Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus aureus*

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Summary

Chemotaxis inhibitory protein of staphylococci (CHIPS) and Staphylococcal complement inhibitor (SCIN) are small, excreted molecules that play a crucial role in the staphylococcal defence against the human innate immune system. Here we show that they both counteract crucial acute responses of our immune system such as complement activation, neutrophil chemotaxis and neutrophil activation. By studying gene expression via promoter–green fluorescent protein fusions, Northern blots and protein expression analyses, we show that SCIN and CHIPS are produced during the early (exponential) growth stages. Although the SCIN and CHIPS genes are expressed simultaneously, they are differently regulated by various *Staphylococcus aureus* regulatory loci. However, the *sae* locus is crucial for upregulation of both SCIN and CHIPS. This is the first study that presents the expression of two extracellular *S. aureus* proteins early during growth. Because SCIN and CHIPS are both efficient modulators of neutrophil chemotaxis, phagocytosis and killing, their early expression is necessary for efficient modulation of the early immune response.

Introduction

Successful eradication of invading pathogens like *Staphylococcus aureus* critically depends on the combined activities of two major components of our innate immune system: neutrophils and the complement system (Fig. 1A) (Beutler, 2004). Activation of complement, which occurs during the first steps of the inflammatory response, results in the formation of bacterium-bound enzyme complexes: C3 convertases (C4b2a and C3bBb) (Xu *et al*., 2001). The C3 convertases cleave the central complement protein C3 and thereby initiate the covalent attachment of C3b to bacterial surfaces (opsonization) and the release of the chemo-attractant C3a (Sahu and Lambris, 2001). Subsequent cleavage of C5 into C5b and C5a is accomplished by the C5 convertases, generated by binding of one C3b molecule to a C3 convertase (Pangburn and Rawal, 2002). The small chemo-attractants C3a, C5a and bacterial formylated peptides trigger neutrophil migration through the blood vessel (diapedesis) but also direct the movement of neutrophils to the site of infection in the tissue, a process called chemotaxis (Gasque, 2004). The uptake of staphylococci by neutrophils (phagocytosis) is largely mediated by the binding of neutrophil receptors (complement receptor 1 and 3, Fc receptor) to opsonic molecules covering the bacterium (C3b, inactivated C3b and antibodies) (Beutler, 2004). Finally, staphylococci are killed in the phagolysosome by antimicrobial peptides and oxygen radicals generated during the neutrophil respiratory burst (Hampton *et al*., 1998).

Although *S. aureus* lacks a thick capsule that protects itself from opsonization, this bacterium has adopted other mechanisms to combat human innate immune defences. In the last few years it has become evident that *S. aureus* resists innate immune defences by producing small soluble molecules that specifically interact with crucial elements of the human immune system (Lee *et al*., 2004; Langley *et al*., 2005; Rooijakkers *et al*., 2005a). Our group described two excreted proteins that efficiently counteract two early steps of the inflammatory response. The chemotaxis inhibitory protein of staphylococci (CHIPS), produced by 65% of clinical *S. aureus* strains, is a 14 kDa protein that blocks neutrophil chemotaxis by binding the formylated peptide receptor and the C5a receptor on neutrophils (Veldkamp *et al*., 2000; De Haas *et al*., 2004; Haas *et al*., 2004; Postma *et al*., 2004; 2005). Staphylococcal complement inhibitor (SCIN), found in 90% of clinical strains, is a 10 kDa protein that specifically interacts with bacterium-bound C3 convertases and thus efficiently prevents C3b deposition and phagocytosis (Rooijakkers *et al*., 2005b). The genes for SCIN and CHIPS are located on an immune evasion cluster (IEC) in *S. aureus*, which can be transferred by β-haemolysin converting bacteriophages (βC-Φ’s) (Fig. 1B) (Van Wamel *et al*., 2006). This IEC also encodes the excreted molecules staphylokinase (SAK) and staphylococcal enterotoxin A (SEA),...
which can modulate later steps of the immune response. SAK is anti-opsonic because it cleaves surface-bound IgG and C3b via the formation of surface-bound plasmin (Rooijakkers et al., 2005c). Moreover, SAK is known to inhibit alpha-defensins and play a role in tissue invasion (Molkken et al., 2002; Jin et al., 2004). SEA is mainly known as a superantigen (Abrahmsen et al., 1995).

