The pathogenic diversity and success of Staphylococcus aureus

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Staphylococcus aureus has several extracellular proteases with proposed roles in virulence. SspA (serine protease), SspB (cysteine protease) and Aur (metalloprotease) have been characterized previously and SspA and SspB were found to be cotranscribed. The coding region for the cysteine protease ScpA has been identified and characterized. It is in a probable bi-cistronic operon with scpA located immediately upstream of a coding region for a 108 aa protein that is a specific inhibitor of ScpA. Using primer extension analysis promoters have been mapped and it was found that σA is the only sigma factor involved in the transcription of scpA, sspABC and aur. The transcription of all the genes occurs maximally at post-exponential phase, being positively regulated by agr (accessory gene regulator) and negatively regulated by sarA (staphylococcal accessory regulator). Furthermore σB represses transcription from the aur and scp operons similarly to the previously shown effect on ssp [Horsburgh, M., Aish, J., White, I., Shaw, L., Lithgow, J. & Foster, S. (2002). J Bacteriol 184, 5457–5467]. Using mutations in each protease gene the proteolytic cascade of activation has been analysed. Aur, SspA, SspB and ScpA are all produced aszymogens, activated by proteolytic cleavage. Although the metalloprotease Aur, does catalyse activation of the SspAzymogen, it is not the sole agent capable of conducting this process. Site-directed mutagenesis revealed that Aur is not capable of undergoing auto-proteolysis to achieve activation. The cysteine protease, ScpA, appears to reside outside this cascade of activation, as mature ScpA was observed in the ay, sspA and sspB mutant strains. Using a mouse abscess model, it has been shown that insertion inactivation of sspA or sspB results in significant attenuation of virulence, whilst mutations in ay or scpA do not. It is likely the attenuation observed in the sspA strain is due to polarity on the sspB gene.

INTRODUCTION

The role and regulation of the extracellular proteases of Staphylococcus aureus is amongst the most versatile and successful of the human pathogens. It has the ability to cause a variety of infections in numerous ecological niches within the host. Infections caused range from superficial lesions, such as wound infections and abscesses, to the more serious systemic and life-threatening conditions such as bacteraemia, endocarditis, meningitis and osteomyelitis. The pathogenic diversity and success of S. aureus is largely due to a vast array of temporally, spatially and environmentally coordinated virulence determinants (Lowy, 1998; Novick, 2000).

Modulation of virulence determinant expression and synthesis is largely controlled by two global regulatory elements, agr (accessory gene regulator) (Abdelinour et al., 1993; Janson & Arvidson, 1990; Novick et al., 1993; Novick, 2000) and sar (staphylococcal accessory regulator) (Cheung & Ying, 1994; Cheung et al., 1992, 1997; Chien et al., 1999; Rechtin et al., 1999). agr functions in a growth-phase-dependent manner, repressing synthesis of cell-wall-associated proteins in the post-exponential phase and activating expression of extracellular proteins (Gillaspy et al., 1998; Novick et al., 1993; Patel et al., 1992). sar also regulates virulence determinant synthesis either directly or indirectly by the modulation of agr expression (Novick et al., 1993). Moreover, characterization of a sarA mutant revealed the major phenotype to be the massive upregulation of exoprotease synthesis (Chan & Foster, 1998).

The major proteolytic enzymes secreted by S. aureus consist of a metalloproteinase (aureolysin, Aur), a serine glutamyl endopeptidase (serine protease, SspA) and two related cysteine proteinases referred to as staphopain (ScpA) and the cysteine protease (SspB) (Arvidson, 2000). The latter enzyme is encoded by the sspB gene, which is located in an operon contiguous to the gene for the serine protease (sspA) (Chan & Foster, 1998; Rice et al., 2001). SspA is secreted...
in a pro-form, which is proteolytically cleaved to produce a mature and functional enzyme, possibly in an Aur-dependent manner (Drapeau, 1978; Lindsay & Foster, 1999). SspB is also found as a proteolytically processed product in culture supernatants, and it is proposed that SspA is the agent responsible for this cleavage (Chan & Foster, 1998; Rice et al., 2001). Characterization of the gene encoding Aur (Sabat et al., 2000) uncovered a putative ORF of 1530 bp, with a resultant gene product of 509 aa. Crystal structure (Banbula et al., 1998) and N-terminal analysis (Chan & Foster, 1998) have revealed that mature Aur is only 301 aa long, indicating the need for post-translational processing. The specific mechanism by which Aur maturation occurs is unknown; however, it is common amongst the thermolysin-like enzymes for this to be achieved via auto-catalysis (Miyoshi & Shinoda, 2000). Little is known about staphopain; it too is produced in zymogen form and must undergo proteolytic cleavage to attain activity (Arvidson, 2000; Hofmann et al., 1993; Ziebandt et al., 2001).

