Loss of hemolytic expression in *Staphylococcus aureus* agr mutants correlates with selective survival during mixed infections in murine abscesses and wounds

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Abstract

During the screening of a *Staphylococcus aureus* signature-tagged mutagenesis library, it was noted that nonhemolytic bacteria became more abundant as time passed in murine abscess and wound models, but not within organ tissues associated with systemic infections. To examine this further, a mixed population of hyperhemolytic, hemolytic, and nonhemolytic *S. aureus* strain RN6390 cells were inoculated into mice using abscess, wound, and systemic models of infection. After 7 days in the abscess, the hyperhemolytic group markedly declined, whereas the nonhemolytic population increased significantly. A similar phenomenon occurred in murine wounds, but not during the systemic infection. Sequencing of several of the signature-tagged mutants indicated mutations in the *agrC* gene or within the *agrA*-agrC intergenic region. Both \(\alpha\)-hemolysin and \(\delta\)-hemolysin activity was curtailed in these mutants, but \(\beta\)-hemolysin activity was unaffected. Single strain comparisons between wild-type strain 8325-4 and strain DU1090 (hla-) as well as between strain RN6911 (agr) and wild-type strain RN6390 were performed using the same three animal models of infection. The *agr* mutant strain and the *hla* mutant strain showed no difference in bacterial counts in murine wounds compared to their respective parent strains. The same held true in murine abscesses at day 4, but strain RN6911 counts then declined at day 7. Considerable clearing of the *hla* mutant strain and the *agr* mutant strain occurred in the systemic model of infection. Mixed infections with the DU1090 and 8325-4 strains in the abscess model showed a slight advantage given to the DU1090 population, but a distinct selection for the parental 8325-4 strain in the liver. These results suggest that *agr* mutations cause reductions in the expression of several secreted proteins, including \(\alpha\)- and \(\delta\)-hemolysin, which in turn contribute to a growth advantage of this *agr* mutant group within a mixed population of *S. aureus* cells residing in abscesses and wounds.

Keywords: *Staphylococcus aureus*; Hemolytic; Abscess; Wound

1. Introduction

The bacterial species *Staphylococcus aureus* is an important pathogen of humans, infecting nearly every tissue and organ within the human body. This species is a significant cause of nosocomial infections, many of which are life-threatening [1,2]. Several surface structures and secreted proteins contribute to the pathogenicity of *S. aureus*. Among these virulence factors are four hemolysins (\(\alpha\), \(\beta\), \(\delta\) and \(\gamma\)). The \(\alpha\)-hemolysin, a pore-forming protein that forms an oligomeric beta-barrel [3], was first identified as being associated with lesion formation in mice [4] and later cloned [5] and mutated [6] to discern its true role in virulence. Several animal models of infection, including pneumonia, systemic, mastitis, and kidney, have shown that \(\alpha\)-hemolysin is an important component of the arsenal the *S. aureus* cells have for contributing to the disease state [5,7–10]. However, there have been other studies that suggest that \(\alpha\)-hemolysin expression may not be a major virulence factors in all types of infections [11,12]. The \(\beta\)- and
δ-hemolysins also have been shown to be important in specific animal infection models [13,14].

The question of whether hemolysin expression is important in all infection models was raised during the screening of a S. aureus signature-tagged mutagenesis library through a murine abscess model [15]. A selection for non-hemolytic variants was apparent. Follow-up work has shown that a nonhemolytic population within a mixed infection was selected for after 7 days in mouse abscesses. This was due to either mutations in the agrC gene or the agrA–agrC intergenic region with subsequent reductions in expression of α- and δ-hemolysin. Furthermore, one on one comparisons between a wild-type strain and an hla mutant strain of S. aureus as well as mixed infections with both strains in murine abscess, wound, and systemic models of infection demonstrated either no significant difference or a slight advantage to the hla mutant in the wound and abscess models, but a significant decline of the hla population in the systemic model. These observations suggest that strains with mutations in the agrC gene may have a growth advantage in mixed S. aureus cultures within an abscess or wound in part due to a loss of α-hemolysin expression, but also more likely due to the loss of other secreted proteins.