The expression of virulence factors by S. aureus is not a static but dynamic and well-coordinated process that depends both on the bacterial growth phase and on environmental host factors. S. aureus uses a number of regulatory mechanisms that control virulence gene expression in response to cell density, energy availability or environmental signals (Novick, 2003; Bronner et al., 2004). Three major classes of regulatory elements have been identified in S. aureus: Firstly, two-component systems (TCS) like accessory gene regulator (agr), staphylococcal accessory element (saeRS) and staphylococcal respiratory response regulator (srrAB) control expression of many virulence genes (Peng et al., 1988; Giraudo et al., 1999; Yarwood et al., 2001). Secondly, the alternative sigma factor B (σB) and finally, the staphylococcal accessory regulator A (SarA) protein family (Cheung and Projan, 1994; Chan and Foster, 1998). In vitro, the combined activities of these and other (as yet unknown) regulators normally result in the transcription of cell-wall proteins during exponential phase and extracellular proteins during post-exponential and stationary growth phases (Novick, 2003; Cheung et al., 2004). Also in vivo, cell-wall proteins are thought to be expressed during the early colonization phase of an infection, while excreted proteins are needed for bacterial invasion later during an infection. In this paper we show that the two excreted proteins SCIN and CHIPS are produced in the early phases of staphylococcal growth. Furthermore, we show that these specific inhibitors can block crucial steps of the early inflammatory response. The early production of these two important innate immune evasion molecules in S. aureus proposes a new bacterial mechanism to induce infection and survive in the human host.

**Results**

**SCIN and CHIPS block essential steps of the acute inflammatory response**

In earlier studies we showed that SCIN efficiently blocks complement-dependent phagocytosis while CHIPS prevents neutrophil chemotaxis (De Haas et al., 2004; Rooijakkers et al., 2005b). However, as SCIN acts early in the complement cascade we explored whether this complement inhibitor also prevents C5a production and chemotaxis of human neutrophils. This was tested by opsonization of heat-killed (to prevent production of bacterial formylated peptides) S. aureus and subsequent analysis of C5a-induced calcium mobilization in human neutrophils by supernatants. We observed that rSCIN is a potent inhibitor of the C5a-mediated activation of human
neutrophils (Fig. 2A). Figure 2B shows that this diminished calcium mobilization by SCIN also prevents chemotaxis of neutrophils. Thus, in addition to our earlier findings, *S. aureus* prevents both neutrophil chemotaxis and phagocytosis by the production of the C3 convertase inhibitor SCIN. Animal experiments using SCIN and CHIPS are not possible because both molecules are highly human-specific (De Haas et al., 2004; Rooijakkers et al., 2005b). However, recently Mollnes and coworkers described an elegant whole-human-blood inflammatory model. Here they proposed that the phagocytosis and subsequent intracellular oxidative burst are both C3b- and C5a-dependent (Mollnes et al., 2002). According to these findings, not only SCIN but also CHIPS should block these two final steps of the immune response. We tested this hypothesis in this human whole blood model of inflammation and observed that not only rSCIN but also rCHIPS efficiently prevented phagocytosis of *S. aureus* (Fig. 2C). Moreover, in the same whole blood set-up we observed that the oxidative burst, essential for killing of staphylococci, was inhibited by rSCIN and rCHIPS (Fig. 2D). Altogether these data demonstrate the crucial role of SCIN and CHIPS in the staphylococcal evasion of innate immunity. Both molecules can act in concert to block crucial steps of the early inflammatory response. The question remains whether these molecules can be produced early enough in infection to coincide with these early innate immune events.

**Early transcription of scn and chp**

The growth phase-dependent transcription of the genes for SCIN (*scn*) and CHIPS (*chp*) was studied *in vitro* using promoter–green fluorescent protein (GFP) fusions. As a control, the IEC genes encoding SAK (*sak*) and SEA (*sea*) were studied. As depicted in Fig. 1B, the *scn* and *chp* promoters are transcribed in different directions and are not part of an operon. Promoter sequences of the individual genes were cloned upstream of the *gfp*uvr gene in shuttle vector pACL1484 and subsequently introduced into *S. aureus* RN6390, a representative of *S. aureus* 8325-4 that lacks IEC. Bacteria were grown in Todd–Hewitt broth (THB) and intracellular GFP levels were measured each hour by flow cytometry. No subpopulations of cells were observed, 100% of gated cells were fluorescent. Consistent with previous studies, the *sak* and *sea* promoters were transcribed during the post-exponential and stationary growth phases (Novick, 2003) (Fig. 3A and B). The
decreasing GFP levels in the exponential phases indicate dilution of GFP from stationary-phase cultures. In contrast to sak and sea, transcription of scn and chp started immediately in the exponential growth phase (Fig. 3C and D). The GFP levels produced by the scn promoter reached a maximum within 2 h of growth. Afterwards, transcription levels dropped by almost 30%, but high GFP levels were still detectable during post-exponential and stationary growth phase (Fig. 3C). Expression of the chp promoter was already maximal after 1 h of growth (Fig. 3D). In the next 2 h chp expression dropped quickly. Almost no GFP could be detected during post-exponential and stationary phase. Comparison of GFP levels produced by the four IEC gene promoters indicated that the scn promoter is five times more active than the sak, sea or chp promoters, at least under the conditions studied here (Fig. 3A–D).