In addition to this inter-dependent proteolytic activity, it has recently become apparent that the proteinases of \textit{S. aureus} may also have modulating activity on other proteins secreted by the pathogen. SspA was shown to effectively cleave fibrinogen-binding protein (McGavin et al., 1997) and surface protein A (Spa) (Karlsson et al., 2001). Little is known about staphopain; it too is produced in zymogen form and must undergo proteolytic cleavage to attain activity (Arvidson, 2000; Hofmann et al., 1993; Ziebandt et al., 2001). In addition to this inter-dependent proteolytic activity, it has recently become apparent that the proteinases of \textit{S. aureus} may also have modulating activity on other proteins secreted by the pathogen. SspA was shown to effectively cleave fibrinogen-binding protein (McGavin et al., 1997) and surface protein A (Spa) (Karlsson et al., 2001). Aur is responsible for the cleavage of the surface-associated clumping factor, C5b (McAleese et al., 2001). In addition, it has been suggested that by the degradation of toxins, such as \( \alpha \)-haemolysin, the toxins may downregulate the virulence of \textit{S. aureus} in specific niches \textit{in vivo}, such as when the bacteria colonize the skin and the nares (Lindsay & Foster, 1999). Furthermore, recent analysis of a transposon mutant of spsA revealed the strain to be attenuated in three separate animal models (mouse abscess, bacteremia and wound infection) (Coulter et al., 1998).

In this paper we describe experiments to characterize the role and regulation of the major extracellular proteases of \textit{S. aureus}.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** \textit{Staphylococcus aureus} and \textit{Escherichia coli} strains and plasmids are listed in Table 1. \textit{E. coli} was grown in Luria–Bertani (LB) medium at 37°C. \textit{S. aureus} was grown in 100 ml BHI (1:2-5 flask/vol. ratio) at 37°C with shaking at 250 r.p.m. in Brain Heart Infusion (BHI) broth (Oxoid) (Chan & Foster, 1998), unless indicated otherwise. When required, antibiotics were added at the following concentrations: ampicillin, 100 mg l\(^{-1}\) (\textit{E. coli}); kanamycin, 50 mg l\(^{-1}\) (\textit{E. coli} and \textit{S. aureus}); neomycin, 50 mg l\(^{-1}\) (\textit{S. aureus}); tetracycline, 5 mg l\(^{-1}\) (\textit{S. aureus}); erythromycin, 5 mg l\(^{-1}\) (\textit{S. aureus}); erythromycin, 300 mg l\(^{-1}\) (\textit{E. coli}); lincomycin, 25 mg l\(^{-1}\) (\textit{S. aureus}).

\textit{S. aureus} strain constructions. Derivatives of plasmid pAZ106, an integrating plasmid conferring resistance to erythromycin and containing a promoterless \textit{laciZ} gene (Kemp et al., 1991), or plasmid pAUL-A, a temperature-sensitive integrating plasmid conferring resistance to erythromycin (Chakraborty et al., 1992), were constructed using PCR with Pwo polymerase (Roche) and standard cloning techniques (Sambrook et al., 1989).

**Construction of aur-lacZ and sspA-lacZ reporter gene fusions.** The promoter regions of \textit{aur} and \textit{sspA} were amplified as 1-2 and 0-75 kb PCR-generated DNA fragments using oligo pairs OG-01/OG-02 and OG-47/OG-48, respectively (Table 1). Both purified DNA fragments were digested with BamHI and EcoRI and cloned into similarly digested pAZ106. \textit{S. aureus} RN4220 was transformed separately with the resulting plasmids, pLES01 (\textit{aur}) and pLES03 (\textit{sspA}), and an integrant from each transformation was confirmed by Southern blot analysis before being transduced into appropriate backgrounds using \#11.

**Construction of insertional inactivations of \textit{aur} and \textit{sspB} using pAUL-A.** Internal fragments of \textit{aur} and \textit{sspB} were amplified as 296 and 446 bp PCR-generated DNA fragments using oligo pairs OG-03/OG-04 and OG-05/OG-06, respectively (Table 1). Both purified DNA fragments were digested with BamHI and EcoRI and cloned into similarly digested pAUL-A. \textit{S. aureus} RN4220 was transformed separately with the resulting plasmids, pLES04 (\textit{aur}) and pLES05 (\textit{sspB}), and incubated at 42°C to select for integrants. Single colonies were then picked and streaked onto BHI agar (containing no antibiotics) before further incubation at 42°C, overnight. This procedure was repeated twice to cure the strains of any unintegrated plasmid. Integrants were then selected on agar containing erythromycin and lincomycin and incubated at 42°C. Integrants were confirmed by Southern blot analysis before being transduced into appropriate backgrounds using \#11.

**Construction of insertional inactivations of \textit{sspA} using pAZ106.** An internal fragment of \textit{sspA} was amplified as a 672 bp PCR-generated DNA fragment using oligo pair OG-35/OG-36 (Table 1). The purified DNA fragment was digested with BamHI and EcoRI and cloned into similarly digested pAZ106. \textit{S. aureus} RN4220 was transformed with the resulting plasmid, pLES06 (\textit{sspA}), and an integrant from each transformation was confirmed by Southern blot analysis before being transduced into appropriate backgrounds using \#11.