2. Materials and methods

2.1. Bacterial strains, plasmids

A library of 1520 S. aureus strain 6390 signature-tagged mutagenesis clones of strain RN6390 [15] were screened as 16 separate groups of 95 in a murine abscess model of infection described below. Hyperhemolytic, hemolytic, and nonhemolytic variants of strain RN6390 [16] were selected for on sheep blood agar plates (Remel). The hyperhemolytic variant had a zone of complete clearing about twice the size of the hemolytic variant. Strain RN6911 (agr-) was obtained from Ambrose Cheung, Dartmouth University [17]. Strains 8325-4 and DU1090 (hla::Erm) were obtained from Timothy Foster, Trinity College [6]. All bacteria were originally grown in brain heart infusion broth. Antibiotics were used at the following concentrations: erythromycin 5 μg ml⁻¹, chloramphenicol 10 μg ml⁻¹ (Sigma Chemical Co., St. Louis, MO, USA).

2.2. Hemolysin expression

To test for the presence of α-, β-, and δ-hemolysin, the S. aureus were passaged on both sheep blood agar plates and rabbit blood agar plates incubated at 37°C for 18–24 h, followed by refrigeration at 4°C for 16–24 h. In addition, hemolysin activity was tested using 24-h culture supernatants from the various strains. Heparinized sheep or rabbit blood (Micronet Medical Inc., White Bear Lake, MN, USA) was washed with phosphate-buffered saline (PBS; pH 7.2) until no visible red color was present in the PBS layer. A 2% concentration of red blood cells was added to two-fold serially diluted culture supernatants in a microtiter plate and the plate refrigerated overnight at 4°C. After 18–24 h, the wells were checked for lysis of the red blood cells.

2.3. Staphylokinase expression

Soluble staphylokinase was tested using the procedure of Behnke and Gerlach [18]. Briefly, culture supernatants were applied to holes in agar layers, which contained skim milk and plasminogen. The plates were incubated for 3–8 h at 37°C with the formation of clearing zones representing a positive result.

2.4. Animal models

Three animal models of infection were used in this study. For screening of the large pools of signature-tagged mutants and single mutant determinations through a murine abscess model, the protocol outlined by Coulter et al. [15] was used. A total of two to four mice per pool or four to eight mice per individual strain were injected at three sites along the back for each time point used. Abscesses were recovered after 3–7 days following in vivo selection and homogenized in 0.85% physiological saline. Serial dilutions of the homogenized abscesses were plated onto sheep blood agar plates and the number of hyperhemolytic, hemolytic, and nonhemolytic colonies were counted.

In the murine wound model of infection [19], SKH-1 mice were branded after administration of 0.17 mg kg⁻¹ of Torbutrol analgesic (Fort Dodge Laboratories). With the single mutant strain testing, four to six mice were injected subcutaneously beneath the burn site with 0.2 ml inoculums of 10⁶ cfu for pooled staphylococci or 10⁴ cfu for single strains of the S. aureus. Wound homogenates were serially diluted in physiological saline and plated onto sheep blood agar plates.

For the systemic bacteremia infection model [11], four to six Beige (C57BL/6J-hg¹⁺; Jackson Laboratory) or BALB/c mice (Jackson Laboratory) were injected intravenously with 0.1–0.2 ml of inoculum at approximately 5×10⁶ cfu ml⁻¹. Spleens and livers were harvested, homogenized, and plated onto sheep blood agar plates.

2.5. Statistics

All P-value determinations were done using Student’s t-test.

3. Results and discussion

In this study, a bank of 1520 signature-tagged Tn917
mutants of strain RN6390 were screened in 16 groups of 95 mutants [15] within abscess, systemic, and wound models of infection. As part of the analysis, the percentage of nonhemolytic colonies in the inocula were counted for each group. Overall, 5.13% of the population was nonhemolytic (Fig. 1). Initially, these pools of signature-tagged mutants were inoculated into the backs of mice in an abscess model of infection and allowed to remain there for 4 or 7 days. Abscess homogenates collected at these two time points were plated onto sheep blood agar plates and the number of hemolytic colonies tabulated. After 4 days in vivo in the abscess, the percentage of nonhemolytic variants rose to 23.13% (P < 0.002) and increased again to 33.13% (P < 0.00001) after 7 days inside the abscesses (Fig. 1).

Examination of the signature-tagged mutant pools in systemic and burn wound models of infection were also performed. They indicated that hemolysin production was favored in the liver and spleen (97.2% and 96% hemolytic, respectively) compared to the initial inocula (95.2% hemolytic), but there was a selection for nonhemolytic variants in the murine burn wound (16.8% nonhemolytic) compared to the initial 5.13% found overall in the inocula. These results suggested a selection for nonhemolytic bacterial cells in the abscess and wound models of infection.

