Molecular Cloning and Genetic Analysis of the Determinant for Gamma-Lysin, a Two-component Toxin of Staphylococcus aureus

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The γ-lysine determinant of Staphylococcus aureus strain Smith 5R has been cloned in phage λ and plasmid vectors in Escherichia coli. Genetic evidence is presented which demonstrates that γ-lysine requires the co-operative action of two polypeptides expressed by the closely linked hlgA and hlgB genes. Recombinants expressed haemolytic activity in agarose medium but not in agar, a known property of γ-lysine. Haemolysis was inhibited by antiserum raised against the 32 kDa component of γ-lysine, but not by anti-α-, anti-β- or anti-δ-lysine serum. Subcloning and transposon Tn5 mutagenesis identified a 3-5 kb region which was necessary for γ-lysine expression in E. coli. Two genes (hlgA and hlgB) were mapped and their polypeptide products identified. Non-haemolytic Tn5 mutants fell into two groups based upon complementation tests done between extracts of mutants in vitro and also between extracts of mutants and components of γ-lysine purified from S. aureus culture supernates. Immunoblotting showed that some mutants in group A (defective in expression of hlgA) did not express a 32 kDa polypeptide which was synthesized by the parental haemolytic recombinant and by mutants in group B. Minicell analysis suggested that the products of the hlgB gene were proteins of 38 kDa and 36 kDa. The smaller molecule co-migrates with a protein in a fraction of the S. aureus culture supernate containing component B of γ-lysine. The 38 kDa polypeptide is probably an unprocessed precursor. Southern hybridization demonstrated that the hlgA and hlgB genes are closely linked in the chromosome of several strains of S. aureus.

INTRODUCTION

Staphylococcus aureus is a Gram-positive pathogenic bacterium which causes a variety of human tissue infections including furuncles, wound sepsis, osteomyelitis and endocarditis. It produces an array of extracellular and cell-bound proteins which are potentially important in pathogenesis (for reviews see McCartney & Arbuthnott, 1978; Easmon & Adlam, 1983). These include several cytolytic toxins, of which γ-lysine is one of the least well characterized. The organism is also the major cause of mastitis, an economically important disease of dairy cattle (Anderson, 1983).

Several groups have reported the purification of γ-lysine, but there is conflicting evidence about the number of polypeptide components, their molecular masses and their isoelectric points. Mollby & Wadstrom (1971) described a single 30 kDa polypeptide of pi 9.5, while Fackrell & Wiseman (1976a, b) reported a single 45 kDa protein of pi 6-0. Others have shown that haemolytic activity requires two polypeptides (Guyonnet & Plommet, 1970; Guyonnet et al., 1968; Taylor & Bernheimer, 1974) of 29 kDa and 26 kDa with pi values of 9-9 and 9-8, respectively (Taylor & Bernheimer, 1974).

Little is known about the mode of action of γ-lysine or of its role in pathogenesis. Elevated titres of antibodies to the lysin have been detected in sera of patients with osteomyelitis (Taylor & Plommet, 1973), showing that γ-lysine is expressed in vivo during chronic infections. More
recently it has been shown that most toxic-shock-syndrome-associated strains of *S. aureus* produce γ-lysin but fail to produce α-toxin or β-toxin (Clyne *et al.*, 1987).

One method of testing the role of a toxin in pathogenesis is to examine the virulence of toxin-deficient mutants in experimental infections (Sparling, 1983; Jonsson *et al.*, 1985; O'Reilly *et al.*, 1986). In order to facilitate the future isolation of γ-lysin-deficient mutants we set out to clone and map the determinant from strain Smith 5R. Another objective was to determine unambiguously the number of polypeptides involved in γ-lysin activity.

**METHODS**

**Bacterial strains, plasmids and phages.** The bacterial strains are listed in Table 1 and the plasmids in Table 2. The replacement vector λA47.1 (Loenen & Brammar, 1980) was used to clone the hlg determinant. λA67 (cI857 rex::Tn5 o29 p80) was obtained from N. Kleckner (Harvard University, Cambridge, USA).