**Site-directed mutagenesis of the putative \textit{aur} active site.** A plasmid for the mutagenesis of the Glu\(^{145}\) active site residue of \textit{S. aureus} was constructed by PCR amplification of a 2-0 kb \textit{aur} fragment using primers OG-64, OG-57, OG-77 and OG-79 (Table 1). Divergent primers OG-77 and OG-79 contained a single base-pair mismatch within their coding sequence, converting the Glu\(^{145}\) residue (GAA) to Ala\(^{145}\) (GCA) by the site-directed mutagenesis method of Higuchi et al. (1988). This fragment was then cloned into the \textit{S. aureus} shuttle vector pMK4 (Sullivan et al., 1984), creating pLES08. As a control the entire \textit{aur} gene was PCR-amplified, intact, using primer pair OG-64 and OG-57, and then cloned into pMK4 to create pLES07. The correct sequence of the plasmid insertion was confirmed by DNA sequencing. \textit{S. aureus} RN4220 was transformed with the resulting plasmids and a transformant from each was confirmed by Southern blot analysis before being transduced into appropriate backgrounds using \#11.

**RNA isolation and transcriptional start site mapping.** Total RNA was isolated from post-exponential cultures of \textit{S. aureus} using the rapid liquid nitrogen chill method of Arnau et al. (1996). Frozen cell pellets, briefly stored at \(-70°C\), were thawed and RNA was rapidly extracted by cell disruption using the Fast-Prep blue kit (Bio-101) and a Fast-Prep system reciprocal shaker (Bio-101). Primer extension reactions were performed on the \textit{aur}, \textit{ssp} and \textit{scp} operons as described by Horsburgh & Moir (1999) using 100 μg RNA and 10 pmol of the corresponding primer (OG-44, \textit{aur}; OG-43, \textit{scp}; OG-45, \textit{scp}). The PCR products used for cloning into
### Table 1. Strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid or primer</th>
<th>Genotype or description</th>
<th>Reference(s) or source</th>
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<td>DH5α</td>
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<tr>
<td><strong>S. aureus</strong></td>
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<td>8325-4</td>
<td>Wild-type strain cured of prophages</td>
<td>Lab stock</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction-deficient transformation recipient</td>
<td>Lab stock</td>
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<tr>
<td>SH1000</td>
<td>Functional rsbU derivative of 8325-4 rsbU+</td>
<td>Horsburgh <em>et al.</em> (2002)</td>
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<td>Chan &amp; Foster (1998)</td>
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<td>PC1839</td>
<td>sarA::kan</td>
<td>Chan &amp; Foster (1998)</td>
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<td>Horsburgh <em>et al.</em> (2002)</td>
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<td><strong>Plasmids</strong></td>
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<td>pAZ106</td>
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<td>pAUL-A</td>
<td>Temperature-sensitive erm integrational shuttle vector</td>
<td>Chakraborty <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>pMK4</td>
<td>cm shuttle vector</td>
<td>Sullivan <em>et al.</em> (1984)</td>
</tr>
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<td>pAZ106 containing a 1-2 kb OG-01/OG-02 <em>aur</em> PCR fragment</td>
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<td>OG-04</td>
<td>CCGGAATTCGGGATCCGGGATAATTACGGATTG</td>
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<td>OG-05</td>
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<td>OG-45</td>
<td>GGCGGATCCAGCGCACTTACGTACATCACC</td>
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</table>

**Staphylococcus aureus** extracellular proteases
pAZ106 were purified using a Qiagen PCR kit and then sequenced with a Sequenase 2.0 PCR kit (Amersham) using the same oligonucleotide used for the primer extension reaction.

β-Galactosidase assays. Levels of β-galactosidase activity were measured as described previously (Horsburgh et al., 2001a). Fluorescence was measured using a Victor plate reader (Wallac) with a 0:1 s count time and calibrated with standard concentrations of 4-methyl umbelliferone (MU). One unit of β-galactosidase activity was defined as the amount of enzyme that catalysed the production of 1 pmol MU min⁻¹ per OD₆₀₀ unit. Assays were performed on duplicate samples and the values averaged. The results presented here were representative of three independent experiments that showed less than 10% variability.

Protein analysis. Exoprotein sample preparation and analysis was conducted using 12% (w/v) SDS-PAGE as described previously (Chan & Foster, 1998). Zymography was conducted as detailed by McAleese et al. (2001).

Western blotting. Proteins were blotted onto PVDF membrane (Bio-Rad) and detected using anti-sera raised against staphylococcal SspA (Lindsay & Foster, 1999), 1: 2500 dilution of the antibody and standard methods (Sambrook et al., 1989). Alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (diluted 1:30 000) was used to detect SspA colorimetrically.

Cysteine protease active site labelling with DCG-04 and affinity purification on avidin-agarose. This method was modified from that described by Greenbaum et al. (2000). Samples (5 ml) of the culture supernatant were pretreated with 1:0 mM dichloroisoureas) (Sigma) and 5 mM EDTA (30 min, 37 °C), incubated with DCG-04 (30 min, 37 °C) and dialysed against 50 mM Tris/HCl, pH 7:6, containing 0:5 M NaCl and 0:1% (v/v) Tween 20 (overnight, 4 °C). Samples were then mixed with 0:5 ml avidin-agarose (Sigma) (3 h, 4 °C) and then centrifuged (2000 r.p.m., 4 °C, 5 min). The pellet was first washed with 0:1 M Tris/HCl, pH 8:5, 1:0 M NaCl, then with 0:5 M sodium acetate, pH 4:5, and finally with 20 mM Tris/HCl, pH 7:5, containing 0:5 M NaCl, 0:05% (v/v) Tween 20. The collected pellet was solubilized in SDS-PAGE reducing sample buffer, denatured by boiling for 5 min, centrifuged and subjected to electrophoresis and electrotransfer as described previously (Greenbaum et al., 2000). Samples were then subjected to N-terminal analysis.

