The hyaluronate lyase of *Staphylococcus aureus* – a virulence factor?

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INTRODUCTION

The human and animal pathogen *Staphylococcus aureus* represents a major cause of infection. This organism possesses a formidable range of gene products which allow it to colonize the host, invade through minor breaks in skin and mucous membranes, disseminate through the body, cause overt pathology, and evade host defence mechanisms. As many as 30 potential virulence determinants have been described for this pathogen, with a wide range of biological activities. It is generally held that no single virulence factor is responsible for the pathogenicity of staphylococci, and that disease occurs due to a complex series of processes, with the appropriate pathogenic factors being present at each stage (Jeljaszewicz, 1983). The enzyme hyaluronate lyase (HL) represents such a factor.

HL, a long-neglected potential virulence determinant of *S. aureus*, is produced by the organism as an extracellular enzyme capable of degrading the acidic mucopolysaccharide hyaluronic acid (HA), a major component of the intercellular ground substance of human and animal connective tissue (Arvidson, 1983). Duran-Reynals (1933) observed that culture extracts of *S. aureus* increased permeability of rabbit tissue to vaccinia virus, toxins and dyes. Subsequently, the ‘spreading factor’ was identified as a HL (Linker et al., 1955). A study (Pritchard & Lin, 1993) with group B streptococci (GBS), indicated that the enzyme, previously reported to be a neuraminidase, was in fact a HL. Clearly, previous reports describing an association of elevated levels of this enzyme with virulent strains of GBS add support to the hypothesis that HL is also a virulence factor of staphylococci. In addition, the observation that 91·2% of *S. aureus* strains produce HL (Choudhuri & Chakrabarty, 1969) strengthens the case for a role in virulence. The production of HL from other staphylococcal strains is either negative or variable, though *Staphylococcus hyicus* is positive for HL production and is also an opportunistic pathogen in animals (Devriese, 1990).

Studies on HL regulation were previously initiated by Taylor & Holland (1991). In addition the HL gene, *hysA*, has been previously cloned from a genomic library of *S. aureus* (Farrell et al., 1995). The present investigation was undertaken in order to assess the importance of HL in the virulence of *S. aureus in vivo*. A mutant deficient in HL activity was compared with wild-type *S. aureus*, using an animal model. The roles of the *S. aureus* global regulators, *agr* and *sar*, on the *in vitro* production of HL were also examined.

The hyaluronate lyase (HL) gene of *Staphylococcus aureus* 8325-4 (*hysA*) was inactivated *in vitro* with the insertion of the erythromycin determinant, *ermC*, from plasmid pE194. The *hysA::ermC* mutation was introduced into *S. aureus* via a temperature-sensitive shuttle vector, where it underwent homologous recombination with the wild-type (w.t.) allele. The insertion of *ermC* in the chromosomal *hysA* locus was confirmed by Southern blot hybridization and the loss of HL activity was demonstrated macroscopically by a plate assay. The importance of HL for pathogenicity was assessed by comparing the virulence of the HL− mutant strain to that of the w.t. in an established mouse abscess model of *S. aureus* infection. A significantly higher cell recovery was obtained from lesions infected with the w.t. strain compared to the lesions infected with the HL− strain ($P = 0·01$). Although the lesion areas from both groups were not significantly different ($P = 0·9$) they were of different morphology. A colorimetric assay was used to measure HL activity from culture supernatants of the *S. aureus* 8325-4 strains w.t., WA250 (agr) and PC1839 (sar) grown in a chemically defined medium. HL activity reached a maximum in the w.t. strain during mid-exponential phase ($t = 5\ h$) and while it showed a 16-fold decrease in the *agr* mutant it increased 35-fold in the *sar* mutant background. These results strongly suggest that HL is a virulence factor which is important in the early stages of subcutaneous infections.

Abbreviations: HA, hyaluronic acid; HL, hyaluronate lyase; NAG, N-acetyl-D-glucosamine; TS, temperature sensitive.

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investigated. In the longer term such studies are relevant to the potential future of research directed at designing agents which interfere with virulence determinant expression in this important pathogen.

