

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

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

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## 28 **Introduction**

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

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

51 Twenty isolates were recovered from patients with device-related bloodstream infections  
52 between January 2002 and June 2005 and twenty isolates were from nasal swabs, collected from  
53 patients that were MRSA positive on first hospital admission (between September 2006-June  
54 2007) but with no apparent signs of infection. The *spa* type of each isolate was used to infer the  
55 sequence type (ST) or clonal complex (CC) from which the isolate originated and the  
56 staphylococcal chromosomal cassette *mec* (SCC*mec*) type was also determined (O'Neill *et al.*,  
57 2007). RNA was isolated from cultures grown to OD = 2 (3-5 h, determined from preliminary  
58 experiments to correspond to the mid-late exponential phase) in brain heart infusion (BHI) broth  
59 supplemented with commercially available pooled human serum (5 %) to mimic the *in-vivo*  
60 environment. Real-time RT-PCR was performed using specific primers for each of the four  
61 genes and expression levels were normalized to the expression of *gyrB* as an internal control.  
62 The absolute quantity of each gene in isolates could not be determined without appropriate  
63 standards, therefore calibrator-normalized relative quantitation was used. The relative expression  
64 level of each gene was the ratio of its normalized value in the test isolates to its normalized value  
65 in isolate BH16. Isolate BH16 was chosen arbitrarily from the collection as the calibrator isolate.  
66 The relative expression levels of the four virulence genes in invasive *vs.* colonizing isolates  
67 (including the gene regulator, *RNAIII*) are shown in Figure 1. There was a high level of variation  
68 in the relative expression of virulence genes among both groups of isolates. Comparing the two  
69 groups, colonizing and invasive, there was no significant alteration in the transcription of  
70 *RNAIII*, in invasive isolates *vs* colonizing isolates (P value = 0.0859 by Mann Whitney test using  
71 GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA).  
72 Transcription of *cna* was up-regulated in colonizing isolates compared to invasive isolates (P  
73 value = 0.0110) whereas transcription of *hlg* was up-regulated in invasive isolates compared to

74 colonizing isolates (P value = 0.0193 by Mann Whitney). Although a greater number of invasive  
75 isolates expressed relatively higher levels of the *sea* transcript than colonizing isolates, this was  
76 not statistically significant (P value = 0.1456). Comparing the relative expression of all four  
77 genes in each isolate (data not shown), the transcription of *RNAIII* was accompanied by a  
78 reduction in transcription of *cna* and increased transcription of *hlg* in only 4 invasive isolates and  
79 none of the colonizing isolates. *RNAIII* did not increase transcription of *sea* but the *sea*  
80 transcript was apparently expressed at low levels in most isolates or was not detectable.  
81 Genotyping of isolates used in this study revealed that both colonizing and invasive groups were  
82 highly clonal, belonging to either ST22-MRSA-IV (100% and 65%) or ST8-MRSA-II (0 % and  
83 35%). Of the four *agr* types that have been described, ST22-MRSA-IV and ST8-MRSA-II  
84 isolates belong to *agr* type 1 (Monecke *et al.*, 2008). Figure 2 shows that within the invasive  
85 isolates group ST8-MRSA-II isolates differed significantly in their transcription levels to ST22-  
86 MRSA-IV, for *cna* (P=0.005) and *sea* (P= 0.019) but not for *hlg* (P=0.064) or *RNA III* (P=  
87 0.062). When the ST8-MRSA-II isolates were omitted from the overall analysis, only the  
88 transcript levels of *sea* were significantly increased (P=0.013) in the invasive group compared to  
89 colonizing. Although in the invasive isolates the transcripts level of *cna* were decreased and that  
90 of *hlg* were increased, these failed to reach significance (P=0.22 and 0.19) when only ST22-  
91 MRSAIV isolates were analyzed (data not shown).