Virulence testing of strains in a murine skin abscess model. These studies were conducted as described previously (Horsburgh et al., 2001a).

RESULTS

In silico characterization of the scpA coding region

Of the four major extracellular proteases secreted by S. aureus, the loci encoding Aur, SspA and SspB have previously been identified (Sabat et al., 2000; Rice et al., 2001). However, the staphopain-encoding region has yet to be identified. To aid this study the scpA coding sequence was retrieved from the S. aureus 8325 genome sequence database (http://www.genome.ou.edu/staph.html) based on its homology to that of sspB, and analysed using both the Visual Cloning and Staden programs. The comparison of the genetic map of the new locus (scp) with the loci encoding other major proteinases (aur and ssp) is presented in Fig. 1. The scp locus is a putative bi-cistronic operon consisting of scpA (staphopain) and a further, smaller gene ( termed scpB) encoding a specific inhibitor of ScpA (Rzychon et al., 2003). No evidence of an internal promoter between the two genes was found. Ribosome-binding sites were evident immediately 5' of both scpA and scpB. A putative ρ-independent terminator exists 112 bp downstream of the scpB termination codon, indicating that this is likely to be a distinct bi-cistronic transcriptional unit. Indeed, it was recently shown that both genes are co-transcribed (Rzychon et al., 2003). The translated product of scpA is a pre-pro-enzyme of 388 aa, with a putative signal sequence of 25 aa. The pro-enzyme (363 aa) is processed between Thr214 and Tyr215 (relative to the methionine initiation codon) to form a 174 aa mature enzyme (Hofmann et al., 1993).

Promoter mapping of the aur, ssp and scp operons by primer extension analysis

To facilitate further studies of the protease genetic loci, RNA was isolated from post-exponential phase cultures of 8325-4 and subjected to primer extension analysis for each of the three proteases. In each case only one individual primer extension product was observed (Fig. 2) and the corresponding promoter regions resemble the σ⁺ consensus sequence (Deora & Misra, 1996).
Analysis of the regulation and expression of the \textit{aur}, \textit{ssp} and \textit{scp} operons

Using information gathered from primer extension analysis, reporter gene fusions were constructed for each protease coding region (see Methods). Analysis of the transcriptional coordination and control of each of the three protease operons revealed them to be coregulated. Under standard conditions expression of the \textit{aur}, \textit{ssp} and \textit{scp} operons occurs in a growth-phase-dependent manner with optimal expression at post-exponential phase ($t=4$ h), respectively, compared with 8325-4. Conversely \textit{agr} upregulates expression of \textit{aur}, \textit{ssp} and \textit{scp} as the \textit{agr} mutation results in a 3-, 40- and 6-fold decrease in expression at $t=4$ h, respectively, compared to 8325-4.

We have previously demonstrated that \textit{ssp} is repressed by \textit{sB} (Horsburgh et al., 2002), as in SH1000 (\textit{rsbU}$^+$) expression is reduced. 8325-4 carries an 11 bp deletion in \textit{rsbU}, which controls \textit{sB} activity. Full \textit{sB} activity is only present in SH1000 (\textit{rsbU}$^+$). The effect of the \textit{rsbU} status of the cells is a \textit{iaB} and \textit{argR}, an arginine transcriptional repressor protein; (b) \textit{ecoH}, an enoyl CoA hydratase, and \textit{amnT}, an aminotransferase; (c) \textit{purB}, adenylosuccinate lyase, and \textit{chp}, a conserved hypothetical protein. The sites and directions of primers used for mutant construction, along with generated PCR products are also shown. Primers are not drawn to scale.

**Fig. 1.** Genetic map of the \textit{aur}, \textit{ssp} and \textit{scp} loci of \textit{S. aureus}. Putative promoters and terminators are shown by arrows and loops, respectively. The genes immediately upstream and downstream of each protease locus are shown. These are as follows: (a) \textit{isaB}, immunodominant antigen B, and \textit{argR}, an arginine transcriptional repressor protein; (b) \textit{ecoH}, an enoyl CoA hydratase, and \textit{amnT}, an aminotransferase; (c) \textit{purB}, adenylosuccinate lyase, and \textit{chp}, a conserved hypothetical protein. The sites and directions of primers used for mutant construction, along with generated PCR products are also shown. Primers are not drawn to scale.

**Fig. 2.** Mapping of the 5’ ends of the \textit{aur} (a) \textit{sspA} (b) and \textit{scpA} (c) transcripts by primer extension analysis (top panels) and potential –35 and –10 regions and transcriptional start sites (+1) for \textit{\sigma} (bottom panels). RNA from post-exponential-phase cultures was used in the reactions. A, C, G and T refer to the dideoxy sequencing ladders obtained using the same primer as used for primer extension. rbs indicates the translational recognition sequence, upstream of the translational start (underlined).
on ssp is confirmed here, and aur and scp are also found to be downregulated in the rsbU+ derivative of 8325-4 by 3-, 40- and 6-fold at t = 4 h, respectively.