METHODS

Bacterial strains, plasmids and culture conditions. *S. aureus* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. For cloning and DNA manipulations in *E. coli*, strain XL-1 Blue (Stratagene) was used. To enable transfer of recombinant DNA to *S. aureus* from *E. coli*, plasmids were passaged through *S. aureus* RN4220 (Kreiswirth et al., 1983), a restriction-deficient strain derived from *S. aureus* 8325-4 (Novick, 1967). *E. coli* was grown at 37°C with shaking at 160 r.p.m. in Luria–Bertani (LB) medium (Oxoid) and at a culture: flask volume ratio of 0:2:1. *S. aureus* was grown at 37°C with shaking at 160 r.p.m. in brain-heart infusion (BHI) medium (Oxoid), or in a modification of the chemically defined medium (CDM) of Taylor & Holland (1989). The components of CDM are (concentrations in mg l⁻¹ in parentheses): L-tryptophan (41), L-cystine (165), L-methionine (124), L-tyrosine (1240), L-histidine (330), L-aspartic acid (1650), L-threonine (1650), L-serine (1650), L-proline (1650), L-threonine (1650), L-serine (1650), L-proline (1650), L-glycine (410), L-lysine (400), L-leucine (400), L-arginine (247), L-glutamic acid (1650), L-tryptophan (41), L-cystine (165), L-methionine (124), L-tyrosine (1240), L-histidine (330).

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics*</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
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<tr>
<td>RN4220</td>
<td>Restriction-system-deficient strain</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>8325-4</td>
<td>Wild-type strain cured of known prophages</td>
<td>Novick (1967)</td>
</tr>
<tr>
<td>PC1839</td>
<td>saraA::km Km⁻²</td>
<td>Chan &amp; Foster (1998)</td>
</tr>
<tr>
<td>WA250</td>
<td>ogra::Tn551 Em⁺</td>
<td>Morfeldt et al. (1988)</td>
</tr>
<tr>
<td>HL-6</td>
<td>HL-deficient 8325-4 (Em⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>HL-13T</td>
<td>Parental 8325-4 transductant 13 carrying hysA mutation (Em⁺)</td>
<td>This study</td>
</tr>
<tr>
<td>HL-16T</td>
<td>Parental 8325-4 transductant 16 carrying hysA mutation (Em⁺)</td>
<td>This study</td>
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<tr>
<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>recA1 endA gyrA96 thi-1 hsdR17 supE44 relA lacF pro AB lacP²Z ΔM15 Tn10(Tc⁺)</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript SK</td>
<td>Cloning vector, Ap⁺</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pCR2.1</td>
<td>PCR cloning vector, Ap⁺ Km⁻²</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUL5032</td>
<td>A 2-6 kb EcoRI fragment from pUL5030 containing a promoterless hysA cloned into pBluescript SK, Ap⁺</td>
<td>Farrell et al. (1995)</td>
</tr>
<tr>
<td>pE194</td>
<td>Class I staphylococcal plasmid containing 1-44 kb ermC gene, Em⁺</td>
<td>Horinouchi &amp; Weisblum (1982)</td>
</tr>
<tr>
<td>pTS2tet</td>
<td>Temperature-sensitive derivative of pE194, Cm⁺ Tc⁻</td>
<td>Fitzgerald &amp; Foster (2000)</td>
</tr>
<tr>
<td>pCU1</td>
<td>Shuttle vector derived from the ligation of pUC19 with pCLP100 (a pCI94 derivative), Ap⁺ Cm⁺</td>
<td>Augustin et al. (1992)</td>
</tr>
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<td>pGM01</td>
<td>pCR2.1 containing PCRed ermC with incorporated NsiI sites, Ap⁺ Em⁺ Km⁻²</td>
<td>This study</td>
</tr>
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<td>pGM02</td>
<td>pUL5032 containing ermC upstream of the active site of hysA, Ap⁺ Em⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGM03</td>
<td>pGM02 fused with pTS2tet, Ap⁺ Cm⁺ Em⁺ Tc⁻</td>
<td>This study</td>
</tr>
<tr>
<td>pGM04</td>
<td>A 7-2 kb HindIII fragment from pUL5030 cloned into pCU1, Ap⁺ Cm⁺</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Abbreviations: Km, Em, Tc, Ap, resistance to kanamycin, erythromycin, tetracycline, ampicillin, respectively.