The early expression of scn and chp observed in S. aureus RN6390 was further analysed in clinical
S. aureus strains. Promoter–gfp fusions were introduced into four clinical blood isolates that all carried IEC with scn and chp. The transcriptional profiles of the scn and chp promoters in these strains were similar to S. aureus RN6390 (Fig. 4A and B). Again, expression of scn and chp was most pronounced during the exponential phase. Overall, these transcriptional analyses revealed that scn and chp were expressed during the early growth phases. The similar expression patterns of scn and chp in the IEC-negative S. aureus RN6390 and in the IEC-positive S. aureus clinical strains demonstrate that scn and chp are not likely to be regulated by components located on IEC carrying bacteriophages. To confirm the GFP data, Northern analyses were performed on the same clinical S. aureus strains. Therefore, the strains were grown for 2 h (exponential phase), 4 h (post-exponential phase) and 6 h (stationary phase) in THB. We observed that scn transcripts were predominantly present during the exponential and post-exponential phases of growth (Fig. 4C). Low amounts of scn RNA were found in the stationary phase, indicating that maximal GFP expression from the scn promoter was actually produced in earlier stages (Fig. 2A). Most of the tested strains produced chp transcripts only in the exponential phase, but one clinical isolate also produced chp in the post-exponential phase (Fig. 4D). Overall, analyses of scn and chp transcripts confirmed that both genes are expressed maximally during the exponential growth phase, which is not only in contrast to the transcription of sak and sea, but also to other genes encoding excreted proteins that are generally expressed most during the later growth stages (Novick, 2003). The Northern analyses of scn and chp transcripts validated the use of our promoter–GFP fusions for further transcriptional analyses.

Early transcription of scn and chp results in early secretion

Growth phase-dependent excretion of SCIN and CHIPS was measured in staphylococcal supernatants by sandwich enzyme-linked immunosorbant assay (ELISA). The four clinical S. aureus strains [all positive for scn and chp by polymerase chain reaction (PCR)] and the negative control SH1000 were grown in THB, and supernatants were collected each hour. Supernatants were incubated with anti-SCIN and anti-CHIPS monoclonal antibodies, coated to microtitre plates. SCIN and CHIPS were detected with specific polyclonal antibodies. SCIN could already be detected in supernatants of exponential and early post-exponential growth cultures (Fig. 5A). CHIPS production started in the early post-exponential phase (Fig. 5B). Maximum production levels were reached within 3 h. As a control, SAK activity was tested by SAK-mediated conversion of human plasminogen into plasmin (Rooijakkers et al., 2005c). As described for other excreted proteins, SAK production started during post-exponential phase and maximum levels were reached after 4 h of growth (Fig. 5C). Comparing proteins in supernatants with their recombinant variants revealed that staphylococci (1 × 10⁸) maximally produced 17–55 ng of SCIN, 11–68 ng of CHIPS and 20–25 ng of SAK. These data clearly demonstrate that the early transcriptional activation of scn and chp also results in fast production and excretion of the mature proteins.
Influence of S. aureus regulatory loci on scn and chp expression

The role of several regulatory loci on scn and chp transcription was studied in RN6390 (rsbU–) and SH1000 (rsbU+; Horsburgh et al., 2002) by using promoter–GFP constructs, with sak transcription assayed as a control. We observed that σB plays an important role in transcription of scn, chp and sak. Transcription of scn, chp and sak was low in SH1000 when compared with RN6390 (Fig. 6A–C). Also, deletion of sigB in SH1000 (MJH502) resulted in higher transcription levels of all three genes compared with SH1000. Interesting is that scn and sak transcription in RN6390 was still much higher than in the sigB mutant, suggesting a role for RsbU independent of σB (Fig. 6A and C). The effects of SarA on the transcription of IEC genes was highly diverse. The transcription of scn was not influenced by mutation of sarA in SH1000, while in RN6390 scn transcription was upregulated at most growth stages (Fig. 6D). Transcription of chp was increased in the sarA mutants of both strains (Fig. 6E). This increase was most pronounced in strain RN6390, which is RsbU-defective and was observed during all growth stages. Transcription of sak was also increased in sarA mutants of SH1000 (Fig. 6F). In contrast to chp, mutation of sarA in RN6390 did not significantly alter sak transcription.

Next we studied the effect of agr on transcription of scn, chp and sak. Transcription levels of scn were lower during post-exponential and stationary phase in agr mutant strains of RN6390 (Fig. 7A). In contrast, transcription of chp was not influenced by agr in both strains (Fig. 7B). As expected, mutation of agr resulted in a reduced expression of sak in RN6390 (Fig. 7C) (Recsei et al., 1986). No effect of agr on scn and sak transcription was observed in SH1000 (data not shown), which can be explained by the fact that Agr is poorly expressed in σB-producing strains (Horsburgh et al., 2002).

Finally, the influence of sae on transcription of scn and chp was studied in SH1000. Deletion of saeR or saeS resulted in a low expression of scn and chp during all growth phases (Fig. 7D and E; saeR mutant is shown). However, the sae locus did not influence transcription of sak (Fig. 7F). In summary, the excreted immune modulator proteins SCIN, CHIPS and SAK are expressed via the actions of several major S. aureus regulatory loci where each gene displays a different pattern of regulation. σB is an important downregulator of all three genes. While the agr locus showed mild upregulation of scn and sak, the sae locus serves a major role in the positive regulation of scn and chp, but not sak.