**Fig. 3.** Expression of protease-encoding operons. Assay of transcription from lacZ fusions during growth. Expression data of the aur (a), sspA (b) and scpA (c) reporter gene fusions in S. aureus 8325-4 (rsbU) (diamonds), SH1000 (rsbU+) (squares), PC1839 (agr) (triangles) and PC6911 (sarA) (circles) backgrounds during growth in BHI. Symbols represent representative of at least three separate experiments. Specific β-galactosidase activity was determined from these samples. Results are representative of at least three separate experiments.

**Analysis of the proteolytic activation of the Aur zymogen**

Having identified the transcriptional regulation mechanisms of the protease loci, we further investigated their regulation by a previously reported post-translational cascade of zymogen activation (Drapeau, 1978; Rice et al., 2001). The cascade of activation of the extracellular proteases of S. aureus is believed to proceed from Aur, through SspA to SspB, and potentially to ScpA (Drapeau, 1978; Rice et al., 2001). The specific agent responsible for Aur activation remains elusive, but autocatalysis of thermolysin family proteases is common (Braun et al., 1998; Miyoshi et al., 1997; Miyoshi & Shinoda, 2000). Therefore, to determine if Aur activation is via autocatalysis, it was decided to alter the active Glu145 to Ala, a so-called ‘safe residue’ for site-directed mutagenesis (Bordo & Argos, 1991). The mutant allele was then introduced in trans into strain LES12 (aur). As a control, unmutated intact aur was also generated and separately introduced in trans into strain LES12 (aur). These strains, along with the relevant parents, were then analysed via the use of SDS-PAGE and zymography (Fig. 4). Zymogram analysis reveals that the introduction of intact aur into LES12 (aur) results in a wild-type profile being generated and the appearance of a band corresponding to Aur activity (Fig. 4a, lane 3). Further analysis revealed this band of activity to be sensitive to 1 mM EDTA, indicating that it is a metalloprotease (data not shown). Introduction of mutated aur (E145A) into LES12 (aur) resulted in no detectable Aur activity. This indicated that the mutation Glu145 to Ala145 has abolished the proteolytic activity of this enzyme. SDS-PAGE analysis of exoprotein revealed a band of approximately 40 kDa (Fig. 4b, lane 1), corresponding to mature Aur protein (as determined by Chan & Foster, 1998). This band was found to be absent in the aur mutant strain (Fig. 4b, lane 2). However, the protein was present in strains complemented with either the wild-type or mutated aur (Fig. 4b, lanes 3 and 4, respectively). Using N-terminal analysis it was demonstrated that this 40 kDa band did indeed correspond to processed mature Aur (data not shown). This indicates Aur activation in the absence of Aur activity, proving maturation does not proceed via auto-proteolysis. It is noted that the exoprotein profiles of LES39 (aur, pMK4::aur E145) (Fig. 4b, lane 3) and LES41 (aur, pMK4::aur E145A) (Fig. 4b, lane 4) display some slight variation in proteins from that of the respective parent strain LES01 (aur); however, LES39 and LES41 were cultured in the presence of chloramphenicol to maintain vector selection, whilst LES01 was cultured in the absence of any antibiotic.

**Proteolytic activation of the serine protease zymogen by Aur**

Exoprotein analysis of the aur mutant (Fig. 5) reveals an altered exoprotein profile in both the 8325-4 and PC1839 (sarA) backgrounds. Most prominent is the alteration in protein at approximately 33 kDa, where a large amount of
Fig. 4. (a) Protease activity of 8325-4 (lane 1), LES01 (aur) (lane 2), LES39 (aur, pMK4::aur E145) (lane 3) and LES41 (aur, pMK4::aur E145A) (lane 4) visualized using a gelatin-containing zymogram gel. X refers to unknown protease activity; the other proteases were verified by comparison with purified proteins and via inhibitor analysis (data not shown). Lanes contain exoproteins from 1·0 OD$_{600}$ unit of culture supernatant. (b) Exoproteins of 8325-4 (lane 1), LES01 (aur) (lane 2), LES39 (aur, pMK4::aur E145) (lane 3) and LES41 (aur, pMK4::aur E145A) (lane 4) from culture supernatants after 15 h growth. Arrows indicate mature Aur as verified by N-terminal analysis. Lanes contain exoproteins from 1·0 OD$_{600}$ unit of culture supernatant.