Antibiotics were added to the media appropriately at the following final concentrations: 100 μg ampicillin (Ap) ml⁻¹, 10 μg chloramphenicol (Cm) ml⁻¹, 10 μg erythromycin (Em) ml⁻¹ and 5 μg tetracycline (Tc) ml⁻¹. LK top and bottom agar were prepared by the addition of 0-7 and 1-0% (w/v) agar no. 1 (Oxoid L11) respectively. Antibiotics were added to the media appropriately at the following final concentrations: 100 μg ampicillin (Ap) ml⁻¹, 10 μg chloramphenicol (Cm) ml⁻¹, 10 μg erythromycin (Em) ml⁻¹ and 5 μg tetracycline (Tc) ml⁻¹. LK top and bottom agar contained 25 and 0-15 μg Em ml⁻¹, respectively. LK top agar was also supplemented with 25 μg lincomycin (Lm) ml⁻¹.

DNA techniques. All chemicals were purchased from Sigma unless otherwise specified. Procedures for DNA restriction, dephosphorylation and ligation, agarose gel electrophoresis and PCR were performed as described by Sambrook et al. (1989). Nucleotide sequencing was achieved by the BigDye Terminator cycle reaction (Lark Technologies). For Southern blot hybridizations, digested DNA was transferred onto a Hybond-N⁺ membrane (Roche). DNA
probes were labelled using digoxigenin (DIG) (Roche). PCR products and linearized plasmid DNA fragments were extracted from agarose using the JETsorb Gel Extraction kit (Genomed), and were purified with the Qiaquick nucleotide removal kit (Qiagen). Plasmid DNA was prepared from E. coli by the method of Birnboim & Doly (1979) and was transformed into the same organism by the method of Hanahan (1983). Staphylococcal plasmid DNA was prepared as before, with the substitution of lysostaphin (Sigma) to 50 μg ml⁻¹ in place of lysozyme. Electropotent S. aureus cells were prepared and transformed with plasmid DNA as described by Augustin & Götz (1990). Staphylococcal genomic DNA was prepared as previously described (Parrett & Neveln, 1975; Lindberg et al., 1972) with the following additions. After harvesting, the cells were resuspended in 0·1 vol. EDTA (10 mM) and incubated on ice for 15 min. Lysostaphin and RNase were added to the resuspension buffer to a final concentration of 50 μg ml⁻¹ and the suspension was incubated at 37 °C with gentle agitation for 1·5 h.

**Construction of a temperature-sensitive (TS) shuttle plasmid.**

Plasmid pUL5032 carries a 2·6 kb EcoRI fragment which contains most of the HL gene’s ORF, hysA, with the start site and an 80 bp segment downstream from it missing (Farrell et al., 1995). A mutation was constructed in this incomplete hysA gene by insertion of the 1·44 kb erythromycin resistance (Em') determinant (ermC) from plasmid pE194 (Villafane et al., 1987). Forward (5'–GATCTAGCTTAGAATTGATCTCTG-3'; nucleotides 3386 to 3373) and reverse (5'-GACATGCTAATCGTACCACAACA-3'; nucleotides 1937 to 1951) primers were designed according to the published sequence of pE194 (Horinouchi & Weisblum, 1982), with the additional restriction site NsiI introduced at the 5' end of each primer (underlined). The tails added to the 5' ends of the primers are shown in bold. The amplified ermC fragment was ligated with the PCR vector pCR2.1 (Invitrogen) and the DNA was transformed into E. coli selecting Ap'. The erythromycin-resistance determinant was excised from positive recombinants with NsiI and was ligated with NsiI-digested and dephosphorylated pUL5032. The ligation products were transformed into E. coli selecting Ap'. The desired recombinant plasmid, designated pGM02, was digested with BamHI, dephosphorylated, and ligated with the BamHI-cleaved TS plasmid pTS2tet (Fitzgerald & Foster, 2000). The DNA was transformed into E. coli selecting Ap’. A 13·5 kb plasmid, designated pGM03, was isolated from putative transformants and was mapped by restriction enzyme analysis.

**Inactivation of the chromosomal hysA gene of S. aureus 8325-4.**

The TS shuttle plasmid pGM03 was electroporated into S. aureus RN4220, selecting Em' at 30 °C for 48 h. Positive recombinants were confirmed by demonstrating Cm' and Tc'. Plasmid pGM03 was prepared from RN4220 and established into S. aureus 8325-4 in a similar manner. Recombinants in which the hysA::ermC mutation had replaced the wild-type (w.t.) chromosomal allele were selected as follows. A single colony of 8325-4(pGM03) was prepared from RN4220 and established into fresh TYEg medium lacking antibiotic. At the end of each generation, the culture was subcultured four times with a final concentration of 50 μg ml⁻¹ and the suspension was incubated at 37 °C with gentle agitation for 1·5 h.