The analysis of the signature-tagged mutant library of strain RN6390 hinted that there might be selection for nonhemolytic clones in a mixed population of S. aureus cells residing in a mouse abscess. To verify this, hyperhemolytic, hemolytic, and nonhemolytic variants of wild-type RN6390 were selected for after in vitro passage on sheep blood agar plates. Pools of each population were prepared and mixed together for inoculation using the murine abscess model of infection. Initially, the division of the inoculum going in was 23.7% nonhemolytic, 32.0% hemolytic, and 44.0% hyperhemolytic (Fig. 2A). Following 3 days inside a murine abscess, the division was 36% nonhemolytic, 37.7% hemolytic, and 26.3% hyperhemolytic. After a total of 7 days within mouse abscesses, 61.0% of the bacteria were nonhemolytic, 29.7% were hemolytic, and 9.3% were hyperhemolytic, thus there was a significant selection (P < 0.0023) for nonhemolytic variants over time in the murine abscesses.

When the same mixed population was put through a wound infection model, the population sorted out into 49.8% nonhemolytic, 28.0% hemolytic, and 22.2% hyperhemo-
hemolytic after 6 days in vivo (Fig. 2B; \( P < 0.049 \)), showing a trend that was similar to the abscess model of infection. However, a systemic model of infection seemed to favor hemolytic bacteria because after 2 days post-inoculation, bacteria from the liver sorted into 13.0% nonhemolytic, 42.5% hemolytic, and 44.5 hyperhemolytic (Fig. 2C), whereas bacteria from the spleen divided into 20.0% nonhemolytic, 38.8% hemolytic, and 39.2% hyperhemolytic (Fig. 2D). These analyses supported the observations that nonhemolytic bacteria appeared to be selected for in both the murine abscesses and wounds, but this population was selected against in the liver and spleen.

Sequencing of several of the nonhemolytic clones showed mutations in the \( agr \) gene or the intergenic region between \( agrC \) and \( agrA \), but no mutations in the genes involved in hemolysis (data not shown). Because Agr affects a multitude of surface-expressed and secreted proteins, including \( \alpha \)- and \( \delta \)-hemolysins [17], a variety of functions could be ascribed to these changes, of which, lack of hemolysis may only correlate. To better characterize the nonhemolytic variety of strain RN6390, hemolysin and staphylokinase assays were performed. Although staphylokinase activity might be linked to survival in murine abscesses [20], no difference in activity was observed between hemolytic and nonhemolytic varieties of \( S. \) aureus strain RN6390, indicating staphylokinase activity was not likely responsible for the difference in survival. On the other hand, both \( \alpha \) - and \( \delta \)-hemolysin activities were abolished in the nonhemolytic strain of RN6390, whereas \( \beta \)-hemolysin activity was unaffected.

The data above indicated that in an initial mixed population of wild-type and \( agr \) mutant subpopulations, nonhemolytic variants missing \( \alpha \)- and \( \delta \)-hemolysin activity seemed to prosper at the expense of the clones that were expressing these hemolysins in murine abscesses and wounds. \( \alpha \)-Hemolysin, the chief hemolysin of \( S. \) aureus strains causing disease in humans, is important in at least some animal models of infections [5,7–10]. Since \( \alpha \)-hemolysin is the prime mediator of hemolysis, an \( hla \) mutant strain of \( S. \) aureus was compared to wild-type \( S. \) aureus in three animal models of infection: abscess, wound, and systemic. In an abscess model of infection, the bacterial counts after 3 days in abscesses were not significantly different for strains 8325-4 and DU1090 (Table 1). After 7 days in the murine abscesses, the bacterial counts for DU1090 were basically the same compared to the wild-type strain 8325-4. However, the bacterial counts for the \( agr \) mutant strain RN6911 were significantly lower compared to the wild-type strain RN6390 (\( P < 0.0092 \)). Agr is a positive global regulator of several \( S. \) aureus virulence factors that include hemolysins [17], which appear to be important in the murine abscess. Within a mixed culture, the missing proteins provided by the wild-type bacteria would likely allow the \( agr \) mutant to grow to higher levels, but this assistance would be absent when the \( agr \) mutant was inoculated by itself into the murine abscess. Furthermore, whatever the factor may be whose expression is missing in the \( agr \) mutant strain, it does not appear to be \( \alpha \)-hemolysin, since the \( \alpha \)-hemolysin mutant fared as well as the wild-type parent in this environment.