Fig. 5. (a) Protease activity of 8325-4 (lane 1), PC1839 (8325-4 sarA) (lane 2) LES01 (aur) (lane 3) and LES15 (aur sarA) (lane 4) visualized using a gelatin-containing zymogram. X and Y refer to unknown protease activity; the other proteases were verified by comparison with purified protein and via inhibitor analysis (data not shown). Lanes contain exoproteins from 1·0 OD$_{600}$ unit of culture supernatant. (b) Exoproteins (top panel) and Western blot analysis (bottom panel) of 8325-4 (lane 1), LES01 (aur) (lane 2), PC1839 (8325-4 sarA) (lane 3) and LES15 (aur sarA) (lane 4) from culture supernatants after 15 h growth. Western blot analysis was conducted using anti-SspA antibodies. Lanes contain exoproteins from 1·0 OD$_{600}$ unit of culture supernatant.
protein is observed in 8325-4, but is far less apparent in strain LES12 (aur). This same result is observed to a greater extent in strain LES15 (sara aur) and corresponds to a dramatic decrease in the intensity of a protein band with the electrophoretic mobility of SspA (Fig. 5b, upper panel). Indeed, Western blot analysis confirmed the identity of this protein as SspA and in addition revealed that two bands of similar size in LES12 (aur) represents two forms of SspA (Fig. 5b, bottom panel). Exactly the same profile was observed in a previous study by Lindsay & Foster (1999) where the upper band was identified as the SspA zymogen, whilst the lower band was mature SspA. Here, the N-terminal analysis of the lower SspA protein band in strain LES12 (aur) and LES15 (sara aur) revealed two distinct sequences. One sequence (VILPNNDR) was identified as the N terminus of the mature enzyme (Chan & Foster, 1998), whilst the second (HANVILPN) was found to represent a form of SspA with three additional amino acids residues. Potentially this form represents aberrantly processed proSspA in the absence of Aur.

Analysis of the protease activity of LES12 (aur) and LES15 (sara aur) revealed very little SspA activity in LES12 (aur), although this effect is less apparent in LES15 (sara aur). This indicates that activation of the SspA zymogen can still occur, although at a decreased efficiency, in the absence of Aur. This suggests another as yet unidentified mechanism for SspA activation. Interestingly, the activity of SspB is also decreased in both aur mutant strains. It has already been shown that SspA is likely to be the agent responsible for the maturation of the SspB zymogen (Rice et al., 2001), thus any decrease in the activity of SspA would potentially exhibit a concomitant decrease in the presence and activity of SspB. Indeed in strain LES12 (aur) no SspB activity is detectable, an effect only partially restored in strain LES15 (sara aur) (Fig. 5a). Thus the loss of Aur activity results in a decrease in SspA activity, which in turn reduces the maturation of the SspB zymogen.

Investigation into the activation of the ScpA zymogen

Analysis of the specific mechanism for ScpA maturation was undertaken using DCG-04 active site labelling. This compound is a biotinylated derivative of E64 which specifically recognizes and covalently modifies the thiol group of compound is a biotinylated derivative of E64 which specifically recognizes and covalently modifies the thiol group of cysteine proteinases, including staphopains (Greenbaum et al., 2000). By using the biotinylated tag it is then possible to purify the labelled protease as described in Methods. Using Western blot and N-terminal sequence analyses, a sequence of YNEQYINK was obtained, corresponding to the first 8 aa of the mature ScpA protease (Hofmann et al., 1993). Thus it was possible to identify mature, processed ScpA in strains Les12 (aur), Les22 (sspA) and Les17 (sspB), indicating that these proteases do not have a role in the proteolytic activation of ScpA. Thus activation of this protease must occur in an as yet unidentified manner.

Role of the proteases in virulence

The virulence of the S. aureus protease mutant strains was investigated in an established murine subcutaneous skin abscess model of infection (Chan & Foster, 1998; Chan et al., 1998; Horsburgh et al., 2001a, b) and compared to the parental strain, 8325-4 (Fig. 6). Strains Les22 (sspA, P=0.030) and Les17 (sspB, P=0.028) were significantly attenuated when compared to 8325-4. Reduced virulence was observed with strain Les12 (aur, P=0.118), although this was not statistically significant. Strain Les27 (scpA, P=0.158) displayed no alteration in virulence in this pathogenesis model.

DISCUSSION

Here we present the identification and analysis of the coding region for the cysteine protease staphopain (ScpA). The scpA gene is in an apparent bi-cistronic operon with a downstream gene (scpB, 108 aa) which encodes a novel inhibitor of the ScpA proteinase (Rzychon et al., 2003a). The organization of this operon is similar to that seen with the staphopain homologue SspB, which is also immediately upstream of a smaller gene, encoding a 109 aa protein, SspC. SspC represents a structural and functional homologue of S. pyogenes SspC.

![Fig. 6. Pathogenicity of S. aureus strains in a subcutaneous lesion model of infection. S. aureus strains 8325-4, Les12 (aurA), Les22 (sspA), Les17 (sspB) and Les 27 (scpA) were inoculated subcutaneously into 8-week-old BALB/c mice (10 per strain). After 7 days the lesions were harvested and the c.f.u. per lesion was determined by duplicate serial dilution on BHI agar. These values, along with the inoculum, were used to calculate the percentage recovery of each strain. The horizontal bars indicate the mean percentage of bacteria recovered per strain.](https://www.microbiologyresearch.org/biometrygraphic/fig6.png)
ScpA and the other major proteases are coregulated. All are transcribed from single $\sigma^B$-controlled promoters in a growth-phase-dependent manner, occurring primarily at post-exponential phase. This upregulation was found to be agr-dependent, characteristic of many extracellular secreted virulence determinants (Chan & Foster, 1998; Dunman et al., 2001; Lindsay & Foster, 1999; Novick et al., 1993; Tegmark et al., 1998). All three operons were also found to be repressed by the pleiotropic virulence determinant regulator SarA. Previous work has demonstrated increased protease production in a sara mutant (Chan & Foster, 1998; Dunman et al., 2001; Lindsay & Foster, 1999). It has been proposed that SarA may control protease activity in response to the environment, to allow a rapid response to stimuli and turnover of specific staphylococcal surface components (Karlsson et al., 2001; Lindsay & Foster, 1999; McAleese et al., 2001).

