Differences in expression of virulence genes amongst invasive and colonizing isolates of methicillin-resistant *Staphylococcus aureus*

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) is an important global concern in healthcare due to potentially life-threatening infections and difficulties in treatment. The organism can colonize the mucosa, e.g. anterior nares, or cause invasive infections, e.g. bloodstream infections, through the coordinated expression of extracellular and cell-bound virulence factors (Goerke *et al.*, 2000; Novick *et al.*, 1993). The transcription profiles of virulence genes vary *in vivo* depending on the dynamic interaction between the host environment and the pathogen. Since the dynamic environment may be differentially altered in MRSA carriage compared to infection, the expression of *S. aureus* virulence genes may also vary. We previously found no significant correlation between the presence of 17 virulence genes and invasiveness of MRSA isolates (O'Donnell *et al.*, 2008) and this non-association of specific genes or combinations of genes with invasive isolates is in agreement with other larger studies (Lindsay *et al.*, 2006). The lack of a definitive correlation between invasive *S. aureus* strains and the carriage of virulence genes suggests that the expression, rather than carriage of virulence determinants *in vivo*, may mediate pathogenicity.

We investigated the *in vitro* expression of selected virulence genes, gamma-haemolysin (*hlg*), collagen adhesion-encoding gene (*cna*) and staphylococcal enterotoxin A (*sea*), in colonizing compared with invasive MRSA clinical isolates, using the same isolate collection used in our previous study (O'Donnell *et al.*, 2008). *hlg* and *sea* were representative of toxin genes and *cna* was representative of adhesion genes. These genes were selected because they were among those present in all isolates from our previous study. We also investigated the expression of one regulatory gene, *RNAIII*, the effector molecule of the accessory gene regulator (*agr*) system which is reported to

upregulate toxin genes and downregulate adhesion genes.

Twenty isolates were recovered from patients with device-related bloodstream infections between January 2002 and June 2005 and 20 isolates were from nasal swabs collected from patients that were MRSA-positive on first hospital admission (between September 2006 and June 2007) but with no apparent signs of infection. The *spa* type of each isolate was used to infer the sequence type (ST) or clonal complex from which the isolate originated and the staphylococcal cassette chromosome *mec* type was also determined (O'Neill *et al.*, 2007). RNA was isolated from cultures grown to an optical density of 2 (3–5 h, determined from preliminary experiments to correspond to the mid-late exponential phase) in brain heart infusion broth supplemented with commercially available 5% pooled human serum to mimic the *in vivo* environment. Real-time RT-PCR was performed using specific primers for each of the four genes and expression levels were normalized to the expression of *gyrB* as an internal control. The absolute quantity of each gene in isolates could not be determined without appropriate standards, therefore calibrator-normalized relative quantification was used. The relative expression level of each gene was the ratio of its normalized value in the test isolates to its normalized value in isolate BH16. Isolate BH16 was chosen arbitrarily from the collection as the calibrator isolate.

The relative expression levels of the four virulence genes in invasive versus colonizing isolates (including the gene regulator *RNAIII*) are shown in Fig. 1. There was a high level of variation in the relative expression of virulence genes among both groups of isolates. Comparing the two groups, colonizing and invasive, there was no significant alteration in the transcription of *RNAIII* in invasive isolates versus colonizing isolates ($P=0.0859$ by

Mann–Whitney test using GraphPad Prism version 4.00 for Windows).

Transcription of *cna* was upregulated in colonizing isolates compared to invasive isolates ($P=0.0110$) whereas transcription of *hlg* was upregulated in invasive isolates compared to colonizing isolates ($P=0.0193$ by Mann–Whitney). Although more invasive isolates than colonizing isolates expressed relatively higher levels of the *sea* transcript, this was not statistically significant ($P=0.1456$). Comparing the relative expression of all four genes in each isolate (data not shown), the transcription of *RNAIII* was accompanied by a reduction in transcription of *cna* and increased transcription of *hlg* in only four invasive isolates and none of the colonizing isolates. *RNAIII* did not increase transcription of *sea* but the *sea* transcript was apparently expressed at low levels in most isolates or was not detectable. Genotyping of isolates used in this study revealed that both colonizing and invasive groups were highly clonal, belonging to either ST22-MRSA-IV (100% and 65%) or ST8-MRSA-II (0% and 35%). Of the four *agr* types that have been described, ST22-MRSA-IV and ST8-MRSA-II isolates belong to *agr* type 1 (Monecke *et al.*, 2008). Fig. 2 shows that within the invasive isolates group, ST8-MRSA-II isolates differed significantly in their transcription levels to ST22-MRSA-IV for *cna* ($P=0.005$) and *sea* ($P=0.019$) but not for *hlg* ($P=0.064$) or *RNAIII* ($P=0.062$). When the ST8-MRSA-II isolates were omitted from the overall analysis, only the transcript levels of *sea* were significantly increased ($P=0.013$) in the invasive group compared to the colonizing isolates. Although in the invasive isolates the transcript levels of *cna* were decreased and those of *hlg* were increased, these failed to reach significance ($P=0.22$ and $P=0.19$) when only ST22-MRSA-IV isolates were analysed (data not shown).