The murine wound model of infection also showed little difference in the survival of the \( hla \) mutant strain of \( S. \) aureus compared to the wild-type strain (Table 1). Moreover, there was no difference between the wild-type strain RN6390 versus the \( agr \) mutant strain in the wound infection model. On the other hand, a systemic model of infection demonstrated that both the \( hla \) mutant and \( agr \) mutant strains had fewer bacteria in the livers and spleens of the infected animals compared to their respective wild-type parents (Table 1).

The analyses with individual strains alluded to the possibility that the loss of production of \( \alpha \)-hemolysin may correlate with the survivability of this nonhemolytic subpopulation in murine abscesses and maybe even murine wounds. To verify this, an inoculum comprising a mixture of 45% DU1090 cells and 55% 8325-4 cells was inoculated...
into the murine abscess and systemic models of infection. After 7 days in murine abscesses, the number of DU1090 cells rose to 60% of the population and the number of 8325-4 cells dropped to 40% of the population (Table 2; \( P < 0.047 \)). Within murine livers at day 2 post-inoculation, 12% of the population was DU1090 cells and 88% of the population was 8325-4 cells (\( P < 0.0004 \)). However, inside murine spleens, there was not as strong a selection for hemolytic cells because only 66% of the population was 8325-4 cells and 34% of the population was DU1090 cells (\( P < 0.191 \)). What these analyses showed was that the hla mutant population was slightly selected for in the murine abscess and selected against in the murine liver and spleen, albeit less so in the spleen. Thus, the huge increase in the nonhemolytic population within murine abscesses and wounds observed using the signature-tagged library is only partly the result of a loss of \( \alpha \)-hemolysin, and the loss of other secreted proteins is likely the major reason for this growth advantage.

Certainly, \( \alpha \)-hemolysin is an important virulence factor at some sites within a mammalian body. Several studies have shown its importance in mastitis, pneumonia, systemic infections, and keratitis [6,7,9,10,21,22]. Our own study demonstrated in a systemic model of infection that \( \alpha \)-hemolysin is an important virulence factor – a USA300 strain with an \( \alpha \)-hemolysin-deficient allele had better survival compared to an \( \alpha \)-hemolysin-positive strain over a 7-day period compared to a wild-type strain (Table 1). The staphylococci from the \( \alpha \)-hemolysin-deficient strain survived just as well over a 7-day period compared to a wild-type strain (Table 1). The staphylococci from the \( \alpha \)-hemolysin-deficient strain survived just as well over a 7-day period compared to a wild-type strain. Moreover, in a rabbit endocarditis model of infection, hemolytic variants were also selected for (data not shown).

However, skin infections, wound infections, and abscesses are the predominant types of infections caused by \( S. aureus \) [23]. These infections affect the epidermidis, dermis, and subcutaneous layers of the skin. In a wound model, there is a lot of tissue destruction as the result of the burn, so tissue damage by the \( \alpha \)-hemolysin or even \( \delta \)-hemolysin may not be necessary to allow for bacterial dissemination. Other virulence factors appear to have greater importance over hemolysins in the murine wound. In a murine abscess, the presence of \( \alpha \)-hemolysin also does not confer a competitive advantage to the \( S. aureus \) cells. An hla mutant strain survived just as well over a 7-day period compared to a wild-type strain (Table 1). The staphylococci living within the abscess would be walled off from the immune system and thus in part protected from elimination, enabling the bacteria to survive for extended periods of time.

Because in vitro passage of certain \( S. aureus \) strains can lead to pleiotropic mutants that can affect agrC or agrA, this in turn can affect a myriad array of secreted proteins, including \( \alpha \)-hemolysin [24]. In some strains, this rate of agr mutation can approach 50% in vitro [25]. Such mutations occurred in the signature-tagged mutant pools, giving rise to a small population of nonhemolytic variants. When such a mixed pool is inoculated to form an abscess or create an infection beneath a wound, the nonhemolytic population of cells no longer expends the energy to produce the secreted proteins, which might offer a competitive advantage during these specific types of infection where \( \alpha \)-hemolysin and more importantly other proteins may not be critical for sustaining the infection. Thus, in vivo selective pressures sort out whether these agr mutant bacteria will flourish (i.e. in a murine wound) or be less fit (i.e. in the liver). The loss of \( \alpha \)-hemolysin activity by the agr mutation is just one of possibly many factors that correlate with survivability within murine abscesses and wounds. It is the lack of these other secreted factors that appear to be primarily responsible for most of the growth advantage observed in murine abscesses and wounds, and the lack of \( \alpha \)-hemolysin is but one small facet of this increased survival in different microniche within the human body.

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