**Construction of a vector carrying the active hysA gene.**

A 7·2 kb HindIII fragment containing the full hysA ORF, including upstream regulatory regions, was excised from pUL5030 (Farrell et al., 1995) and following dephosphorylation it was ligated with HindIII-cleaved pUC1 shuttle vector (Augustin et al., 1992). The ligation mix was transformed into E. coli selecting Ap'. A 12·2 kb plasmid, designated pGM04, was isolated from putative transformants and was mapped by restriction enzyme analysis. Plasmid pGM04 was passaged through S. aureus RN4220 and was then electroporated into electrocompetent HL-deficient cells selecting Em' and Cm'. Several colonies were screened for HL production on Cm plates containing HA.

**Phage transduction.**

This was performed as described by Foster (1998) using φ11 (Novick, 1991) as the transducing phage. Briefly, 50 μl of an overnight w.t. S. aureus 8325-4 culture was transferred to a plastic universal containing 500 μl phage lysate (10⁻⁸ p.f.u. ml⁻¹) and 1 ml CK broth. The lysate/cell mixture was incubated at 37 °C without shaking for 25 min, followed by 15 min with shaking at 250 r.p.m. Then 1 ml ice-cold sodium citrate (0·02 M) was added and the cells were pelleted at 5000 r.p.m. for 10 min, resuspended in 1 ml ice-cold sodium citrate (0·02M) and incubated on ice for 2 h. The lysate/cell mixture was spread on LB bottom agar plates containing 0·05% (w/v) sodium citrate and 0·15 μg Em ml⁻¹, and was incubated at 37 °C for 90 min. The plates were overlaid with 5 ml CK top agar containing 25 μg Em ml⁻¹ and 25 μg Lc ml⁻¹, and following setting, they were incubated at 37 °C until the appearance of putative phage transductants (18–72 h).

**HL assays.**

All chemicals were purchased from Sigma unless otherwise stated. HL-deficient isolates were screened from strains with the w.t. phenotype by the plate assay of Grenier & Michaud (1993). Briefly, BHI agar plates supplemented with HA (0·4 mg ml⁻¹) were spot inoculated with several candidate colonies and incubated at 37 °C. Following growth, the plate was flooded with 10% (w/v) cetylpyridinium chloride (BDH) and was left to stand at room temperature for 20–30 min. The cetylpyridinium chloride was then rinsed off the plate with distilled water. Colonies producing HL were surrounded by a zone of clearing.

HL in culture supernatants was assayed by measuring the production of N-acetylamino end-group sugars by the method of Reissig et al. (1955). A standard curve was constructed for the subsequent determination of HL activity units. Briefly, 0·1 ml potassium tetraborate solution (0·8 M, pH 9·1) was mixed with 0·5 ml N-acetyl-D-glucosamine (NAG) standard sample (0·05–0·5 μmol ml⁻¹) and with 0·5 ml distilled water. The samples were boiled for exactly 3 min and cooled immediately under cold water. Colour production was initiated by the addition of 3 ml 0·1% DMAB reagent [10% (w/v) p-dimethylaminobenzaldehyde, 12·5% (v/v) 10 M HCI (BDH), 87·5% (v/v) glacial acetic acid (BDH)] and was completed by incubating the resulting solutions at 37 °C for exactly 20 min. The A₅₄₄ of each sample was measured using an SP6-450 UV/VIS spectrophotometer (Pye Unicam). A standard curve of A₅₄₄ against NAG concentration (μmol ml⁻¹) was plotted using a best-fit straight line. The procedure was repeated with 0·5 ml of test sample or blank (30 mM sodium acetate, pH 5·2). Each sample was mixed with 1 ml HA solution [200 mM NaCl, 1% (w/v) sodium azide, 0·6% (w/v) HA] and allowed to react at 37 °C. Reactions were stopped immediately (t₀) or exactly 15 minutes (t₁₅) following the addition of the substrate by mixing 0·5 ml of the reaction mixture with 0·1 ml potassium

