Staphylococcus aureus clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization

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Summary

Staphylococcus aureus is an important cause of sepsis in both community and hospital settings, a major risk factor for which is nasal carriage of the bacterium. Eradication of carriage by topical antibiotics reduces sepsis rates in high-risk individuals, an important strategy for the reduction of nosocomial infection in targeted patient populations. Understanding the mechanisms by which S. aureus adheres to nasal epithelial cells in vivo may lead to alternative methods of decolonization that do not rely on sustained antimicrobial susceptibility. Here, we demonstrate for the first time that the S. aureus surface-expressed protein, clumping factor B (ClfB), promotes adherence to immobilized epidermal cytokeratins in vitro. By expressing a range of S. aureus adhesins on the surface of the heterologous host Lactococcus lactis, we demonstrated that adherence to epidermal cytokeratins was conferred by ClfB. Adherence of wild-type S. aureus was inhibited by recombinant ClfB protein or anti-ClfB antibodies, and S. aureus mutants defective in ClfB adhered poorly to epidermal cytokeratins. Expression of ClfB promoted adherence of L. lactis to human desquamated nasal epithelial cells, and a mutant of S. aureus defective in ClfB had reduced adherence compared with wild type. ClfB also promoted adherence of L. lactis cells to a human keratinocyte cell line. Cytokeratin 10 molecules were shown by flow cytometry to be exposed on the surface of both desquamated nasal epithelial cells and keratinocytes. Cytokeratin 10 was also detected on the surface of desquamated human nasal cells using immunofluorescence, and recombinant ClfB protein was shown to bind to cytokeratin K10 extracted from these cells. We also showed that ClfB is transcribed by S. aureus in the human nares. We propose that ClfB is a major determinant in S. aureus nasal colonization.

Introduction

Staphylococcus aureus is a major cause of both community-acquired and nosocomial infection (Schalberg, 1991; Kluytmans, 1998). A key risk factor for such disease is S. aureus carriage, the principal ecological niche for which is the moist squamous epithelium adjacent to the nasal ostium in the anterior nares (Cole et al., 2001). Approximately 20% of the population persistently carry S. aureus at this site, whereas about 60% of individuals are intermittent carriers (Kluytmans, 1997). Staphylococcus aureus can also be found at other body sites including the axilla and the perineum (Williams, 1963), but nasal carriage is a prerequisite for carriage at such sites (Kluytmans, 1998; von Eiff et al., 2001). Temporary eradication of carriage reduces the risk of S. aureus infection, a strategy that is being adopted to reduce nosocomial sepsis in individuals at high risk such as those receiving haemodialysis via a temporary intravenous device. Whereas this approach is effective it may ultimately prove to be short-lived, given its reliance on sustained susceptibility of S. aureus to topical antibiotics.

The development of alternative control measures could depend on understanding the determinants of carriage at the cellular and molecular level, a central component of which is the mechanism by which S. aureus adhere to nasal epithelial cells in vivo.

The adherence of S. aureus cells to host proteins such as fibrinogen, fibronectin, collagen, elastin, von Willebrand factor, vitronectin and bone sialoprotein is mediated by bacterial cell wall-associated proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Staphylococcus aureus can express up to 20 different potential MSCRAMMs that are anchored to cell wall peptidoglycan following sortase-
mediated cleavage of the C-terminal LPXTG motif (Navarre and Schneewind, 1994; Mazmanian et al., 1999; Roche, F.M., Massey, R., Peacock, S.J. and Day, N.P.J., submitted). However, only the fibrinogen-binding proteins ClfA and ClfB (McDevitt et al., 1994; Ni Eidhin et al., 1998), the fibronectin-binding proteins FnBPA and FnBPB (which also bind to fibrinogen) (Signas et al., 1989; Jönsson et al., 1991; Wann et al., 2000), the collagen-binding protein Cna (Patti et al., 1992) and protein A, which binds to IgG and to von Willebrand factor (Sjödahl, 1977; Moks et al., 1986; Hartlieb et al., 2000) have been characterized in detail.

*Staphylococcus aureus* adhere to desquamated human nasal epithelial cells in vitro (Aly et al., 1977; Bibel et al., 1982). Squamous cells undergo shape change, increasing keratinization and loss of the nucleus as they move from the basal layer to the exposed surface in vivo. Adherence of *S. aureus* in vitro is greatest to the most mature cell type (Bibel et al., 1982). The factors on the surface of squamous epithelial cells that promote bacterial attachment are not known. One possible candidate is the heterodimeric protein keratin, which appears to be exposed on the surface of epithelial cells (Hembrough et al., 1995; Sajjan et al., 2000, 2002). Keratins are major structural proteins and are members of a superfamily of proteins known as intermediate filaments (reviewed in Fuchs, 1995). They are divided into one of two different types based on isoelectric point and sequence. Type II keratins include K1 and K8 and are generally more basic and larger (~53–67 kDa) than the type I molecules. Type I keratins include K10 and K18 and tend to be smaller (~40–63 kDa) and more acidic. Keratin filaments are composed of heterodimeric multimers of a type I and a type II keratin. Thus, K1, a type II keratin, pairs with K10, a type I keratin. As epithelial cells differentiate and migrate towards the surface of the epidermis, a significant change in keratin expression occurs. Cells in the basal layer express K5 and K14, while the more differentiated cells of the supra-basal layer contain large amounts of K1 and K10. Whereas K8 and K18 are typical of simple epithelial cells, they are not associated with stratified squamous epithelia.