Virulence factors that promote binding to the mucosal layers are important in *S.*

Fig. 1. Fold change in transcript levels of *RNAIII*, *cna*, *hlg* and *sea* in MRSA colonizing isolates (black circles) and invasive isolates (grey triangles) using real-time PCR with relative quantification with respect to an arbitrarily chosen isolate. Horizontal lines indicate the median value for each dataset. Statistically significant changes in transcript levels are indicated.

aureus colonization of the host, whereas factors that promote tissue damage and provoke damaging immune responses are important during *S. aureus* infection (Cheung *et al.*, 2004; Novick *et al.*, 1993). In the present study, we found significant differences in the relative expression levels of virulence genes in colonizing versus

invasive MRSA isolates when the clinical isolate was grown to mid-late exponential phase in the presence of human serum in an attempt to mimic the *in vivo* conditions. In the presence of human serum, a greater number of invasive isolates expressed lower levels of the cell-associated transcript *cna* and higher levels

of the toxin gene *hlg* but not of *sea*. A greater number of colonizing isolates expressed relatively high transcript levels of *cna* with *hlg* transcripts also being relatively high in some, and *sea* transcription was low or beyond the limit of detection. Our data also indicate that the origin of the isolate cannot be considered independently of other genetic factors relating to the isolate as these may make a significant contribution to pathogenicity. It has been shown that the carriage of virulence genes is highly clonal but here we have also demonstrated an association between clonal type and transcription of virulence genes (Fig. 2). Although the differences in the pattern of expression of virulence-associated genes may contribute to the pathogenicity of MRSA in invasive versus colonizing isolates, this study shows that colonizing isolates may also have the capacity to promote host damage in some situations. Alterations in the local environment, such as pH or proteolytic activity, may affect the activity of expressed proteins, or differences in the immune response to the organism may determine the overall pathogenesis of MRSA.

Expression of *RNAIII*, in the late-exponential phase, positively correlated with the expression of *hlg* and negatively correlated with the expression of *cna* for only a small number of invasive isolates. Similarly, other authors have shown that positive correlation between transcription of the toxin genes and the regulator gene *RNAIII* may be isolate-specific (Papakyriacou *et al.*, 2000; Sabersheikh & Saunders, 2004) or may vary with the phase of growth studied as *RNAIII* is a quorum-sensing effector. Alternatively, expression of *RNAIII* may not be critical for expression of *S. aureus* toxins and this has been confirmed in one of the few studies that have measured *in vivo* transcription of *RNAIII* in an animal model of infection (Yarwood *et al.*, 2002). It is also possible that virulence gene expression may be regulated by other regulator genes or a combination of these. Alternative regulatory systems identified in *S. aureus* include *sar*, *sigB*, *rot*, *arlR/S*, *svrA* and *saer/S* (Bronner *et al.*, 2004; Novick & Jiang, 2003).

We have found that under *in vitro* conditions using human serum that may

Fig. 2. Fold changes in transcript levels of *RNAIII*, *cna*, *hlg* and *sea* in MRSA invasive isolates when correlated with the clonal type of the isolate, ST22 (black triangles) and ST8 (grey triangles). Horizontal lines indicate the median value for each dataset. Statistically significant changes in transcript levels are indicated.

partly mimic what occurs in patients (at least in infection), variations in the transcription of some virulence genes occurred that correlated with the isolate source (colonizing or from infection), and within the invasive group, ST8-MRSA-II isolates showed decreased transcription of *cna* and increased transcription of *sea* compared to ST22-MRSA-IV isolates. Because environmental conditions in the nares of patients differ from those in the bloodstream, further work in this area should aim to mimic more closely the local host environment during colonization and infection (e.g. bacterial growth phase, pH, osmolarity and/or host-niche-specific factors, e.g. presence of polymorphonuclear neutrophils and/or platelets) or to investigate transcription of virulence genes when isolates are grown in the presence of the patient's own serum. These studies should also be extended to other virulence genes and other gene regulators. Accurate direct measurement of gene expression levels *in vivo* presents a challenge, but this research area may benefit from recent improvements in microgenomic detection of bacterial RNA in human tissues (Klitgaard *et al.*, 2007). These approaches should further our understanding of the properties of *S. aureus* that allow it to transform from transient commensal to pathogen.

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