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Hyaluronate lyase

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duplicate and the enzyme specific activity was expressed as $10^3 \times \mu$mol NAG released ml$^{-1}$ min$^{-1}$ per OD$_{600}$ unit and was calculated according to the following formula:

$$10^3 \times \Delta A_{644} \times \frac{1}{m} \times \frac{1}{15} \times \frac{1}{OD_{600}}$$

where $\Delta A_{644}$ represents the mean difference in absorbance between the two $t_{15}$ and the two $t_{6}$ readings from each sample, $m$ represents the slope of the calibration curve, $3$ is the dilution of the enzyme test sample (0.5 ml) in substrate solution (1 ml) and $1/15$ represents the reciprocal of the amount of time the enzyme test samples were incubated with sodium hyaluronate at 37°C (15 min). If samples were diluted before assaying, the enzyme activity was multiplied by the dilution factor. OD$_{600}$ was the measurement of cell culture density.

**Pathogenicity study.** The virulence of *S. aureus* strains was tested in an established murine abscess model of infection (Chan et al., 1998). *S. aureus* strains were grown in BHI to stationary phase (20 h) as described above. The bacteria were harvested and washed twice in an equal volume of ice-cold sterile PBS (pH 7.4). The concentration of the bacterial cells was adjusted to approximately $1 \times 10^7$ c.f.u. ml$^{-1}$ using a counting chamber (Thoma). Female, 5- to 8-week hairless mice (Harlan) were injected subcutaneously with 0.2 ml of bacterial suspension. Serial dilutions of the samples were plated on BHI agar. The precise sizes of the inocula were confirmed by counting the resulting growth of colonies. After 7 days, the mice were killed and skin lesions were aseptically removed. Lesions destined for histological examination were measured and stored in sterile PBS supplemented with 10% (v/v) formalin. The remaining lesions were measured, chopped and homogenized in 3 ml sterile ice-cold PBS in a mini-blender for approximately 5 min. The total number of bacteria recovered from the lesions was determined by viable cell count on BHI agar. Statistical significance of the data was evaluated by ANOVA. All values are reported as means $\pm$ 95% confidence limits.

**Histology.** Skin lesions were fixed in 10% (w/v) formalin saline solution for 48 h and were then embedded in paraffin wax (Paraplast). Each wax-embedded lesion was sectioned (6 μm) using stainless steel microtome blades (RaLamb). A total of 10 sections per lesion were prepared, stained with haematoxylin and eosin using routine histological procedures (Bancroft & Stevens, 1996) and analysed by microscopy (Olympus BX40).

**RESULTS AND DISCUSSION**

**Isolation of an *S. aureus* HL-deficient mutant**

A Clustal W alignment of *S. aureus* HysA with the HLs from *Streptococcus pneumoniae* (L20670; Li et al., 2000) and *Streptococcus agalactiae* (U15050; Pritchard et al., 2000) showed that essential residues for the activity of the streptococcal enzymes are conserved in the *S. aureus* enzyme (data not shown). The ORF of *hysA* was disrupted in vitro by insertion of the 1.44 kb *ermC* gene, conferring erythromycin resistance, upstream of the putative HL active site. Following transformation of the TS shuttle vector pGM03 into *S. aureus* RN4220 and subsequently into 8325-4, its temperature sensitivity was checked by measuring the efficiency of plating on medium containing erythromycin at 30°C ($1.45 \times 10^7$ c.f.u. ml$^{-1}$) relative to 45°C ($9.4 \times 10^7$ c.f.u. ml$^{-1}$). Previous studies have shown that the pE194 *ts* replicon used in this work can be eliminated from cultures of *S. aureus* 8325-4 at temperatures of 40–43°C (Greene et al., 1995; Luchansky et al., 1989). Although it has previously been shown that plasmid pTS2tet, which also carries pE194 *rep ts*, can be eliminated from *S. aureus* RN4220 cells following growth at 43°C for 15 mean cell generations (Adams, 1999), plasmid pGM03 could still be prepared from 8325-4 cultures following the same incubation conditions (results not shown). Consequently, it was hypothesized that a higher incubation temperature would result in greater instability of pGM03. No substantial difference in growth rate was observed at 45°C versus 43°C ($\mu=1.2$ h$^{-1}$ vs 1.3 h$^{-1}$; $t_{0}=0.55$ h vs 0.54 h, respectively). Putative *hysA::ermC* mutants of 8325-4 were identified on HA agar plates following elimination of the TS pGM03 (Fig. 1). The structure of the *hysA::ermC* locus was examined from mutant strains by Southern blot hybridization (Fig. 2) and PCR (results not shown). One isogenic mutant, HL-6, was chosen for further studies. Supernatants from hourly samples of strain HL-6, which was grown in batch culture, tested negative for HL production with the colorimetric assay (data not shown).