**Bacteriological media.** *Escherichia coli* strains were grown in L broth and on L agar (Miller, 1972). *S. aureus* was cultured in Trypticase Soy Broth and Agar (Oxoid). λ phages were propagated in λ base and top agar (Miller, 1972). Brain heart infusion broth (Oxoid) was used for culturing *E. coli* DS410 strains for producing minicells (Dougan & Kehoe, 1984).

**Chemicals and enzymes.** Chemicals were obtained from Sigma or were the best grade available from BDH. Ampicillin (Ap), kanamycin (Km) and tetracycline (Tc) were purchased from Sigma. Restriction enzymes and T4 DNA ligase were obtained from the Boehringer Corporation and were used according to the manufacturer's instructions.

**Antisera.** Rabbit antisera prepared against γ-lysin were from two sources. Dr T. H. Birkbeck (Microbiology Department, Glasgow University, UK) kindly provided the serum which was used in early experiments. More recently serum against the purified 32 kDa component of γ-lysin was kindly provided by M. Clyne (Microbiology Department, Trinity College, Dublin). Anti-α-, anti-β- and anti-δ-lysin sera were generously supplied by Dr C. Adlam (Wellcome Biotechnology, Beckenham, UK).

**Assays for haemolytic activity.** *E. coli* strains were tested for haemolysis by stabbing colonies onto L agarose plates containing 5% (v/v) washed rabbit erythrocytes and incubating at 37°C for 24 h. Haemolytic titrations were...
performed by making doubling dilutions of E. coli sonicates (25 μl volumes) in microtitre trays using phosphate-buffered saline (0.067 M-sodium phosphate, 0.07 M-NaCl, pH 7.0; PBS). An equal volume of washed 2% (v/v) rabbit erythrocytes in the same buffer was added and incubated at 37 °C for 45 min. One haemolytic unit (HU) was defined as the reciprocal of the dilution giving 50% lysis.

Complementation tests were done in vitro by pipetting 25 μl volumes of E. coli cell lysates into 4 mm wells in agarose containing 5% (v/v) washed rabbit erythrocytes. Extracts were either placed separately into adjacent wells (diffusion test) or were mixed together in the same well. Plates were incubated at 37 °C for 18 h and at 4 °C for a further 2–3 d. Tests were also done using purified γ-lysin components (about 2 μg protein per well, provided by M. Clyne, Microbiology Department, Trinity College, Dublin).

Immunchemical analysis of γ-lysin. Proteins produced in λhlg lysates were prepared as described previously (O'Toole & Foster, 1986a). Lysates of pJC-plasmid-carrying strains of E. coli were prepared by sonication of cells from 100 ml L broth cultures grown to stationary phase in 250 ml flasks shaken at 150 r.p.m. at 37 °C for 18 h. Cells were concentrated 30-fold in PBS. Proteins were separated by SDS-PAGE (Laemmli, 1970) and subjected to immunoblotting (Burnette, 1981). Filters were incubated with rabbit antiserum prepared against the purified 32 kDa component of γ-lysin followed by peroxidase-conjugated protein A. Colony immunoblotting was done by the method of Helfman et al. (1983).

Transformation. E. coli strains were transformed with plasmid DNA as described by Maniatis et al. (1982).

Molecular cloning. DNA manipulations were performed using standard procedures (Maniatis et al., 1982). A library of S. aureus Smith 5R DNA was constructed in λλ47.1 as described previously (O'Toole & Foster, 1986a), except that the genomic DNA was cleaved partially with Sau3AI and the λ replacement vector was cut with BamHI. Recombinant phages from the library were plated on E. coli C600 and plaques were screened by overlaying with agarose containing rabbit erythrocytes. Plaques producing a zone of haemolysis after a further 4–8 h incubation at 37 °C were picked and purified. They were replated and the identity of the haemolysin was tested by placing small drops of antisera specific for S. aureus haemolysins over the plaques prior to incubation with the erythrocyte overlay. Only anti-γ-lysin serum inhibited haemolysis, whereas anti-β-, anti-δ- and anti-α-lysin sera had no effect. In addition, substitution of agar for agarose in the erythrocyte overlay inhibited complementation of the γ-lysin determinant by E. coli C600 cells containing the recombinant plasmids. Concentrated lysates from the recombinant phages expressing γ-lysin formed colonies on L agarose erythrocyte medium which were surrounded by haemolysis after 18 h incubation, and lysates contained 128 HU ml⁻¹.