In this paper, we expressed ClfB in the heterologous host *Lactococcus lactis* and used a mutant of *S. aureus*, defective in ClfB expression, to show that ClfB promoted adherence to squamous nasal epithelial cells, most likely through a specific interaction with the type I cytokeratin molecule, K10. We show that K10 is expressed on the surface of desquamated human nasal epithelial cells, and that the clfB gene is transcribed by *S. aureus* in the human nares in vivo. We also demonstrate that ClfB promotes bacterial adherence to immobilized purified epidermal keratin. We propose that ClfB is a major determinant in *S. aureus* nasal colonization.

### Results

*S. aureus* cells adhere to immobilized epidermal cytokeratin

Previous studies have demonstrated that *S. aureus* adheres to keratinized human nasal epithelial cells (Aly et al., 1977; Bibel et al., 1982). Here we studied the bacterial surface components involved and examined the role of host keratins in this process.

*Staphylococcus aureus* Newman, 8325-4, P1, Cowan, MSSA and EMRSA-16 (MSSA and EMRSA-16 are the *S. aureus* strains that are currently being sequenced at the Sanger Center, UK), grown to exponential phase or to stationary phase, were tested for adherence to immobilized human epidermal keratin. Cells grown to exponential phase adhered strongly (Fig. 1A). With the exception of strain Newman, *S. aureus* adherence to keratin was restricted to cells from mid-exponential phase. Stationary phase cells of other strains adhered very poorly to immo-

**Fig. 1.** A. *S. aureus* strains were grown to exponential phase (shaded) or stationary phase (unshaded) and tested for binding to immobilized epidermal keratin (5 μg ml⁻¹). B. *L. lactis* cells containing the empty pKS80 vector, or a pKS80 construct expressing one of the *S. aureus* surface proteins indicated, were tested for their ability to adhere to immobilized keratin (5 μg ml⁻¹). Results shown are the mean of triplicate samples. Error bars show standard deviations.
Adherence to immobilized human epidermal cytokeratin by L. lactis cells expressing S. aureus surface proteins

In order to identify the receptor(s) responsible for mediating the adherence of S. aureus cells to immobilized human epidermal keratin, L. lactis cells expressing ClfB, SdrC or SdrD (O’Brien et al., 2002) were tested for adherence to ELISA wells coated with the protein. Previous studies have shown that each of these strains expresses the heterologous protein at levels similar to the native host (O’Brien et al., 2002). Expression of ClfB by L. lactis conferred the ability to adhere to keratin \( (P = 0.0007) \) (Fig. 1B). However, adherence of L. lactis expressing SdrC or SdrD (other SD repeat-containing S. aureus adhesins) was not significantly different from that of L. lactis carrying the expression vector alone \( (P = 0.6 \) and \( P = 0.4, \) respectively). These data indicated that ClfB was at least partly responsible for promoting binding of S. aureus cells to epidermal keratin.

Because ClfB is a clumping factor that binds to the \( \alpha \)-chain of fibrinogen (Perkins et al., 2001), it was necessary to show that the epidermal cytokeratins used in these studies were not contaminated with fibrinogen. Purified epidermal keratin \( (20 \mu g) \) did not react with polyclonal anti-fibrinogen antibodies by Western immunoblotting, but did react, showing a single \( 56.5 \) kDa band, with a monoclonal anti-K10 antibody (data not shown). In addition, the anti-fibrinogen antibodies did not block the adherence of S. aureus cells to keratin, whereas they completely inhibited the adherence of S. aureus to fibrinogen (data not shown). Thus, the epidermal cytokeratins used here are not contaminated with fibrinogen.

S. aureus mutants deficient in ClfB do not adhere to immobilized human epidermal cytokeratin

To further investigate the role of ClfB in mediating the adherence of S. aureus cells to keratin, exponential phase cells of S. aureus Newman wild type and the mutants Newman clfB, and Newman clfB complemented with clfB’ carried on a plasmid (pCU1:clfB’) were tested for keratin binding. ClfA is a related fibrinogen-binding protein and its role was also evaluated using the double-defective mutant Newman clfBclfA. Newman wild-type cells adhered in a dose-dependent manner (Fig. 2A). Adherence of both the clfB mutant and the clfBclfA double mutant was markedly reduced, whereas binding of the clfB mutant complemented with pCU1:clfB’ approached wild-type levels. These data indicated that ClfB was responsible for mediating the adherence of S. aureus cells to epidermal cytokeratin. Furthermore, these data showed that ClfA was not responsible for S. aureus binding to keratin, as the level of adherence for Newman clfB and Newman clfActclfB was comparable.