Discussion

In this paper we demonstrate the crucial role of SCIN and CHIPS in staphylococcal evasion of innate immunity. Not
only does CHIPS inhibit chemotaxis and activation (De Haas et al., 2004), but also it strongly influences phagocytosis in a whole blood system of inflammation. SCIN not only inhibits opsonization (Rooijakkers et al., 2005b) but also strongly inhibits both chemotaxis and phagocytosis. Both molecules do act in concert to block essential steps of the early inflammatory response.

In order to be effective immune modulators, SCIN and CHIPS are most beneficial to the bacterium if they are produced in the early growth stages. We could demonstrate that the genes encoding SCIN and CHIPS are indeed transcribed during the early stages of staphylococcal in vitro growth. Transcriptional analyses using promoter–GFP constructs and Northern analyses indicated that *scn* and *chp* are predominantly expressed during exponential phase and at lower levels during post-exponential phase, in both laboratory and clinical strains. Low amounts of *scn* and *chp* transcripts were detected in the stationary growth phase. By ELISA we determined that the early transcriptional activation also led to the early excretion of SCIN and CHIPS. Earlier we showed that these excreted proteins are produced in vivo as we found antibodies against SCIN, CHIPS and SAK in human sera (Rooijakkers et al., 2005b). Although virtually all important studies on staphylococcal virulence regulation have been performed in vitro, the co-ordinate expression of virulence genes in vitro is believed to resemble an in vivo infection (Cheung et al., 2004). That is, the colonization phases of an infection is related to the exponential growth phase, in which cell-wall proteins facilitate attachment of the bacte-
Early expression of staphylococcal innate immune modulators

Fig. 7. Effect of Agr and Sae on the transcription of scn, chp and sak.
A–F. Flow cytometric analysis of the scn, chp and sak promoters upstream of gfpuvr in pALC1434 in S. aureus.
A–C. Effect of the agr locus on transcription of scn, chp and sak in RN6390. A. The agr locus upregulates transcription of scn in RN6390.
B. No effect of the agr locus on chp transcription in RN6390. C. The agr locus upregulates transcription of sak in RN6390.
D–F. Effect of the sae locus on transcription of scn, chp and sak in SH1000. D. The sae locus strongly upregulates transcription of scn in SH1000.
E. The sae locus strongly upregulates transcription of chp in SH1000. F. No influence of the sae locus on transcription of sak in SH1000. Data shown represent mean ± SE of three separate experiments.
*, P < 0.05; **, P < 0.005 versus control.

rrium to the host tissue and immune evasion. Later, extracellular toxins and proteases are produced that initiate local bacterial invasion by degradation of host tissues and cells. Here we show that two excreted immune modulators SCIN and CHIPS are indeed produced early during in vitro growth. The early expression of proteins like SCIN and CHIPS seems logical from a bacterial point of view because the inflammatory mediators of the innate immune system act within the first minutes after onset of an infection. Therefore, bacterial survival is greatly aided with a fast production of innate immune evasion molecules like SCIN and CHIPS.

Although SCIN and CHIPS are expressed simultaneously during exponential growth we found that they are differently regulated; the agr locus was involved in upregulation of scn but not chp, while SarA is involved in the downregulation of chp. The sae locus was shown to fulfill a crucial role in upregulation of both scn and chp during all growth phases. This is an interesting finding as increasing evidence is presented that sae is a major regulator of virulence genes in vivo, while agr was found to be repressed in many in vivo models (Goerke et al., 2001; Benton et al., 2004; Harraghy et al., 2005). Because sae upregulates scn and chp, but not sak, this locus could be important for the early regulation of scn and chp. σB was identified as a downregulator of all three IEC-encoded immune modulators SCIN, CHIPS and SAK. Taken together, transcription of scn, chp and sak in S. aureus is controlled by a complex regulatory network. Our transcription analyses also showed that the IEC-carrying bacte-
riophage is not essential for transcription of its genes. *scn* and *chp* were equally well transcribed in the presence and absence of bacteriophages (Fig. 4A and B). Although earlier studies have suggested a role for the bacteriophage life cycle in expression of phage-encoded virulence genes SAK and SEA (Sumby and Waldor, 2003), our data show that IEC genes are nicely integrated into the regulatory mechanisms of *S. aureus* and do not require active phages. Altogether, the way these immune modulatory genes can be transferred between strains by bacteriophages and subsequently integrated into the regulatory mechanisms of the host provides *S. aureus* with a highly efficient mechanism to increase its survival chances.