In order to construct a vector plasmid which lacked the β-lactamase determinant, for use in minicell experiments, the 1-07 and 0.92 kb PstI fragments of Tn5 (which carry the Km' marker) were cloned into the PstI site of pBR322, forming pJC10 (Fig. 1). Subsequently a 6 kb EcoRV fragment was subcloned from pJC10 into pBR322, forming pJC8 (Fig. 1). E. coli C600 cells containing either plasmid produced haemolytic activity when concentrated lysates were tested (4 and 64 HU ml⁻¹, respectively) but colonies were not haemolytic when grown on L agarose incorporating rabbit erythrocytes. Consequently, a 6 kb HindIII fragment was subcloned from pJC8 into the expression vector pKK233-2 (Amann & Brosius, 1985), forming pJC9 (Fig. 1). pKK233-2 has the strong trc promoter located adjacent to the HindIII cloning site. In the lac+ strain C600 the trc promoter is expressed constitutively, presumably because the small number of lac repressor molecules are titrated out by multiple copies of the lac operator. E. coli C600 carrying plasmid pJC9 formed colonies on L agarose erythrocyte medium which were surrounded by haemolysis after 18 h incubation, and lysates contained 128 HU ml⁻¹.

In order to construct a vector plasmid which lacked the β-lactamase determinant, for use in minicell experiments, the 1-07 and 0.92 kb PstI fragments of Tn5 (which carry the Km' marker) were cloned into the PstI site of pBR322, forming pJC10. A 7-2 kb PvuI–BanHI fragment from pJC9 carrying the entire hlg insert linked to the trc promoter (Fig. 1) was subcloned into pJC10 cleaved with PvuI and BanHI to form pJC11. This plasmid expressed γ-lysin at the same level as pJC9.

Transposon mutagenesis. Transposon Tn5 mutagenesis was performed by infecting E. coli strain XACSu-carrying pJC9 with A67 as described previously (Coleman & Foster, 1981).

Minicells. Polypeptides specified by hlg plasmids were detected using the minicell system. E. coli strain DS410 transformed with plasmids and minicells were prepared, labelled and fractionated as described by Dougan & Kehoe (1984). Samples were chased for 45 min at 37 °C with unlabelled methionine after the labelling step in order to reduce the level of the β-lactamase precursor (Dougan & Kehoe, 1984).

Southern hybridization. Probes were labelled by nick-translation in vitro (Rigby et al., 1977) and Southern blot hybridization (Southern, 1975) was carried out as described in Maniatis et al. (1982). The 1-4 kb XbaI fragment which spans hlgA, and the 1-2 kb EcoRI–XbaI fragment covering hlgB (Fig. 1), were used as probes.

RESULTS

Analysis of the cloned γ-lysin determinant

The γ-lysin determinant, cloned in the expression vector pKK233-2 (pJC9), was mapped with restriction enzymes and analysed by transposon Tn5 mutagenesis. Ten independent Tn5 insertions which abolished the ability of pJC9 to express haemolysis around colonies were
isolated and mapped (Fig. 1). The insertions spanned 3.5 kb. One mutant (number 1) mapped in the small piece of pBR322 DNA located between the cloned hlg sequences and the vector. This implies that Tn5 is preventing expression of haemolytic activity in this mutant (and possibly some others) by polarity. Extracts of cells carrying mutants 3, 5 and 6 had 128, 32 and 32 HU ml⁻¹ respectively whereas the other mutants were devoid of detectable haemolytic activity.