To determine if ClfB promoted keratin binding of other S. aureus strains, 8325-4 and P1 wild type and their clfB mutants were tested for adherence to immobilized keratin. Adherence of both mutants was significantly reduced compared with that for wild type \( (8325-4, \ P = 0.002; \ P1, \ P = 0.003, \) Fig. 2B).

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Recombinant ClfBN2-3 protein binds to immobilized human epidermal cytokeratin and blocks adherence of ClfB-expressing S. aureus cells

Several recombinant truncated variants of the A region of ClfB (Fig. 3A) have been constructed (Perkins et al., 2001). Binding of ClfBN1-3, ClfBN1-2 and ClfBN2-3 to immobilized human epidermal keratin was measured. Both ClfBN1-3 and ClfBN2-3 bound to keratin in a dose-dependent manner, whereas ClfBN1-2 did not bind (Fig. 3B). These data indicate that the keratin-binding site is located within the N2-3 domain of ClfB.

The ability of each recombinant ClfB truncate to block the adherence of S. aureus cells to immobilized keratin was measured. There was no significant inhibition with either ClfBN2 or ClfBN3, and a small reduction at only the highest concentration of recombinant protein for ClfBN1-2 (Fig. 3C). This contrasted with the effects of ClfBN1-3 and ClfBN2-3; ClfBN1-3 caused significant inhibition at both 50 and 100 µM recombinant protein (P = 0.002 and < 0.0001 respectively), whereas ClfBN2-3 protein gave almost complete inhibition of S. aureus binding to keratin at 10 µM (P < 0.0001). The reason for the difference in inhibition capabilities between ClfBN1-2 and ClfBN2-3 is unclear but may be caused by a difference in protein folding. These data agreed with the direct recombinant protein binding experiments, which showed strongest keratin binding by ClfBN2-3. They also show that relative keratin binding affinities of recombinant domains mirrors their fibrinogen binding activity (Perkins et al., 2001), suggesting that keratin and fibrinogen might bind to similar regions within the ClfB protein.

Anti-ClfB A domain antibodies inhibit adherence of S. aureus to immobilized human epidermal cytokeratin

Polyclonal antibodies that had been raised against the ClfB A domain (N1-3) were tested for their ability to inhibit

![Fig. 3. A. Schematic representation of the mature ClfB protein, showing the three subdomains (N1, N2 and N3), located within the unique ligand-binding region (A domain). B. Serial dilutions of recombinant ClfB proteins: rClfBN2-3 (open circles), rClfBN1-3 (closed circles) and rClfBN1-2 (diamonds), ranging from 2 µM to 4 nM, were tested for their ability to interact with human epidermal keratin (5 µg ml⁻¹). C. Recombinant ClfB proteins; ClfBN2 (diamonds), ClfBN3 (crosses), ClfBN1-2 (triangles), ClfBN2-3 (closed circles) and ClfBN1-3 (open circles) were diluted to concentrations ranging from 100 to 4 µM. These proteins were incubated in 96-well ELISA dishes coated with 5 µg ml⁻¹ keratin and tested for their ability to block S. aureus Newman spa from binding to keratin. D. Dose–response inhibition of S. aureus Newman spa cells binding to immobilized keratin (5 µg ml⁻¹) following incubation with serial dilutions of anti-ClfBN1-3 antibodies (4 µM–2 nM). Results shown are the mean of triplicate samples. Error bars show standard deviations. © 2002 Blackwell Science Ltd, Cellular Microbiology, 4, 759–770.](image-url)
adherence of a protein A-deficient mutant of S. aureus Newman (spa) to immobilized keratin. This strain was used instead of the wild type to avoid the interaction between protein A and the Fc region of IgG. Bacterial adherence was inhibited in a dose-dependent manner following preincubation with anti-N1-3 antibodies (Fig. 3D). Both anti-N2 and anti-N3 antibodies also reduced adherence while pooled anti-N2 and anti-N3 antibodies gave almost complete inhibition of adherence, when compared to the background level (data not shown). In contrast, anti-N1 antibodies did not block adherence (data not shown). These data support the notion that the keratin-binding domain is located within the N2-3 region (aa 197–542) of the protein.