92 Virulence factors that promote binding to the mucosal layers are important in *S. aureus*  
93 colonization of the host, whereas factors that promote tissue damage and provoke damaging  
94 immune responses, are important during *S. aureus* infection (Cheung *et al.*, 2004; Novick *et al.*,  
95 1993). In the present study we found significant differences in the relative expression levels of  
96 virulence genes in colonizing vs. invasive MRSA isolates when the clinical isolate was grown to

97 mid-late exponential phase, in the presence of human serum in an attempt to mimic the *in-vivo*  
98 conditions. In the presence of human serum, a greater number of invasive isolates expressed  
99 lower levels of the cell-associated transcript, *cna* and higher levels of the toxin gene *hlg* but not  
100 *sea*. A greater number of colonizing isolates expressed relatively high transcript levels of *cna*  
101 with *hlg* transcripts also being relatively high in some and *sea* transcription was low or beyond  
102 the limit of detection. Our data also indicate that the origin of the isolate cannot be considered  
103 independently of other genetic factors relating to the isolate as these may make a significant  
104 contribution to pathogenicity. It has been shown that the carriage of virulence genes is highly  
105 clonal but here we have also demonstrated an association between clonal type and transcription  
106 of virulence genes (Fig. 2). Although the differences in the pattern of expression of virulence  
107 associated genes may contribute to the pathogenicity of MRSA in invasive *vs.* colonizing  
108 isolates, this study shows that colonizing isolates may also have the capacity to promote host  
109 damage in some situations. Alterations in the local environment, such as pH or proteolytic  
110 activity, may affect the activity of expressed proteins, or differences in the immune response to  
111 the organism may determine the overall pathogenesis of MRSA.

112       Expression of *RNAIII*, in the late exponential phase, positively correlated with the  
113 expression of *hlg* and negatively correlated with the expression of *cna*, for only a small number  
114 of invasive isolates. Similarly, other authors have shown that positive correlation between  
115 transcription of the toxin genes and the regulator gene *RNAIII* may be isolate-specific  
116 (Papakyriacou *et al.*, 2000; Saberssheikh & Saunders, 2004) or may vary with the phase of growth  
117 studied as *RNAIII* is a quorum sensing effector. Alternatively, expression of *RNAIII* may not be  
118 critical for expression of *S. aureus* toxins and this has been confirmed in one of the few studies  
119 that has measured *in-vivo* transcription of *RNAIII* in an animal model of infection (Yarwood *et*

120 *al.*, 2002). It is also possible that virulence gene expression may be regulated by other regulator  
121 genes or a combination of these. Alternative regulatory systems identified in *S. aureus* include  
122 *sar*, *sigB*, *rot*, *arlR/S*, *svrA*, *saeR/S* (Bronner *et al.*, 2004; Novick & Jiang, 2003).

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

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#### 146 **Legend Figure 1**

147 Fold change in transcript levels of *RNAIII*, *cna*, *hlg* and *sea* in MRSA colonizing isolates (●) and  
148 invasive isolates (▲) using real-time PCR with relative-quantitation with respect to an arbitrarily  
149 chosen isolate. Horizontal lines indicate the median value for each data set. Statistically  
150 significant changes in transcript levels are indicated.

151

#### 152 **Legend Figure 2.**

153 Fold changes in transcript levels of *RNAIII*, *cna*, *hlg* and *sea* in MRSA invasive isolates when  
154 correlated with the clonal type of the isolate, ST-22 (▲), ST-8 (▲). Horizontal lines indicate the  
155 median value for each data set. Statistically significant changes in transcript levels are indicated.