Complementation tests

Samples of lysates of E. coli C600 carrying pJC09hlg : :Tn5 mutant plasmids were tested in all paired combinations for enhanced haemolysis. One example of a complementation test is shown in Fig. 2(a). The mutants fell into two classes based on complementation of haemolytic activity. Mutants 1, 2, 3, 4 and 5, mapping in the trc-promoter-proximal region, each complemented mutants 6, 7, 8, 9 and 10, which are clustered distally to the trc promoter. The mutants thus formed two complementation groups, which we designated A and B, respectively. Mutant 6 also complemented mutants 7–10 in a diffusion test that took 7 d to give a result.

Complementation tests were also performed between extracts of mutants from groups A and B and γ-lysin components purified from culture supernates of S. aureus. Lysates of group B mutants underwent complementation with a fraction containing the 36 kDa protein, while group A mutants were complemented by the 32 kDa component (Fig. 2b). This suggests that group A mutants are deficient in expression of the 32 kDa protein while group B strains lack the 36 kDa peptide.

Analysis of hlg-specified polypeptides

Immunoblotting was done with extracts of strains carrying pJC09hlg : :Tn5 plasmids using antiserum raised against the purified 32 kDa component of γ-lysin. Mutants 3, 5, 6, 7, 8, 9 and 10 produced an immunoreactive protein of 32 kDa while mutants 1, 2 and 4 did not (Fig. 3). The
two complementation group A mutants which expressed immunoreactive protein were those that also had haemolytic activity in lysates. All group B mutants expressed an immunoreactive protein.

Polypeptide expression of the cloned hlg determinant was analysed using the E. coli minicell system. In addition to pJC09 and the pJC09hlg::Tn5 mutants used in the experiments described above, an additional plasmid carrying hlg (pJC11) which lacked a β-lactamase
Minicell analysis of polypeptides expressed by \textit{hlg} plasmids. Samples were separated by SDS-PAGE on a 7\% (w/v) gel. Protein samples were stained with Coomassie Brilliant Blue (tracks A–C). Minicells were analysed by autoradiography (tracks D–K). The filled arrows indicate the positions of molecular mass markers. The open arrows indicate the 36 kDa and 32 kDa components of \textit{\textgamma}-lysin. The 36 kDa protein band in tracks B, E, G, I and K is also marked with an arrow. Track A, molecular mass standards; track B, partly purified 36 kDa component B of \textit{\textgamma}-lysin (the band of lower molecular mass is a contaminant); track C, 32 kDa component of \textit{\textgamma}-lysin; track D, pKK233-2; track E, pJC09; track F, pJC10; track G, pJC11; track H, pJC09\textit{hlg} ::\textit{Tn5} mutant 8; track I, pJC09\textit{hlg} ::\textit{Tn5} mutant 4; track J, pJC09\textit{hlg} ::\textit{Tn5} mutant 10, track K, pJC09\textit{hlg} ::\textit{Tn5} mutant 6. The photograph of track K has been overexposed compared to tracks D–J.

determinant was studied. Wild-type plasmids pJC09 and pJC11 and mutant 4 in complementation group A expressed polypeptides of 38 and 36 kDa which were absent in vector controls (Fig. 4, tracks E, G and I). The other group A mutants also expressed these proteins (data not shown). The 36 kDa protein comigrated with a polypeptide in the fraction of an \textit{S. aureus} Smith 5R culture supernate containing the \textit{\textgamma}-lysin B component (Fig. 4, track B).

\textit{Tn5} mutants 8 and 10 in complementation group B were tested in the minicell system and were shown to lack the 38 and 36 kDa polypeptides while for mutant 6, trace amounts were observed (Fig. 4, tracks H, J and K). (Mutant 6 was the only group B mutant to express haemolytic activity in lysates.) These results suggest that the 38 kDa and 36 kDa proteins are products of the \textit{hlgB} gene. It seems likely that the 38 kDa protein is a precursor of the 36 kDa component B of \textit{\textgamma}-lysin.