**Experimental procedures**

**Bacterial strains and plasmids**

*Escherichia coli* and *S. aureus* strains or plasmids used in this study are listed in Table 1. *E. coli* and *S. aureus* were grown in Luria–Bertani (LB) broth and THB (Difco, Detroit, MI) respectively. When necessary, ampicillin (100 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹), erythromycin (3–5 µg ml⁻¹), tetracycline (5 µg ml⁻¹) was added. Antibiotics were purchased from Sigma Chemical, St. Louis, MO. Strains were tested for the presence of *hbl, scn, chp, sak, sea* by PCR as described previously (Van Wamel et al., 2006). The 11 bp deletion in *rsbU* was tested by PCR using primers up- and downstream of the deletion (Kullik and Giachino, 1997).

**Calcium mobilization and chemotaxis**

*Staphylococcus aureus* strain Cowan EMS (protein A deficient mutant of *Cowan I* provided by A. Forsgren, Malmö University, Malmö, Sweden) was grown in LB medium to an *A₆₆₀* of 0.5 and subsequently killed by incubation at 60°C for 30 min. For C5a generation, washed bacteria (5 × 10⁷) were pre-incubated with 10% serum for 30 min at 37°C in a total volume of 100 µl RPMI/1% HSA, in the presence of recombinant SCIN or CHIPS, after which samples were placed on ice. Bacteria were spun down and supernatant-induced calcium mobilization with isolated human neutrophils was performed as described (Veldkamp et al., 2000). Briefly, basal calcium levels of Calcein-loaded cells were mea-

### Table 1. Strains, plasmids and primers used.

| Strain, plasmid or primer | Genotype or description | Source |
|---------------------------|-------------------------|--------|
| *S. aureus*               |                         |        |
| 8325-4                    | Wild-type strain cured of prophages | A. Cheung |
| RN6390                    | Laboratory strain, derivative of 8325-4 | A. Cheung |
| RN4220                    | Restriction-deficient derivative of 8325-4 | A. Cheung |
| SH1000                    | Functional *rsbU* derivative of 8325-4 *rsbU* | S. Foster |
| SH1001                    | SH1000 *agr*:tet | S. Foster |
| MJH502                    | SH1000 *rsbU* *sigB*:tet | S. Foster |
| ALC1342                   | RN6390 *sara*:erm | A. Cheung |
| RN6911                    | RN6390 *agr*:tet | A. Cheung |
| UMCU502                   | SH1000 *sara*:erm | This study |
| JLA562                    | SH1000 *saer*:erm | S. Foster |
| JLA564                    | SH1000 *saer*:erm | S. Foster |
| UMCU13                    | Isolate from a patient with bloodstream infection at UMCU | This study |
| UMCU24                    | Isolate from a patient with bloodstream infection at UMCU | This study |
| UMCU58                    | Isolate from a patient with bloodstream infection at UMCU | This study |
| UMCU61                    | Isolate from a patient with bloodstream infection at UMCU | This study |
| Cowan EMS                 | Laboratory strain | ATCC |
| **E. coli**               |                         |        |
| TOP10F⁺                  | Host strain for cloning His-tagged protein | Invitrogen |
| BL21(DE3)                | Host strain for the expression of His-tagged protein | Invitrogen |
| **Plasmids**             |                         |        |
| pRSET-B                   | Expression vector for His-tagged recombinant protein | Invitrogen |
| pACL1484                  | Promotorless *gfp*, shuttle vector | A. Cheung |
| pACLsacr                  | sca Promoter cloned into EcoR1-Xba1 site of pACL1484 | This study |
| pACLchp                   | chp Promoter cloned into EcoR1-Xba1 site of pACL1484 | This study |
| pACLsak                   | sak Promoter cloned into EcoR1-Xba1 site of pACL1484 | This study |
| pACLssea                  | sea Promoter cloned into EcoR1-Xba1 site of pACL1484 | This study |
| **Primers**              |                         |        |
| *scn*-1                   | cgg aat tct tag aca cag cca tgc ttt gtt c |        |
| *scn*-2                   | gct cta gat gct tag taa tta gta cta a |        |
| *chp*-1                   | cgg aat tca tat taa tta cta ata g |        |
| *chp*-2                   | gct cta gat cct taa att cta tta a |        |
| *sak*-1                   | cgg aat tca tca tag tgg gag agg cgg t |        |
| *sak*-2                   | gct cta gat cca tat ata tat tgg t |        |
| *sea*-1                   | cgg aat taa tgg tat tag cca a |        |
| *sea*-2                   | gct cta gac tct taa gca taa ttc cat a |        |
| *rsbU*-5'                 | gctctagagttcaagacattag |        |
| *rsbU*-3'                 | tattctaccaaccttgaatactctgataac |        |

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sured in the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), after which undiluted supernatants or recombinant human C5a (Sigma, St. Louis, MO, USA) were added. The relative calcium increase of each sample was determined by dividing stimulus-induced fluorescence by the initial fluorescence levels. Calcium flux by supernatants was exclusively by C5α, as pre-incubation of neutrophils with rCHIPS lacking the first 30 amino acids (which has no formylated peptide receptor (FPR)-blocking activity) completely blocked the response (Haas et al., 2004). Chemotaxis of human neutrophils towards staphylococci in a serum environment was determined as described with minor adjustments (Veldkamp et al., 2000). BICECF-labelled neutrophils (5 x 10^6 cells in 100 µl) were added to the upper compartment of a Transwell system (3 µm; Costar, Cambridge, MA, USA), which was placed into a 24-well plate containing 600 µl HBSS/1% HSA, or 3 x 10^6 heat-killed S. aureus in the presence of 1% serum and 1 x 10^-6 M rSCIN or rCHIPS. After 45 min incubation at 37°C/5% CO₂, the inlays were removed and the fluorescence of the well was read in a Cytotuor II multwell fluorometer (PerSeptive Biosystems, Framingham, MA, USA).