L. lactis cells expressing ClfB adhere to squamous nasal epithelial cells and keratinocytes

Highly keratinized stratified squamous epithelial cells are the predominant cell type at the exposed surface of the site of S. aureus colonization in the anterior nares. We examined the ability of ClfB to promote bacterial adherence to squamous cells, removed by swabbing the anterior nares of healthy individuals. Lactococcus lactis cells expressing either ClfB or ClfA were tested for their ability to adhere to squamous cells. Lactococcus lactis containing the empty pKS80 vector adhered poorly (Fig. 4A). Adherence of L. lactis expressing ClfA (pKS80:clfA) was not significantly different from that of L. lactis carrying pKS80 alone (P = 0.13). In contrast, a significant increase in the adherence to squamous cells was observed with L. lactis cells expressing ClfB (P = 0.001).

In addition, preincubation of the squamous epithelial cells with recombinant ClfBN1-3 caused a 93% reduction in binding by L. lactis cells expressing ClfB. For L. lactis cells expressing ClfB, a bacterial count of 865.5 ± 44.5 cells per 100 squamous cells was observed. However, when the squamous cells were preincubated with recombinant ClfBN1-3, only 62 ± 45 L. lactis cells expressing ClfB adhered (P = 0.0004), thus indicating that the interaction between ClfB and the squamous epithelial cell was specific.

ClfB was tested for its ability to mediate adherence to HPV-G keratinocytes. Keratinocytes are the progenitors of squamous epithelial cells. Lactococcus lactis cells carrying the empty pKS80 vector showed very low levels of adherence to the keratinocytes, whereas only a slight increase was seen with L. lactis cells expressing ClfA (Fig. 4B). However, L. lactis cells expressing ClfB adhered strongly to the keratinocytes, showing a highly significant increase in the numbers of bacteria attached (P < 0.0001). These data show that ClfB is capable of mediating bacterial adherence to squamous epithelial...
cells from the anterior nares and to undifferentiated human keratinocytes.

A S. aureus clfB mutant has reduced adherence to squamous nasal epithelial cells

Adherence of S. aureus strain Newman wild-type, Newman clfB, 8325–4 wild-type and 8325–4 clfB cells to squamous epithelial cells was compared. The 8325–4 clfB mutant had a 1.45-fold reduced level of adherence compared with wild type \((P = 0.0002)\), whereas the Newman clfB mutant had a 1.98-fold reduced level of adherence compared to the wild type \((P = 0.0007)\) (Fig. 4C). These data indicate that ClfB plays a role in mediating the attachment of S. aureus to nasal squamous epithelial cells. However, it is clear that other factors are also involved.

Recombinant ClfBN\(_{2-3}\) binds to type I cytokeratin K10 from nasal squamous epithelial cells

To examine the host receptor involved in the interaction between ClfB and squamous epithelial cells, desquamated cells were harvested and lysed, the proteins separated by SDS-PAGE and analysed by silver staining. Two major protein bands corresponding to the predicted size of the type II keratin, K1 (67 kDa) and the type I keratin, K10 (56.5 kDa) were observed (Fig. 5A).

Squamous cell lysates were also probed with recombinant ClfBN\(_{2-3}\) protein in ligand affinity blots. ClfBN\(_{2-3}\) bound to a 56.5 kDa protein (Fig. 5B). Lysates were also analysed by Western immunoblotting using monoclonal antibodies specific for the human type I keratin, K10. K10 was detected as a single immunoreactive band, which migrated at 56.5 kDa, the same size as the protein band recognized by ClfBN\(_{2-3}\) (Fig. 5C). These data strongly suggest that the S. aureus ClfB protein binds specifically to the type I keratin K10.

To ensure that fibrinogen was absent in the squamous cell lysates, blots were probed with HRP-labelled polyclonal anti-human fibrinogen antibodies. No fibrinogen was detected (data not shown). This, coupled with the fact that ClfB binds to the 70 kDa fibrinogen α-chain (Perkins et al., 2001), ruled out the possibility that ClfB bound to fibrinogen in this assay.

The type I keratin K10 is detected on the surface of human desquamated nasal epithelial cells and cultured keratinocytes

To confirm that cytokeratin 10 molecules were present on the surface of desquamated nasal epithelial cells and on the surface of keratinocytes, cells were probed with specific FITC-labelled anti-cytokeratin 10 monoclonal antibodies. Cells were then analysed by flow cytometry in order to measure the fluorescence intensity of each sample. For both the squamous epithelial cells and the keratinocytes, an increase in fluorescence was observed compared to the normal mouse FITC-labelled antibody control (Fig. 6). Thus, these data showed that keratin 10 was exposed on the surface of both the squamous epithelial cells and the keratinocytes.

Confocal microscopy

Having established, by flow cytometry, that K10 is expressed on the surface of intact squamous cells, we decided to examine in more detail ClfB+ L. lactis cells adhering to squamous epithelial cells by confocal micros-
S. aureus clfB binds keratin 10

copy. Squamous cells were isolated, immobilized on glass slides and then incubated with L. lactis ClfB+. Cells were fixed and then incubated with a monoclonal antibody specific for K10. Adherent K10 antibodies were labelled using a FITC-labelled secondary antibody, whereas adherent bacterial cells were recognized by labelling with rhodamine-labelled anti-ClfB antibodies. K10 is expressed on the surface of the squamous epithelial cell in a non-uniform fashion, with some areas of the membrane staining more heavily than others (Fig. 7A). Lactococcus lactis ClfB+ adhered strongly to the squamous cells (Figs 7B–D).