**In vitro complementation of the HL deficiency**

To verify whether the observed phenotype of strain HL-6 was due to the insertional inactivation of *hysA* by *ermC*, plasmid pGM04, a shuttle carrying the active *hysA* gene, was transformed into HL-6. All the isolates that grew on Cm+Em plates and were subcultured on HA-containing

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**Fig. 1.** HL plate assay. HL produced by an *S. aureus* culture carrying the active *hysA* gene diffuses into the medium surrounding the growth, breaking down the HA present in the medium. Upon addition of cetylpyridinium chloride (10%, w/v), HA precipitates, leaving a clear zone around the growth (w.t.). No clearing zone is observed around the growth arising from mutant strain HL-6.
medium tested positive for HL production (data not shown).

Transduction of the \textit{hysA}::\textit{ermC} allele

The \textit{hysA}::\textit{ermC} mutation present on the chromosome of HL-6 was transduced to the w.t. 8325-4 strain via \textit{w}11. The expression of the \textit{ermC} gene was initially induced with low levels of Em. However, in order to minimize the selection of point mutations for Em, the cells were screened on Lm as well as Em. Two transductants, HL-13T and HL-16T, were isolated and their identity was verified using the Pastorex Staph-Plus (PSP) latex agglutination test (Bio-Rad). Culture supernatants from both isolates were HL negative when tested with the plate and the colorimetric assays (data not shown).

Growth characteristics of staphylococcal strains

The growth characteristics, i.e. maximum specific growth rate (\(r_{\text{max}}\)) and growth yield, of HL-6, HL-13T and HL-16T were compared to those of the w.t. strain during growth in BHI medium. All four strains reached a maximum specific growth rate of 1·4 h\(^{-1}\). In addition, they reached similar growth yields, which ranged between 10 and 12 OD\(_{600}\) units.

Virulence studies with HL-deficient mutants

In order to determine whether HL is a virulence factor of \textit{S. aureus}, mice were challenged subcutaneously with strains 8325-4 (w.t.) and HL-6. Following the 7 day infection period the majority of the animals challenged with the w.t. strain developed a red, open and crusty lesion. In contrast, the HL-negative strain caused a less severe white, raised pustule (Fig. 3). These results suggested a role for HL in subcutaneous infections.

Microscopic examination of stained sections from infected areas revealed clear histopathological signs of inflammatory response, characterized by the presence of pus in the centre of the lesion and cellular infiltration mainly to the periphery (Fig. 4a). Closer inspection showed that in some lesions the infiltrate was composed mainly of mononuclear cells while in others of numerous neutrophils (Fig. 4b). Despite

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**Fig. 2.** (a) Construction of HL-6 (8325-4 \textit{hysA}::\textit{ermC}). A double crossover event introduces the mutated allele from pGM03 (double line) into the w.t. 8325-4 chromosome (solid line). (b) Southern blot analysis of the \textit{hysA} loci of w.t. and HL-6, confirming the insertion of \textit{ermC} in \textit{hysA} of strain HL-6. Chromosomal DNA from HL-6 (lanes 1 and 3) and w.t. (lanes 2 and 4) strains was digested with HindIII and SacI, electrophoresed on 0·8 % (w/v) agarose gel and transferred onto a nylon membrane. The transferred DNA was hybridized with DIG-labelled \textit{ermC} (lanes 1 and 2) and with a 0·7 kb \textit{NsiI–NcoI} probe corresponding to a DNA fragment in \textit{hysA} downstream from the point of insertion of \textit{ermC} (grey rectangle in a) (lanes 3 and 4). The presence of the 8·6 kb fragment on lanes 1 and 3 indicates incomplete digestion by SacI.