**Whole blood inflammation model**

Freshly isolated human blood was treated with 50 µg ml⁻¹ lepirudin (Refudlan, Schering, Kenilworth, NJ) as anticoagulant (Mollnes et al., 2002). Phagocytosis was measured by incubation of FITC-labelled S. aureus Cowan EMS (3 x 10^7) (Rooijakkers et al., 2005c) with 50% blood in a final volume of 200 µl for 30 min at 37°C, after which samples were incubated with 1 ml BD Lysis Reagent (Beckton Dickinson) for 10 min at room temperature. Cells were spun down and washed twice with PBS. Bacterial uptake by 10 000 neutrophils was determined by flow cytometry. Oxidative burst was measured by chemiluminescence. Heat-killed S. aureus (15 µl of 5 x 10^6 bacteria) and 100 µl of luminol were automatically injected into microtitre plates containing 12.5% blood and inhibitors, in a luminometer (Berthold Technologies, Bad Wildbad, Germany) at 37°C, 2004. Chemiluminescence was measured every 30 s during 60 min and the area under curve was determined.

**Construction of promoter-gfp_uvr constructs**

pA.CL1484 is a pSK236-derived shuttle plasmid that contains the gene for GFP_uvr, a derivative of GFP_C (Wolz et al., 2000). PCR primers used to clone promoter sequences of IEC genes in pA.CL1484 are listed in Table 1. Chromosomal DNA from S. aureus strain Newman was used as the template for PCR. Amplified regions were ligated into the EcoR1 and Xba1 site of pA.CL1484 and cloned into E. coli. Recombinant plasmid was isolated (Qiagen PCR purification kit, Qiagen), electroproporated into RN4220, and cm-resistant colonies were selected. Plasmids from RN4220 were transferred to S. aureus strains RN6390, clinical S. aureus UMCO strains, SH1000, SH1001, MJH502, ALC1342, RN6911, UMCU502, JLA562 and JLA564 via standard electroporation or transduction techniques (Schenk and Laddaga, 1992).

**Transcriptional analyses of IEC promoters linked to gfp_uvr**

After overnight culture, S. aureus strains harbouring recombinant vectors were diluted to an A₆₆₀ of 0.08 in 3 ml THB and grown at 37°C. Every hour, A₆₆₀ was assayed and culture aliquots were taken. Bacteria were washed and GFP production of 10 000 bacteria was quantified by flow cytometry. The control vector with no promoter was used as a negative control.

**Northern analysis**

Clinical S. aureus strains were grown in THB as described above. Every hour, A₆₆₀ was assayed and bacteria and supernatants were collected after 2 (exp), 4 (ppx) and 6 (sta) h. For Northern analysis, total RNA from bacterial pellets was isolated using a TRIzol isolation kit according to the manufacturer’s instructions (Gibco BRL, Gaithersburg, MD). Total RNA concentrations were measured at OD₂₆₅, and 7 µg of RNA was electrophoresed through a 1.2% agarose-0.66 M formaldehyde gel in 20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, pH 7.0. RNA was transferred onto a Hybond-N membrane (Amersham) under mild alkaline conditions and fixed to the membrane at 80°C for 2 h. The scn and chp genes were amplified by PCR and gel-purified DNA probes were radiolabelled with [%-³²P]-dCTP by using the random-primed DNA labelling kit (Roche Diagnostics GmbH). Blots were hybridized with the probes under aqueous-phase conditions at 65°C, washed and autoradiographed.