Figure 7C appears to show K10 staining in some areas of the cell that do not have any bacterial cells bound. However, because the squamous cell is three-dimensional, a proportion of bound bacteria are sometimes not observed when the cell is viewed in a single focal plane. In fact, the squamous cell shown here does have bound bacteria in most areas, but some are not obvious in the plane shown. This is clearly demonstrated in Fig. 8 where a Z-stack has been performed. Here, images of the same cell shown in Fig. 7, taken in four different planes ranging from upper through lower levels (Fig. 8A–D), are shown. This clearly shows that bacterial cells are distributed across most areas of the squamous cell. This is a typical representation of all squamous cells observed.

The clfB gene is transcribed in S. aureus cells isolated from human nasal carriers

To examine whether the clfB gene is transcribed in bacteria colonizing the nasal cavity of a human carrier, Reverse transcription polymerase chain reaction (RT-PCR) was performed with RNA isolated from bacteria collected directly from a nasal swab. This was compared with RNA isolated from the equivalent number of bacteria grown aerobically to stationary phase, conditions where the clfB gene is not transcribed (McAleese et al., 2001). The RT-PCR amplification of the constitutively expressed gyr gene was included as a control. Transcription of the gyr gene was detected in both preparations of RNA, whereas transcription of the clfB gene was only detected in bacteria harvested from the carrier (Fig. 9). These data indicate that clfB is transcribed by S. aureus present in the nasal cavity.

Discussion

Staphylococcus aureus attaches to a very specific region in the nasal vestibule, namely the moist squamous epithelium which is devoid of hair, cilia and microvilli (Cole et al., 2001). Attachment of S. aureus to these cells is likely to be a critical step in both initiation and perpetuation of colonization. This study has shown that S. aureus adheres to desquamated stratified squamous epithelial cells isolated from the nares of volunteers. Adherence occurs by a mechanism that appears likely to be at least partly due to the interaction between the S. aureus ClfB protein and the host type I cytokeratin, K10. This interaction was demonstrated by analysis of S. aureus attachment to both squamous epithelial cells and to purified epidermal cytokeratin.
We show that keratin binding is a widespread property of *S. aureus* strains, including laboratory strains and natural isolates. A common factor was that the bacterial receptor responsible was expressed primarily during the exponential phase of growth. Expression in exponential phase is characteristic of the fibrinogen-binding clumping factor ClfB (McAleese et al., 2001). *Lactococcus lactis* cells expressing various *S. aureus* surface proteins were tested for their ability to adhere to human epidermal cyto-keratin. ClfB-expressing *L. lactis* cells adhered to immobilized keratin, whereas SdrC- and SdrD-expressing cells did not. The observations made with the heterologous expression system were further investigated using isogenic mutants of *S. aureus*. ClfB was shown to be entirely responsible for mediating the attachment of *S. aureus* cells to immobilized keratin.

The region of the ClfB protein responsible for binding to epidermal keratin was examined by testing binding to keratin by recombinant domains and inhibition of binding by antibodies specific to different regions of the protein. These experiments identified the keratin-binding domain in domains N2 and N3 between aa 197–542. The affinity of the recombinant ClfB proteins for keratin mirrors their affinities for fibrinogen, suggesting that each of these host...
proteins binds to similar regions in ClfB. This will be investigated by locating the binding sites precisely with synthetic peptides and by mutagenesis.

In addition to its ability to promote attachment of *S. aureus* cells to epidermal keratin, we have shown that ClfB is also partly responsible for mediating bacterial attachment to the highly keratinised squamous epithelial cells from the nasal cavity. Our results indicate that ClfB binds to these cells via an interaction with the type I keratin molecule, K10, the predominant type I keratin in squamous epithelial cells. We have also shown that ClfB is expressed by *S. aureus* cells isolated from the nasal cavity of a healthy carrier, suggesting that this interaction can occur in vivo. Thus, we propose that the interaction between ClfB and K10 is important in nasal colonization. ClfB also mediates bacterial attachment to cultured keratinocytes, possibly through an interaction with keratin 10. We have shown, by flow cytometry and immunofluorescence, that cytokeratin 10 is exposed on the surface of desquamated epithelial cells and keratinocytes. Others have demonstrated that cytokeratin 13 is expressed on the surface of buccal epithelial cells (Sajjan et al., 2000) and squamous bronchial epithelial cells (Sajjan et al., 2002) and that cytokeratin 8 is expressed on the surface of hepatocytes and other epithelial cell lines (Hembrough et al., 1995). Although keratin has classically been known as an intracellular molecule, recent evidence has suggested that this may not necessarily be true. Our studies indicate that a certain amount of keratin 10 molecules are indeed expressed on the surface of squamous epithelial cells and keratinocytes. The mechanism by which this occurs is not understood.