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**Fig. 3.** Skin lesions in mf-1 hairless mice following a 7 day infection period. Images show (a) a red, open, crusty, lesion following inoculation with w.t. \textit{S. aureus}; (b) a white, raised pustule obtained following inoculation with the HL-6 strain. Mice inoculated with sterile PBS had no sign of infection (not shown).
Fig. 4. Haematoxylin- and eosin-stained sections of mouse skin. (a) Infiltration of inflammatory cells (A) surrounding the lesion (B) 7 days after inoculation with $1.59 \times 10^7$ c.f.u. of w.t. *S. aureus* and $2.02 \times 10^7$ c.f.u. of HL-6 *S. aureus*. Bars, 200 μm. (b) In certain lesions the infiltrate was neutrophilic (C) and in others it was composed of mainly mononuclear cells (D). Bars, 20 μm.
the differences in the gross appearance of the lesions produced by the w.t. and HL-6 strains, no clear differences in the types of inflammatory infiltrate were observed between the lesions of the two animal groups (Fig. 4a). This observation might be explained by the capacity of the w.t. to degrade the ground substance and cause an open lesion.

The HL-producing strain (w.t.) was significantly more virulent than the isogenic mutant as demonstrated by the total number of viable bacteria recovered from skin lesions. The mean total viable bacterial count recovered from the skin of mice inoculated with the w.t. strain was $2.1 \times 10^7$ c.f.u. (approx. 135% recovery) whereas that recovered from mice challenged with the HL-6 strain was $7.8 \times 10^5$ c.f.u. (approx. 4% recovery). This difference was significant ($P=0.01$). In addition, HL-6 appeared less virulent than the w.t. as demonstrated by the size of lesions it produced, with a mean lesion area of 13.5 mm$^2$ vs 14.1 mm$^2$ for the w.t.; however this difference was not significant ($P=0.9$).

These results strongly suggest that the disruption of the hysA gene of *S. aureus* has a negative effect on the degree of pathology caused by the organism.

**Role of global regulators *agr* and sar on HL production**

Although, to date, there are no reports on the role of the *agr* and *sar* loci in the regulation of HL, the results of previous studies by Taylor & Holland (1991) and Rogers (1954) suggest that its regulation is distinct from that of other *S. aureus* exoproteins, such as toxic shock syndrome toxin-1 (TSST-1), which are expressed during late exponential phase.

The w.t. *S. aureus* 8325-4 strain and its derivatives, WA250 (*agr*) and PC1839 (*sar*), were grown in CDM in the absence of selection. HL activity and $OD_{600}$ were measured as described in Methods. Although the three strains had similar growth kinetics (Fig. 5), strain WA250 demonstrated approximately a 16-fold reduction in HL activity compared to the w.t. (0.9 vs 14.0 specific activity units per $OD_{600}$ unit). In contrast, strain PC1839 exhibited a 35-fold (487 specific activity units per $OD_{600}$ unit) increased activity compared to the w.t. It is of interest that the activity from all three strains decreased to undetectable levels during the post-exponential phase of growth. It is possible that proteases in the supernatant fluid may cleave HL post-translationally, thus reducing its activity. This effect was exaggerated in the *sar* strain, in which although HL specific activity was the highest of the strains tested, it diminished at the highest rate. Previous investigations have reported that proteases, which are overexpressed in the *sarA* mutant strain PC1839, are responsible for the inactivation of z-haemolysin in the same strain (Lindsay & Foster, 1999). However, additional factors may be responsible for this observed decrease in HL specific activity since it occurred even in the *agr* mutant strain, which is expected to have a reduced protease production.

These observations indicate that *sar* and *agr*, two global virulence modulators, downregulate and upregulate respectively HL production. This provides further evidence of the involvement of HL in virulence of *S. aureus*. However, HL production, as observed by *in vitro* experiments, appears to be different from that of other staphylococcal exoprotoins in that it occurs during the early to mid-exponential phases of growth. This observation strengthens the argument for involvement of HL in the early stages of infection.

The results presented in this work were obtained using the standard laboratory *S. aureus* strain 8325-4, which is
defective for rsbU, the gene required for the activation of σ^B. The results were therefore obtained in the absence of σ^B function and hence may not be representative of the behaviour of a true w.t. clinical strain. However, since strain 8325-4 has been used for much work on the regulation of virulence of S. aureus, it is important that the regulation of HL be analysed in this background for comparative purposes. Furthermore, the results obtained from in vivo experiments using the isogenic hysA− mutant are valid because the positive control w.t. 8325-4 strain was of an identical genomic background apart from the hysA gene. Future work should investigate the regulation of HL in an rsbU+ derivative of S. aureus 8325-4. Such a derivative has recently been described by Horsburgh et al. (2002).

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