The 32 kDa product of the \textit{hlgA} gene was also discernible in the pJC11 sample, which lacked TEM \textbeta-lactamase (Fig. 4, track G), but it migrated too close to \textbeta-lactamase to be identified in pJC09 or the \textit{Tn5} mutants.
The method used to construct the library of *S. aureus* Smith 5R DNA allowed the possibility that different genomic fragments could have been mixed in the same vector. In order to confirm that the *hlgA* and *hlgB* genes are linked in the chromosome of *S. aureus* in the same way as in pJC09 (Fig. 1), Southern blot hybridization analysis was done with DNA of strains Smith 5R, 8325-4 and M60. Genomic DNA was cleaved with restriction endonucleases *Hind*III and *Pst*I, enzymes that do not cleave within the cloned Smith 5R hlg determinant (Fig. 1). Samples were hybridized with probes specific for the *hlgA* and *hlgB* genes. In strain Smith 5R the two probes hybridized to *Hind*III fragments of the same size (Fig. 5, tracks N and T). Similar results were obtained when DNA from strains M60 and 8325-4 was tested (Fig. 5, tracks L, J, R and P). Interestingly, the 8325-4 and M60 samples revealed the presence of an additional *Hind*III site obtained when DNA from strains M60 and 8325-4 was tested (Fig. 5, tracks L, J, R and P). The same type of result was obtained when the chromosomal DNA was cut with *Pst*I (Fig. 5, tracks I, K, M, O, Q and S). Thus the *hlgA* and *hlgB* probes hybridized to genomic fragments of the same size (with the exception of 8325-4 and M60 DNA cleaved with *Hind*III and probed with *hlgA*). These results are consistent with the genes being closely linked in the *S. aureus* chromosome.

The cloned *hlgA* and *hlgB* genes are located on contiguous *XbaI* fragments of 1-4 and 1-45 kb in pJC09 (Fig. 1). Fragments of 1-4 kb hybridized when genomic DNA of the three strains was cleaved with *XbaI* and probed with the *hlgA* specific probes (Fig. 5, tracks A–D). A 1-45 kb fragment hybridized to the *hlgB* probe (Fig. 5, tracks E–H).

**DISCUSSION**

In this paper we present molecular genetic evidence that the γ-lysin determinant of *S. aureus* requires the synergistic activity of two proteins. The gene products were identified by immunoblotting and in the minicell system as proteins of 32 kDa and 36 kDa. The genes which code for the proteins (*hlgA* and *hlgB*, respectively) were mapped. *In vitro* complementation tests between extracts of *E. coli* strains carrying mutant plasmids provide convincing evidence that γ-lysin involves two components. This was supported by the complementation that occurred between extracts of mutants and purified components of γ-lysin.

Physical mapping of the *hlg*: Tn5 insertions allowed the complementation groups to be correlated with two discrete clusters of mutants. Mutants 6, 7, 8, 9 and 10 in complementation group B define the *hlgB* gene. Group A comprises mutants 1, 2, 3, 4 and 5. However, the group A mutants span 2-5 kb of DNA, which is much longer than is required to code for the putative *hlgA* gene product of 32 kDa. Mutation 1 is actually located in a small section of pBB322 DNA located between the *trc* promoter of pKK233-2 and the cloned sequences. It is thus likely that Tn5 mutants 1 and 2 fail to express haemolysin due to polarity. If this is so, the promoter for the *hlgA* gene must be poorly expressed in *E. coli*. This is consistent with the level of expression of haemolysin by pJC01, pJC08 and pJC09. Plasmid pJC01 expresses a very low level of haemolysin which is detectable only in extracts. In subcloning *hlg* to form pJC08, the *hlgA* gene was moved closer to the *tet* promoter of pBB322 and a higher level of expression was measured. When the fragment was recloned so that *hlgA* was downstream from the *trc* promoter, a higher level of haemolysin was observed. In addition, pJC09-carrying cells formed colonies which were haemolytic on blood agar. These results imply that the *hlgA* promoter is not active in *E. coli* and furthermore the direction of transcription of *hlgA* can be inferred to be from left to right as indicated in Fig. 1.