Clearly, there are other factors involved in the attachment of *S. aureus* cells to nasal epithelia, because ClfB-deficient mutants of *S. aureus* strains 8325-4 and Newman could still adhere strongly. However, the ClfB-independent adherence to squamous cells is unlikely to be due to keratin binding, because several *S. aureus* clfB mutants that were tested could not adhere to immobilized epidermal keratin. Characterizing the protein(s) responsible for the ClfB-independent binding of *S. aureus* cells to squamous epithelial cells will be the focus of future work in this laboratory. It should also be noted that previous studies have shown that *S. aureus* cells can also bind to the type I keratin, cytokeratin 8 (Tamura and Nittayajarn, 2000). The implications for *S. aureus* binding to K8 are not clear, but it is unlikely that they are related to colonization. This is because K8 is associated with simple epithelial cells and not stratified squamous epithelia or the upper layers of the dermis.

Our findings lead us to propose that binding to K10 via ClfB contributes to *S. aureus* colonization in the nose. Furthermore, this interaction may contribute to carriage of *S. aureus* on the skin including the hands, which is the carriage site central to host-to-host transmission. It is also possible that this interaction could contribute to the pathogenesis of skin infections caused by *S. aureus*. The interaction between ClfB and keratin is unlikely to define the carriage state (that is, non-carriage versus carriage), but rather be one component in the host–bacterial interaction in carriers. Our findings provide important leads towards the development of new therapeutic agents capable of eradicating *S. aureus* carriage in individuals at risk of staphylococcal infection.

**Experimental procedures**

**Materials and reagents**

Human epidermal cytokeratins were from the Sigma Chemical, St Louis, MI, USA. Human fibrinogen was from Calbiochem, Nottingham, UK. Mouse anti-human K10 monoclonal antibodies (FITC-conjugated and unlabelled) were from Research Diagnostics, NJ, USA. Other antibodies were from Dako, Denmark. Enzymes were from Roche or New England BioLabs, unless otherwise stated.

**Bacterial strains and growth conditions**

*Staphylococcus aureus* was grown in Brain–Heart Infusion (BHI) broth (Oxoid) (50 ml volumes were grown in a 250 ml conical flask). Stationary phase cultures were grown for 16 h. Exponential phase cultures were established by a 1 in 50 dilution of washed stationary phase cultures and grown to an OD600 of 0.6–0.8 (1.5–2 h) at 37°C in air with shaking at 200 r.p.m. *Lactococcus lactis* strain MG1363 was used for heterologous expression of the *S. aureus* surface proteins (O’Brien et al., 2002). *Lactococcus lactis* MG1363 was grown on M17 agar or broth (Difco) incorporating 0.5% glucose at 30°C in air. The following antibiotics were incorporated where appropriate: ampicillin (100 µg ml⁻¹), erythromycin (5 or 10 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹) and tetracycline (2 µg ml⁻¹).

*Staphylococcus aureus* Newman defective in protein A (spa:Ka; DU5971) (Ni Eidhin et al., 1998), clumping factor A (clfA2::Tn917; DU5876) (McDevitt et al., 1994), clumping factor

![Fig. 9. RT-PCR of total RNA isolated from stationary phase *S. aureus* strain Newman cells (lanes 1 and 2) or *S. aureus* cells isolated directly from the anterior nares (lanes 3 and 4). Lanes 1 and 3 show the product obtained with primers specific to the gyrA gene, whereas lanes 2 and 4 show the product obtained with primers specific to the clfB gene.](image-url)
The reaction was stopped with 2 M H$_2$SO$_4$ (50 mM phosphate citrate buffer, pH 5.0) was added per well for 10 min.

Previously described (Ni Eidhin et al., 1998) and the clfB: Tc$^{\text{R}}$ mutant complemented by clfB present on a multicopy plasmid (pCU1::clfB) (Ni Eidhin et al., 1998) were described previously. Staphylococcus aureus 8325-4 deficient in clfB (clfB::Em$^{\text{R}}$; DU5961) was also described previously (McAleese et al., 2001). Staphylococcus aureus P1 (Sherertz et al., 1993) defective in clfB was constructed by transduction of the clfB::Em$^{\text{R}}$ mutation from strain DU5961 to generate strain DU5994 (F. McAleese, unpublished data). Strains MSSA and EMRSA-16 are currently being sequenced at the Sanger Center, UK, (http://www.sanger.ac.uk/Projects/Microbes/).