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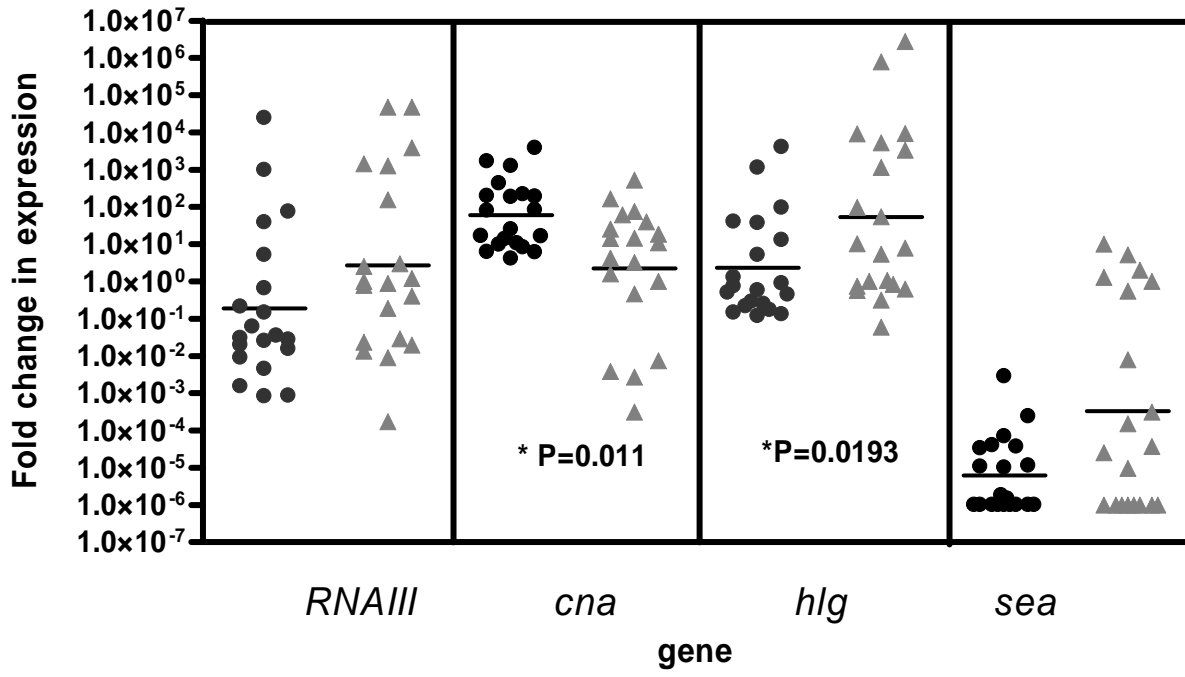
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217 Figure 1.

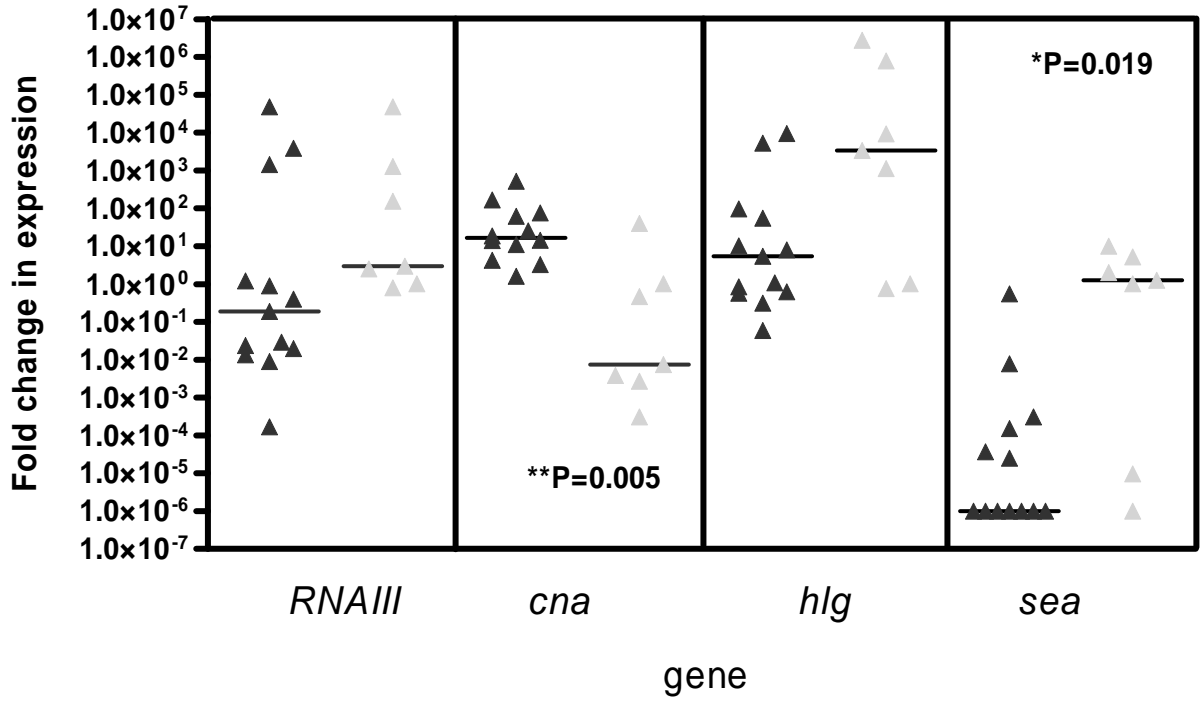


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Figure 2.



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