**Detection of IEC proteins in supernatants by sandwich ELISA**

Overnight cultures of clinical S. aureus strains and SH1000 (negative control) were inoculated in 3 ml of THB to achieve an initial OD₆₆₀ of 0.08. Cultures were allowed to grow for 6 h and bacteria were pelleted each hour. Collected supernatants were frozen at –20°C. Mouse-anti-SCIN, mouse-anti-CHIPS and rabbit-anti-CHIPS antibodies were prepared as described earlier (Haas et al., 2004; Rooijakkers et al., 2005b). Microtitre plates (Greiner immunoplates, Frickenhausen, Germany) were coated with 0.15 µg anti-CHIPS (2H7) or anti-SCIN (1G1) monoclonal antibody, diluted in PBS, and left overnight at 4°C. Wells were blocked with 4% bovine serum albumin (BSA, Sigma) in PBS with 0.05% Tween-20 for 1 h at 37°C. Between all subsequent incubations wells were washed three times with PBS containing 0.05% Tween-20 (Sigma). Supernatants were pre-incubated with 100 µg ml⁻¹ Goat IgG (Jackson, West Grove, PA, USA) in 1% BSA to block protein A. Antibody-coated wells were incubated with blocked supernatant or recombinant SCIN or CHIPS (De Haas et al., 2004; Rooijakkers et al., 2005b) as a reference for 1 h at 37°C. CHIPS was detected with rabbit-anti-CHIPS followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Southern, Birmingham, AL, USA) in 1% BSA for 1 h at 37°C. SCIN was detected with a biotinylated monoclonal antibody (2F4) followed by HRP-conjugated streptavidin (Southern, Birmingham, AL, USA) in 1% BSA for 1 h at 37°C. HRP was visualized with 0.1 mg ml⁻¹ tetra-methyl-benzidine substrate (TMB, Sigma) and 0.15 mg ml⁻¹ urea hydrogenperoxide (Sigma) in 0.1 M acetate buffer. The reaction was stopped with 0.5 M H₂SO₄ and optical density was measured at 450 nm. SAK-mediated conversion of plasminogen (PLG) was tested as described (Rooijakkers et al., 2005c). In short, supernatants were incubated with 60 µg ml⁻¹ human Glu-PLG (Calbiochem, EMD Biosciences, Darmstadt, Germany) and the PL-specific substrate H-Val-Leu-Lys-Paranitroanalide (S-2251) (Bachem, Bubendorf, Switzerland) (3.8 mg ml⁻¹, pH 7.8) at 37°C for 1 h. Digestion of the sub-
strate by PL resulted in the release of para-nitro-analide that was measured at OD 405. rSAK (Rooijakkers et al., 2005c) was used as a reference. To calculate the protein production by a fixed amount of staphylococci, supernatant derived quantities of 1 × 10^8 bacteria were used.

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References

Abrahmsen, L., Dohlsen, M., Segren, S., Bjork, P., Jonsson, E., and Kalland, T. (1995) Characterization of two distinct MHC class II binding sites in the superantigen staphylococcal enterotoxin A. EMBO J 14: 2978–2986.

Benton, B.M., Zhang, J.P., Bond, S., Pope, C., Christian, T., Lee, L., et al. (2004) Large-scale identification of genes required for full virulence of Staphylococcus aureus. J Bacteriol 186: 8478–8489.

Beutler, B. (2004) Innate immunity: an overview. Mol Immunol 40: 845–859.

Bourner, S., Monteil, H., and Prevost, G. (2004) Regulation of virulence determinants in Staphylococcus aureus: complexity and applications. FEMS Microbiol Rev 28: 183–200.

Chan, P.F., and Foster, S.J. (1998) Role of SarA in virulence determinant production and environmental signal transduction in Staphylococcus aureus. J Bacteriol 180: 6232–6241.

Cheung, A.L., and Projan, S.J. (1994) Cloning and sequencing of sarA of Staphylococcus aureus, a gene required for the expression of agr. J Bacteriol 176: 4168–4172.

Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H., and Xiong, Y.Q. (2004) Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. FEMS Immunol Med Microbiol 40: 1–9.

De Haas, C.J., Veldkamp, K.E., Peschei, A., Weerkamp, F., van Wamel, W.J.B., Heeze, E.C., et al. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. J Exp Med 199: 687–695.

Gasque, P. (2004) Complement: a unique innate immune sensor for danger signals. Mol Immunol 41: 1089–1098.

Giraud, A.T., Calzolari, A., Cataldi, A.A., Bogni, C., and Nagel, R. (1999) The sae locus of Staphylococcus aureus encodes a two-component regulatory system. FEMS Microbiol Lett 177: 15–22.

Goerke, C., Fluckiger, U., Steinhuber, A., Zimmerli, W., and Wolz, C. (2001) Impact of the regulatory loci agr, sarA and sae of Staphylococcus aureus on the induction of alpha toxin during device-related infection resolved by direct quantitative transcript analysis. Mol Microbiol 40: 1439–1447.

Haas, P.J., de Haas, C.J., Kleibeuker, W., Poppelier, M.J., van Kessel, K.P.M., Kruijtzer, J.A., et al. (2004) N-terminal residues of the chemotaxis inhibitory protein of Staphylococcus aureus are essential for blocking formylated peptide receptor but not C5a receptor. J Immunol 173: 5704–5711.

Hampton, M.B., Kettle, A.J., and Winterbourn, C.C. (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92: 3007–3017.

Harraghy, N., Kormanec, J., Wolz, C., Homerova, D., Goerke, C., Ohlsen, K., et al. (2005) Sae is essential for expression of the staphylooccal adhesins Eap and Emp. Microbiology 151: 1789–1800.