Bacterial adherence to immobilized epidermal cytokeratin

Adherence assays were performed as described previously (O’Brien et al., 2002). Briefly, 96-well plates were coated with keratin or fibrinogen ranging from 0.15 to 5 μg ml$^{-1}$ in phosphate-buffered saline (PBS) overnight at 4°C. Keratins were supplied dissolved in 8 M urea at a concentration of 9.5 mg ml$^{-1}$, and diluted in PBS for coating. Following bacterial staining with crystal violet, adherence was measured by ELISA plate reader (Lab-systems Multiskan Plus) at 570 nm.

Recombinant ClfB truncate anti-ClfB antibodies

Recombinant ClfB proteins representing subdomains from within the A region were described previously (Perkins et al., 2001). They were designated N$_1$, N$_2$, N$_3$, N$_L$, and N$_E$ corresponding to rClfB44-542, rClfB44-375, rClfB197-542, rClfB44-196, rClfB197-375 and rClfB376-542 respectively (see Fig. 3A). Antibodies to N$_1$, N$_2$ and N$_3$ were raised in young New Zealand white rabbits (2 kg) whose preimmune sera showed no reaction with S. aureus antigens by Western blotting. Anti-N$_{1-3}$ antibodies were previously described (Ni Eidhin et al., 1998).

Binding of recombinant ClfB to immobilized epidermal cytokeratin

Ninety-six-well plates were coated with keratin (5 μg ml$^{-1}$) and blocked with 5% BSA. Recombinant proteins were serially diluted from 2 mM to 4 mM and added to each well. Plates were incubated for 2 h at room temperature, washed with PBS and incubated with anti-N2 antibodies (1:2000) for 1 h. After washing, HRP-labelled goat anti-rabbit IgG was added (1:2000) for 1 h at room temperature. After washing, 100 μl of chromogenic substrate (1 mg ml$^{-1}$ tetramethylbenzidine, 0.006% H$_2$O$_2$ in 0.05 M phosphate citrate buffer, pH 5.0) was added per well for 10 min. The reaction was stopped with 2 M H$_2$SO$_4$ (50 μl well$^{-1}$) and plates were read at 450 nm.

Inhibition of bacterial cell adherence to immobilized epidermal cytokeratin by rClfB

Ninety-six-well plates were coated with keratin (5 μg ml$^{-1}$) and blocked with 5% BSA. Different concentrations of recombinant proteins, ranging from 100 μM to 6 μM (50 μl well$^{-1}$), were added and incubated for 2 h at room temperature. A bacterial cell suspension (50 μl, 2 × 10$^8$ CFU ml$^{-1}$) was added and incubated for 90 min at room temperature. Plates were washed with PBS and adherent cells detected by crystal violet staining, as described above.

Antibody inhibition of bacterial adherence to immobilized epidermal cytokeratin

Staphylococcus aureus Newman spa cells were grown to exponential phase, washed in PBS and resuspended to an OD$_{600nm}$ of 1.0. Cells (350 μl) were incubated with 4 μg of antibody for 1 h at 37°C (anti-N1, anti-N2, anti-N3 or anti-N$_{2-3}$ antibodies). In the case of the anti-N$_1$ antibodies, dilutions were made and added to 350 μl of bacterial cells for 1 h at 37°C. Adherence of cells to 5 μg ml$^{-1}$ immobilized keratin was measured, as described above.

Bacterial adherence to squamous nasal epithelial cells and keratinocytes

Squamous cells were harvested from the anterior nares of healthy donors by vigorous swabbing of the moist squamous epithelium on the inside of the nasal septum. Swabs from both nostrils were taken and agitated in 3 ml of PBS to suspend the cells. Squamous cells were harvested by centrifugation at 3000 r.p.m. for 5 min and washed twice in PBS. HPV-G keratinocytes were obtained as a kind gift from C. Mothersill, Dublin Institute of Technology, Ireland. They are immortalized but not transformed human keratinocytes derived from foreskin (Piriasi et al., 1988). They were routinely cultured in DMEM:F$_12$ (1:1) containing 10% fetal bovine serum (FBS), 1% penicillin:streptomycin, and hydrocortisone (Sigma). All cultures were used for experiments when 85–90% confluent. Cells were removed from flasks by subculturing using a solution of equal volumes of 0.25% trypsin and 1:5000 versene solution (Gibco). For experimental purposes, keratinocytes were removed from the flasks by scraping in 10 ml of incomplete medium and were passed through a 26 GA needle. Cells were harvested by centrifugation at 750 g and washed twice in incomplete medium. The number of squamous cells and keratinocytes were quantified using a Neubauer Improved counting chamber and adjusted to 5 × 10$^6$ cells ml$^{-1}$ in PBS. Bacterial cells were grown to either exponential phase or stationary phase, were harvested, washed, and adjusted to 1 × 10$^7$ cells ml$^{-1}$. 100 μl of bacterial cells were then mixed with 100 μl mammalian cells and incubated at 37°C for 1 h with occasional shaking. Following this incubation, PBS (800 μl) was added to each tube to minimize further bacterial attachment. Mammalian cells were then collected under vacuum onto a 12 mm Isopore$^{\text{TM}}$ polycarbonate filter (Millipore) using the BioDot$^{\text{TM}}$ apparatus (Bio-Rad) and washed five times with 300 μl of PBS. Cells were fixed onto these filters using the cytological fixative Cytofix$^{\text{TM}}$ (Cellpath) and allowed to dry for 10 min. Filters were stained with 5% crystal violet (45 s), washed in H$_2$O and dried. They were then mounted on glass slides under 25 × 50 mm coverslips using the mountant D.P.X. (BDH).

