products of *S. aureus* which have been implicated as a virulence factor. Many independent studies have demonstrated that the α-hemolysin may be the most important of these products in causing tissue damage and in establishing and maintaining infections (13). For this reason, we decided to study in detail the structure of the genetic determinant which encodes for the *S. aureus* α-hemolysin. Although almost all isolates of *S. aureus* produce a substance with α-hemolytic activity, the levels of production are known to vary under different growth

TABLE 2. Levels of hemolysin expressed by α-hemolytic plasmids in *E. coli* K-12

Plasmid or strain	Hemolytic titer (hemolytic units per ml)[a]	Hemolytic units per mg of protein[b]
C600	<2	<20
pDU1148	32	264
pDU1150	32	228
pDU1194	32	244
pDU1195	32	231
pDU1191	<2	<20
pXY311	<2	<25
pXY312	64	423
Pure α-hemolysin	8	21,333

[a] Determined from cell lysates as described in the text.

[b] pDU1148 expresses approximately 0.01 ng of α-hemolysin per μg of cell lysate.

conditions, and individual isolates can alternate between phases of high- and low-level α-hemolysin production (13). In common with other gram-positive virulence factors, virtually nothing is known about how *S. aureus* cells regulate the expression of α-hemolysin. Since it is very difficult to study the mechanism of α-hemolysin production in *S. aureus*, which expresses several other hemolysins, the α-hemolysin determinant was cloned into *E. coli* where it could be easily characterized. This approach greatly simplified the isolation of specific mutations in the α-hemolysin gene. It is intended to introduce specific mutations into the α-hemolysin gene and to transfer these back into virulent strains of *S. aureus* either by using plasmid vectors or by using homologous recombination with residual wild-type α-hemolysin determinants. The cloned α-hemolysin gene has already been successfully reintroduced into *S. aureus* by plasmid transformation (12).

The phage vector system proved very useful for the purpose of isolating the *S. aureus* α-hemolysin determinant from strain Wood 46 genomic DNA. Since the products expressed by recombinant phage in *E. coli* are released extracellularly, they can be detected without the need to artificially lyse whole cells. We have used the cloning system to clone a number of determinants which encode for hemolysins, including a phospholipase C from *Pseudomonas aeruginosa* (7) and streptolysin O from *Streptococcus pyogenes* (M. Kehoe and K. Timmis, unpublished data). Both of these products were expressed at readily detectable levels in *E. coli* K-12, which greatly simplified their identification.

The α-hemolysin determinant was localized to a small region of DNA of approximately 1,620 bp. So far we have been unable to implicate the involvement of more than one cistron in α-hemolysin production, although more detailed mapping will be required to eliminate this possibility. The *E. coli* α-hemolysin requires other cistrons in addition to that encoding the hemolysin structured protein for full expression of active protein to be achieved (22). These other proteins are associated with the cell envelope and are involved in transporting the hemolysins out of the cells. Such complex systems may not be required to transport proteins across the gram-positive cell envelope, which lacks an outer membrane barrier. Unlike in *S. aureus*, α-hemolysin production was clearly cell associated in *E. coli*. This is also the case for an extracellular *P. aeruginosa* hemolysin when it is expressed in *E. coli* (7). Apparently the active protein is only released when the cells begin to lyse in late-stationary phase.

Although the α-hemolysin was expressed in a stable manner by *E. coli* C600 cells, difficulties were encountered when transforming plasmid DNA encoding a functional α-hemolysin gene into a number of different *E. coli* strains. Further results obtained during attempts to localize α-hemolytic activity to subcellular fractions suggest that *E. coli* cells may undergo physiological adaptation when expressing the α-hemolysin. As yet we cannot explain these observations, although they could be related either to the levels of α-hemolysin expressed or tolerated by different strains. The expression of high levels of the membrane-active α-hemolysin in *E. coli* may interfere with normal membrane functions.

α-Hemolytic plasmids have been found to direct the synthesis of two novel polypeptides in *E. coli* minicells (11) of molecular weights 34,000 and 33,000 (unpublished data). At present we do not know whether these polypeptides represent a precursor and processed form of the same polypeptide or are two distinct gene products. Polypeptides of similar molecular weights can be precipitated by using anti-hemolysin antibodies if the cloned α-hemolysin gene is introduced into *Bacillus subtilis* (12).

We intend to further localize the position of the α-hemolysin gene on the cloned *S. aureus* DNA by DNA sequencing analysis. Determination of the DNA sequence may give insight into how the α-hemolysin protein interacts with eucaryotic cell membranes, how it is exported from bacterial cells, and how the expression is regulated at the level of transcription.

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