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Lineage associated expression of virulence traits in bovine-adapted *Staphylococcus aureus*

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ABSTRACT

Bovine mastitis is the most costly disease to the dairy industry worldwide with *Staphylococcus aureus* commonly associated with intramammary infections that are persistent and refractory to treatment. The strains of *S. aureus* that cause mastitis predominantly belong to a number of well-described bovine-adapted lineages. The objective of this study was to determine if a variety of potential virulence traits were associated with lineage. Bovine-adapted *S. aureus* isolates (n = 120), belonging to lineages CC97, CC151 and ST136, were tested for their ability to adhere to and internalise within cultured bovine mammary epithelial cells (bMEC), to bind bovine fibronectin, to form a biofilm in TSB, TSB + 1% glucose and TSB + 4% NaCl, and to induce an immune response from bMEC. There were no significant differences between the lineages in ability to adhere to or internalise within bMEC although there were significant differences between individual isolates. For lineages CC97 and ST136, mammalian cell adherence was correlated with the ability to bind bovine fibronectin, however isolates from CC151 could not bind bovine fibronectin *in vitro*, but adhered to bMEC in a fibronectin-independent manner. There were significant differences between the lineages in ability to form a biofilm in all three growth media with ST136 forming the strongest biofilm while CC151 formed the weakest biofilm. Lineages also differed in their ability to elicit an immune response from bMEC with CC97 eliciting a stronger immune response than CC151 and ST136. These data indicate the potential for both lineage and strain-specific virulence and a strain-specific response to infection *in vivo* and caution against extrapolating an effect from a single strain of *S. aureus* to draw conclusions regarding virulence or the host response to infection in unrelated lineages.

1. Introduction

Staphylococcus aureus is among the most common pathogens associated with bovine intramammary infection (IMI), a disease of global importance with negative consequences for animal health and welfare, and milk quality (Keane et al., 2013; Peton and Le Loir, 2014; Wickstrom et al., 2009). *S. aureus* is associated with both clinical and more commonly sub-clinical mastitis, both of which frequently result in persistent and recurrent infections with a low cure rate after antibiotic therapy (Schukken et al., 2011).

The mechanism of persistence of *S. aureus* is still not fully elucidated, although a number of strategies have been postulated. One mechanism used by *S. aureus* to evade the immune system is internalisation into host cells. *S. aureus* can invade a variety of non-professional phagocytes (Haggar et al., 2003) and persistent infections may be related to the ability of *S. aureus* to invade and survive within certain types of host cells. It is well established that *S. aureus* can adhere to and internalise into mammary gland epithelial cells (MEC). The bacteria may also evade phagocytosis by persisting in the form of metabolically inactive small colony variants (Atalla et al., 2008). The best understood mechanism for *S. aureus* internalisation is provided by fibronectin binding proteins (FnBPs). Fibronectin acts as a bridging molecule which binds *N*-terminally to *S. aureus* FnBPs and via an RGD motif to the host cell integrin $\alpha5\beta1$ and signals bacterial uptake (Fowler et al., 2000).

Chronic, persistent and antibiotic-refractory *S. aureus* infections have also been associated with growth of the bacteria in a biofilm (Dunne, 2002). Biofilms are a community of bacterial cells arranged in a structured manner, enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton et al., 1999). *S. aureus* can produce a number of molecules which facilitate biofilm formation including an extracellular polysaccharide adhesin, extracellular DNA and a variety of proteins such as the biofilm associated protein (Bap). Biofilm-

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associated bacteria show an innate resistance to antibiotics and clearance by host defense mechanisms (Melchior et al., 2006) and a *bap*$^{+}$ *S. aureus* strain had a significantly enhanced ability to colonize and persist in the ovine mammary gland compared to a *bap*$^{-}$ strain (Cucarella et al., 2004).

The innate immune system is the first line of defense against an invading pathogen. Pattern recognition receptors on the surface of host cells recognize non-specific pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide or lipoteichoic acid. This induces a signaling cascade which results in the production of cytokines, chemokines and the recruitment of somatic cells to the mammary gland (Brenaut et al., 2014). MEC are the most abundant cell type to initially encounter intramammary pathogens and are an important source of pro-inflammatory cytokines and chemokines that attract neutrophils early in infection, with professional immune cells such as dendritic cells and macrophages also essential for immune surveillance. The ability to mount a rapid and effective immune response is critical to resolving a mastitis infection (Thompson-Crispi et al., 2014). However, *S. aureus* has acquired a variety of immune evasion strategies to attenuate the host immune response and facilitate pathogen persistence (Foster, 2005; Vrieling et al., 2015).