The work conducted here also concurs with the recent study by Horsburgh et al. (2002), which demonstrated a role for $\sigma^B$ in the repression of expression of sspA. Here we show that this occurs for aur and scp as well. The specific mechanism for this phenomenon is unclear; however, it has been proposed that this is a sarA-mediated effect, conducted via the $\sigma^B$-dependent P3 promoter of the sar locus (Bischoff et al., 2001; Giachino et al., 2001). However, SarA levels do not alter between 8325-4 (rsbU) and SH1000 (rsbU$^+$) (Horsburgh et al., 2002). The Horsburgh et al. (2002) study revealed that agr transcription in SH1000 was severely decreased when compared to strain 8325-4, which may account for the reduced protease expression. As $\sigma$ factors essentially function as transcriptional activators, it seems reasonable to assume that $\sigma^B$ is potentially upregulating a repressor of agr, thus giving concomitant repression of protease expression.

An unusual feature of the staphylococcal extracellular proteolytic system is the cascade of zymogen activation. Previous investigation has postulated that Aur activates SspA (Drapeau, 1978), which in turn activates SspB, and possibly ScpA (Rice et al., 2001). Here we present evidence providing a deeper understanding of this cascade and the mode in which it is executed. Through the use of an aur mutant we have confirmed that Aur has a role in the post-translational activation of SspA. Exoprotein and zymogram analysis of this mutation in the 8325-4 background reveals a decrease in the amount of secreted mature SspA and a significant decrease in its activity. However, although protein levels and activity were decreased, they were not entirely abolished. It has already been noted that naturally occurring aur mutants hyper-accumulate the inactive pro-precursor of SspA (Drapeau, 1978). Using Western blot analysis, the presence of pro-SspA in the aur mutant was revealed; however, we also noted the presence of mature SspA, which demonstrated proteolytic activity.

Analysis of the aur mutation in a sarA-null mutant background also showed the presence of mature SspA protein and concomitant activity. Therefore, although Aur is responsible for modulation of serine protease activity, it is not the sole agent of this process. Other known proteolytic exoenzymes of S. aureus include the staphopains (SspB and ScpA) and six putative serine proteases referred to as Spl (Reed et al., 2001). Analysis of mutations in both sspB and scpA revealed absolutely no alteration in the protein levels or activity of SspA (data not shown), indicating no role in SspA processing. Previous analysis of an isogenic spl mutant revealed no significant alteration of the secreted exoprotein profile (Reed et al., 2001), thus suggesting that maturation of pro-SspA occurs in an spl-independent manner. Although pro-SspA has been demonstrated to be proteolytically inactive (Drapeau, 1978; Lindsay & Foster, 1999) homologous glutamyl-endopeptidases secreted from numerous Bacillus and Streptomyces species are post-translationally activated via auto-catalysis (Birkoft & Breddam, 1994). Thus if even a small percentage of the population of pro-SspA molecules were capable of limited proteolytic activity, it could trigger exponential auto-proteolysis. This would also explain the observation that SspA processing in the absence of Aur is significantly less efficient.

Using DCG-04 active site labelling it was possible to determine that mature ScpA was present in culture supernatants of the other three protease mutants (aur, sspA and sspB) (data not shown). This therefore indicates that the catalytic maturation of ScpA occurs independently of these three proteases. Analysis of the amino acid sequence surrounding the pro-ScpA to mature ScpA cleavage sites reveals that processing occurs between a threonine and a tyrosine residue. Such a cleavage site would tend to exclude the possibility of the processing of ScpA by one of the Spl proteases as they are catalytically related to SspA and are glutamyl preferential serine proteases (Reed et al., 2001). Indeed, results from a preliminary experiment clearly indicated the presence of fully active and processed ScpA in the medium of the spl mutant (E. Golonka and others, unpublished data). In the absence of any other specific candidates it is possible that ScpA achieves activation in an auto-catalytic manner. ScpA has a very broad substrate range with no preference in cleavage recognition site (Bjorklund & Jornvall, 1974). Furthermore, it is relatively common for papain-like proteases to undergo auto-catalytic maturation (Potempa et al., 2000), as is the case with the cysteine protease streptopain from Streptococcus pyogenes (Elliott, 1945; Liu & Elliott, 1965; Rasmussen & Bjorck, 2002).