The number of bacteria adherent to desquamated nasal epithelial cells or keratinocytes was quantified using a visual method. Using oil immersion and a 100 × magnification lens, the number of bacteria associated with 100 epithelial cells or 100 keratinocytes was enumerated. Only anucleate squamous cells

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Samples were incubated with anti-cytokeratin 10 antibodies with 3% paraformaldehyde for 10 min at ambient temperature. (5 min each wash, with gentle shaking). Cells were then bacterial cells were removed by washing six times in TS buffer and stained by incubating with a rhodamine-labelled anti-ClfB N 1-3 antibody (2μg ml⁻¹) for 1 h at 37°C. Following this incubation, 2% paraformaldehyde was added and the fluorescence intensity of each sample analysed using a FACScan cytometer and the cellquest software.

**Flow cytometry**

Squamous epithelial cells and keratinocytes were harvested and washed as before. Monoclonal FITC-labelled anti-keratin 10 antibodies (Research Diagnostics) or polyclonal FITC-labelled normal mouse antibodies (Dako) were then added and the cells incubated for 1 h at 37°C. Following this incubation, 2% paraformaldehyde was added and the fluorescence intensity of each sample analysed using a FACScan cytometer and the cellquest software.

**Confocal microscopy**

Squamous cells were collected onto poly-L-lysine coated glass slides (Sigma) by centrifugation at 500 r.p.m. for 5 min using a Shandon Cytospin 2. Cells were blocked with 2% BSA for 1 h at 37°C in a humidified chamber, and then incubated with approximately 1 × 10⁵ L. lactis ClfB⁺ cells for 1 h at 37°C. Non-adherent bacterial cells were removed by washing six times in TS buffer (5 min each wash, with gentle shaking). Cells were then fixed with 3% paraformaldehyde for 10 min at ambient temperature. Samples were incubated with anti-cytokeratin 10 antibodies (2 μg ml⁻¹) for 1 h at 37°C and then washed three times for 10 min with TS buffer. Cells were then incubated with fluorescein-conjugated rabbit anti-mouse antibodies (1:20; Dako) for 1 h at 37°C. Slides were washed with TS buffer. Adherent bacteria were stained by incubating with a rhodamine-labelled anti-ClfB N1-3 antibody (2 μg ml⁻¹) for 1 h at 37°C. (Approximately 200 μg of anti-ClfB N1-3 antibody was labelled with rhodamine using the Pierce EZ-label™ rhodamine protein labelling kit, according to the manufacturer’s instructions.) Following washing four times for 5 min in TS buffer with gentle shaking, slides were dipped in H₂O and mounted under a glass coverslip using the vectashield mountant (Vector Laboratories). Cells were then viewed under a 40 × magnification lens using a Zeiss LSM 510 Axioplan 2 confocal microscope and the LSM version 2.8 software.

**Isolation of RNA**

Bacteria were either grown to stationary phase or isolated directly from the anterior nares of a healthy carrier as before. Bacteria from nasal swabs were washed in sterile PBS and an aliquot was plated onto mannitol salt selective agar to measure the number of S. aureus cells (taken as mannitol fermenting colonies). The RNA was isolated using a QiAamp® Viral RNA kit (Qiagen) in accordance with the manufacturer’s instructions except 10 μg ml⁻¹ lysostaphin (AMBI, New York) was added to the AVL buffer and incubated at 37°C for 20 min. Contaminating DNA was removed (Goerke et al., 2001).

**Detection of gyr and clfB transcript by RT-PCR**

Reverse transcription polymerase chain reaction was carried out using the TITAN One Tube RT-PCR system (Roche) in accordance with the manufacturer’s instruction. The concentration of RNA was normalized according to the number of CFU ml⁻¹ in each preparation. The primers for the gyr gene were as described previously (Goerke et al., 2001). The sequence of the primers for the clfB gene were: 5’-TGCAAGATCAACGCTTCC-3’ and 5’-TCGGTCTGTAAATAGGTATGTA-3’. To exclude the possibility of DNA contamination, control samples were subjected to PCR amplification without prior reverse transcription. Samples were analysed on a 0.9% agarose gel.

**Statistical analysis**

Statistical analysis was carried out using the Statview 4.5 statistical software package (Abacus). Comparison between bacterial strains of the mean absorbance (microtitre plate adherence assays), or bacterial count (epithelial cell adherence assays), was performed using a t-test.

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