The data also suggest that the *hlgA* and *hlgB* genes are expressed from different promoters in *E. coli*. This may also be the case in *S. aureus*. Strongly polar Tn5 insertions in group A still express the 36 kDa protein product of the *hlgB* gene, as detected in minicells and in complementation tests. Indeed the direction of transcription of *hlgB* relative to *hlgA* is unknown.

Two mutants in complementation group A produced measurable amounts of haemolysin and a protein that cross-reacted immunologically with the 32 kDa component of γ-lysin. The most
Fig. 5. Southern blotting. Genomic DNA of *S. aureus* strains 8325-4 (tracks A, E, I, O, P), M60 (tracks B, F, K, L, Q, R) and Smith 5R (tracks C, G, M, N, S, T) was cleaved with restriction endonucleases *Xba*I, *Pst*I (*PI*) or *Hind*III (*HI*III) as indicated. The probes were the *hlgB*-specific fragment (tracks A–D; I–N) or the *hlgA*-specific fragment (tracks E–H; O–T). Tracks D and H have *Xba*I-cleaved pJC09.
likely explanation is that mutation 3 is weakly polar in comparison to mutations 1 and 2 (Tn5 polarity is variable: Berg et al., 1980). Mutations 4 and 5 are probably located in the hlgA structural gene since they are the group A mutations most distal to the trc promoter. However, mutant 5 also produces a protein which cross-reacts with the 32 kDa γ-lysin component and is weakly haemolytic. Perhaps Tn5 has inserted in the 3′ end of the hlgA coding sequence resulting in a partly functional protein.

All mutants in complementation group B failed to produce 38 kDa and 36 kDa proteins and were non-haemolytic, with the exception of mutant 6, which had weak haemolytic activity and expressed reduced levels of the proteins in minicells. In addition, mutant 6 complemented mutants 7–10 in a very slow reaction. We suggest that the transposon is located in the intercistronic region between hlgA and hlgB, possibly in the promoter or regulatory region of the hlgB gene, and reduces expression of the 38 kDa and 36 kDa proteins. It seems likely that the 38 kDa protein is the unprocessed form of the 36 kDa protein which normally appears in S. aureus culture supernates. The latter protein comigrated with component B of γ-lysin from S. aureus. Both proteins are missing in hlgB: ::Tn5 mutants.

The results imply that hlgB is expressed from its own promoter in E. coli. According to complementation tests the promoter-proximal insertions 1–5 express the 36 kDa protein and they all complement mutants in group B. Thus we can conclude that the hlgA and hlgB genes are expressed from separate promoters in E. coli. The hlgA promoter may be similar to the promoters of the epidermolytic toxin B (O'Toole & Foster, 1986b) and enterotoxin B genes (Ranelli et al., 1985) of S. aureus, which are not expressed in E. coli. The possibility that the hlgA promoter was lost during the cloning seems unlikely unless the hlgA gene is part of a larger transcription unit.

Southern blot hybridization showed that the hlgA and hlgB genes were linked in the chromosomes of S. aureus, thus eliminating the possibility that linkage was generated during cloning in the phage vector. Two restriction enzymes which did not cleave within the hlg locus cloned in pJC09 were used to cut genomic DNA. The hlgA and hlgB probes hybridized to HindIII fragments of the same size in S. aureus strain 8325-4. A similar result was obtained for strains M60 and Smith 5R and when genomic DNA was cut with PstI. Thus the hlgA and hlgB genes are probably located on the same fragments (in M60 and 8325-4 an additional HindIII site is present in the hlgA gene). Further evidence for linkage was obtained from hybridization of XbaI-cleaved DNA. The hlgA and hlgB genes are located on adjacent XbaI fragments of 1-4 kb and 1-45 kb, respectively, in pJC09. Chromosomal DNA fragments of the same size hybridize, which also supports the notion that close linkage of the genes was not created during cloning. If the genes were not linked then at least one XbaI fragment of different size would be expected in hybridizations.

There is no compelling evidence for an important role for γ-lysin in pathogenesis of S. aureus infections. We propose to examine this question by isolating specific mutants in the hlgA and hlgB genes using allele-replacement (O'Reilly et al., 1986) and by testing their virulence in experimental infections.

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