Site-directed mutagenesis of Aur has revealed that the catalytic residue Glu$^{145}$ is absolutely required for proteolytic activity. This confirms the earlier work conducted by Banbula et al. (1998) which proposed that this Glu residue functions as the catalytic residue of this protease. Furthermore, it was found that in the presence of a
mutation converting Glu\textsuperscript{145} to Ala\textsuperscript{145}, processed Aur protein was still observed in culture supernatants. Thus catalytic maturation of Aur is not achieved in an autoproteolytic manner, and as such is processed by an as yet unknown agent. Analysis of the strains with mutations in the \textit{sspA}, \textit{sspB} or \textit{scpA} genes reveals the presence of processed Aur in the culture supernatants, excluding them from a role in this process. Analysis of the cleavage site of mature Aur reveals that it is cleaved between a Glu and an Ala residue. This is characteristic of the cleavage preference of the glutamyl-specific endopeptidase SspA and the Spl proteases. As mutations in \textit{sspA} do not affect the activity of Aur, it is possible that Aur achieves catalytic maturation in an Spl-protease-dependent manner. Interestingly, the Spl proteases have no requirement for proteolytic maturation and possess only pre-fragments required for secretion (Reed et al., 2001). Thus it is feasible that the activation of Aur, and thus the commencement of the activated cascade through to SspA and SspB, is instigated upon secretion of one, or more, of the Spl proteases. Indeed, transcriptional analysis of the \textit{spl} operon reveals similar temporal regulation to that of the four proteases described here, with maximal expression occurring in an agr-dependent manner during post-exponential phase (Reed et al., 2001). Fig. 7 contains a summary of the proposed mechanism for post-translational activation of the major extracellular proteases of \textit{S. aureus}.

Analysis of the virulence of the four protease mutations in the 8325-4 background was conducted using a mouse murine abscess model. It was found that mutations in \textit{sspA} and \textit{sspB} resulted in significant attenuation in the model, with the mutation in \textit{aur} also producing decreased virulence by approximately half (as measured by percentage cellular recovery against 8325-4), although this was not a statistically significant effect. Mutations in the \textit{scpA} gene had no effect on the virulence of \textit{S. aureus}. The data for the \textit{scpA} mutant correlates with a previous study where an \textit{sspA} transposon mutant was shown to be attenuated in three separate animal models (Coulter et al., 1998). However, due to the organization of the \textit{ssp} locus it is almost certain that this insertion results in polar mutations on the downstream genes, \textit{sspB} and \textit{scpC}. Thus this strain is an \textit{sspABC} mutant and specific attribution to the agent responsible for this phenotype is difficult. Virulence studies described here using an \textit{sspB} mutant revealed that the level of attenuation observed was almost identical to that of the \textit{sspA} mutant. As levels of SspA are unaffected in the \textit{sspB} mutant it is probable that any effect in virulence seen with an \textit{sspA} mutant will largely be due to the loss of \textit{sspB} function. Indeed a recent study using strains shown to be mutants of \textit{sspA} but not \textit{sspB} revealed that virulence of this strain did not vary from the wild-type (Rice et al., 2001). Thus we propose that it is \textit{sspB}, not \textit{sspA}, that is important for infection in \textit{S. aureus}.

Such attenuation is unsurprising as it has recently become apparent that the proteinases of \textit{S. aureus} have modulating activity on other proteins secreted by the pathogen. Accepted dogma suggests that \textit{S. aureus} must change phenotype from adhesive to invasive in order to disseminate (Lowy, 1998). In such a scenario proteolytic enzymes, the expression of which is increased in the post-exponential phase of growth, are likely to be important factors in the shedding of surface proteins. For example, SspA was shown to effectively cleave fibrinogen-binding protein (McGavin et al., 1997) and surface protein A (Spa) (Karlsson et al., 2001). It appears that Aur is responsible for the cleavage of the surface-associated clumping factor, ClfB (McAleese et al., 2001). The subsequently released N-terminal domain of this protein was then promptly degraded with the remaining cell-associated truncated form of ClfB found to be non-functional as a fibrinogen-binding protein. In addition, it has been suggested that by the degradation of toxins, such as the z-haemolysin, the proteases may downregulate the virulence of \textit{S. aureus} in specific niches \textit{in vivo}, such as when the bacteria colonize the skin and the nares (Lindsay & Foster, 1999).

Furthermore, these staphylococcal exoproteinases have all been shown to actively degrade human protease inhibitors, including \textit{\alpha-1}-proteinase inhibitor (Potempa et al., 1986; Rapala-Kozik et al., 1999). The result of these proteolytic digestions is the deregulation of host proteolytic activity and its control which ultimately may lead to abnormal connective tissue degradation. In addition to this, Aur has been shown to modulate immunogenic reactions by affecting the stimulation of both T and B lymphocytes, as well as inhibiting immunoglobulin production (Prokesova et al., 1991). Moreover, \textit{in vitro} SspA has been shown to cleave the heavy chains of all the human immunoglobulin classes (Prokesova et al., 1992). Staphopain also possesses elastinolytic activity, allowing for speculation that the enzyme may participate in the tissue invasion and destruction associated with staphylococcal ulceration (Potempa et al., 1988).

The extracellular proteases of \textit{S. aureus} form a complex interactive network of components with pleiotropic roles in pathogenesis. They are instrumental both in host damage and microbial adaptation to the host, thus providing a rapid

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**Fig. 7.** Diagrammatic representation of the post-translational cascade of activation of the extracellular proteases of \textit{S. aureus}. Dotted lines represent putative proteolytic steps in the cascade.
response to environmental signals allowing \textit{S. aureus} to be such a versatile and adaptable pathogen.

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