Horsburgh, M.J., Aish, J.L., White, I.J., Shaw, L., Lithgow, J.K., and Foster, S.J. (2002) SigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from Staphylococcus aureus 8325-4. J Bacteriol 184: 5457–5467.

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J., and Tarkowski, A. (2004) Staphylococcus aureus resists human defensins by production of staphylolokinase, a novel bacterial evasion mechanism. J Immunol 172: 1169–1176.

Kullik, I.I., and Giachino, P. (1997) The alternative sigma factor sigmaB in Staphylococcus aureus: regulation of the sigB operon in response to growth phase and heat shock. Arch Microbiol 167: 151–159.

Langley, R., Wines, B., Willoughby, N., Basu, I., Proft, T., and Fraser, J.D. (2005) The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria. J Immunol 174: 2926–2933.

Lee, L.Y., Hook, M., Haviland, D., Wetsel, R.A., Yonter, E.O., Syrbeys, P., et al. (2004) Inhibition of complement activation by a secreted Staphylococcus aureus protein. J Infect Dis 1: 571–579.

Molkken, T., Tyynela, J., Heini, J., Kalkkinen, N., and Kuusela, P. (2002) Enhanced activation of bound plasminogen on Staphylococcus aureus by staphylinokinase. FEBS Lett 24: 1587–1590.

Mollnes, T.E., Brekke, O.L., Fung, M., Fure, H., Christiansen, D., Bergseth, G., et al. (2002) Essential role of the C5a receptor in E. coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. Blood 100: 1869–1877.

Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol 48: 1429–1449.

Pangburn, M.K., and Rawal, N. (2002) Structure and function of complement C5 convertase enzymes. Biochem Soc Trans 30: 1006–1010.

Peng, H.L., Novick, R.P., Kreiswirth, B., Kornblum, J., and Schlievert, P. (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in Staphylococcus aureus. J Bacteriol 170: 4365–4372.

Postma, B., Poppelier, M.J., van Galen, J.C., Proft, T., van Strijp, J.A.G., De Haas, C.J., and Van Kessel, K.P.M. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus binds specifically to the C5a and formylated peptide receptor. J Immunol 172: 6994–7001.

Postma, B., Kleibeuker, W., Poppelier, M.J., Boonstra, M., van Kessel, K.P.M., van Strijp, J.A.G., and De Haas, C.J. (2005) Residues 10–18 within the C5a receptor N terminus compose a binding domain for chemotaxis inhibitory protein of Staphylococcus aureus. J Biol Chem 280: 2020–2027.

Recepi, P., Kreiswirth, B., O’Reilly, M., Schlievert, P., Gruss, A., and Novick, R.P. (1986) Regulation of exoprotein gene expression in Staphylococcus aureus by agr. Mol Gen Genet 202: 58–61.
Early expression of staphylococcal innate immune modulators

Rooijakkers, S.H.M., Van Kessel, K.P.M., and Van Strijp, J.A.G. (2005a) Staphylococcal innate immune evasion. Trends Microbiol 12: 596–601.

Rooijakkers, S.H.M., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., et al. (2005b) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nature Immunol 6: 920–927.

Rooijakkers, S.H.M., Van Wamel, W.J.B., Ruyken, M., van Kessel, K.P.M., and Van Strijp, J.A.G. (2005c) Anti-opsonic properties of staphylokinase. Microbes Infect 7: 476–484.

Sahu, A., and Lambris, J.D. (2001) Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev 180: 35–48.

Schenk, S., and Laddaga, R.A. (1992) Improved method for electroporation of Staphylococcus aureus. FEMS Microbiol Lett 73: 133–138.

Sumby, P., and Waldor, M.K. (2003) Transcription of the toxin genes present within the Staphylococcal phage phiSa3ms is intimately linked with the phage’s life cycle. J Bacteriol 185: 6841–6851.

Van Wamel, W.J.B., Rooijakkers, S.H.M., Ruyken, M., Van Kessel, K.P.M., and Van Strijp, J.A.G. (2006) The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of Staphylococcus aureus are located on β-hemolysin-converting bacteriophages. J Bacteriol 188: 1310–1315.

Veldkamp, K.E., Heezius, H.C., Verhoef, J., Van Strijp, J.A.G., and Van Kessel, K.P.M. (2000) Modulation of neutrophil chemokine receptors by Staphylococcus aureus supernate. Infect Immun 68: 5908–5913.

Wolz, C., Pohlmann-Dietze, P., Steinhuber, A., Chien, Y.T., Manna, A., Van Wamel, W., and Cheung, A. (2000) Agr-independent regulation of fibronectin-binding protein(s) by the regulatory locus sar in Staphylococcus aureus. Mol Microbiol 36: 230–243.

Xu, Y., Narayana, S.V., and Volanakis, J.E. (2001) Structural biology of the alternative pathway convertase. Immunol Rev 180: 123–135.

Yarwood, J.M., McCormick, J.K., and Schlievert, P.M. (2001) Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of Staphylococcus aureus. J Bacteriol 183: 1113–1123.