Particular lineages of bovine-adapted *S. aureus* are responsible for the majority of cases of bovine infections worldwide (Smith et al., 2005). There is substantial genetic variation between lineages, with gene and allele content of a variety of virulence factors lineage-specific (Budd et al., 2015; McCarthy and Lindsay, 2010) suggesting lineages may differ in their molecular mechanisms of pathogenicity. An improved understanding of the association between lineage and virulence traits would be valuable for the development of *S. aureus* IMI control and treatment strategies. A previous study found that the lineages associated with clinical mastitis in Ireland were CC97, CC151 and ST136 (Budd et al., 2015). The objective of this study was to determine if lineage was a predictor of a number of virulence attributes, including the ability to adhere to and internalize within MEC, form a biofilm and modulate the host response to infection.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

All *S. aureus* bovine-adapted isolates used in this study were recovered from milk samples taken from cows presenting clinical mastitis between February 2010 and February 2011 from 26 farms in Ireland. Sample collection, bacterial isolation and identification methods and isolate genotyping have been described previously (Budd et al., 2015; Keane et al., 2013). A brief description of the lineages used in the study can be found in Table 1. Reference strains SH1000, SH1000Δ*fnbAB*Δ*clfAB* and Cowan (ATCC 12598) were a kind gift from Prof Tim Foster (TCD). All strains were cultured in trypticase soy broth (TSB) or trypticase soy agar (TSA) at 37 °C unless otherwise stated.

2.2. Adherence and internalisation

Quantification of *S. aureus* adherence to and internalisation within mammalian cells by flow cytometry was previously described (Trouillet et al., 2011). The bovine mammary epithelial cell line, MAC-T, was grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 38 °C with 5% (v/v) CO_2. Cells were disassociated from the flask using Trypsin-EDTA (0.25%), and centrifuged at 118*g* for 8 min. The supernatant was removed and the cells gently resuspended in 10 mL of DMEM + FBS. The centrifugation and resuspension steps were repeated twice more. All bacterial isolates were grown in 5 mL TSB for 16 h at 37 °C. The bacterial cells were washed by centrifugation at 10,000*g* for 5 min, supernatant removed and resuspended in 5 mL sterile phosphate buffered saline (PBS). The optical density (OD) of the suspension was adjusted to an OD_{600nm} of 1 using sterile PBS. Bacterial cells were stained with Calcein AM (Merck Millipore, UK) for 30 min at a 1:500 dilution. The cells were subsequently washed 3 times with sterile PBS. MAC-T cells were stained for 30 min with a 1:500 dilution of 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Aldrich UK) and washed with sterile DMEM + FBS. Stained bacterial and MAC-T cells were counted on the Attune flow cytometer (Life technologies, Germany) before co-incubation at a multiplicity of infection (MOI) of 10:1 for 3 h at 38 °C with 5% CO_2. This MOI was chosen as it has previously been reported to lie in the mid-range of values for which there is a linear relationship between internalisation and MOI (Bayles et al., 1998). Cells were then washed 3 times with PBS, following which the murine primary antibody mAb55 (Hycult biotech, USA) was incubated with the cell/bacteria suspension at 1:150 for 30 min. Cells were subsequently washed three times with sterile DMEM + FBS. To detect bound mAb55, Qdot 655 donkey anti-mouse IgG was diluted 1:100 in the cell/bacteria suspension and incubated for 30 min before washing with DMEM + FBS three times. Aliquots of the suspension were added to the wells of a 96 well round bottom plate (Starstedt, Germany) and mammalian cells counted on the Attune flow cytometer. The number of MAC-T cells with bacteria internalized was calculated by subtracting the number of cells stained with mAb 55 (adhered only) from the number stained with calcein AM (adhered and internalized). Results are the mean of triplicate experiments.

2.3. Fibronectin binding

Fibronectin-binding was determined as described previously (Zapotoczna et al., 2013). Flat bottom 96-well ELISA plates (Starstedt, Germany) were coated with 100 μL of 0.625 μg/mL bovine fibronectin (Calbiochem, Merck Chemicals, Germany) overnight at 4 °C. Wells were then washed 3 times with PBS, blocked with 5% BSA for 2 h at 37 °C and again washed 3 times with PBS. Bacterial strains were grown overnight in TSB at 37 °C, diluted 1:200 in pre-warmed TSB and grown to an OD_{600nm} of 0.3, washed and resuspended to an OD_{600nm} of 0.45 and added to triplicate

Table 1
Genotypic and phenotypic characteristics of the bovine-adapted *S. aureus* lineages.

Lineage	Clonal Complex	Number of isolates	Key genotypic characteristics	Key phenotypic characteristics
CC71	CC97	42	*cap8 agrI bap*$^{-}$ *ica*$^{-}$ *bla*$^{+}$	FnBP^{+} SpA^{+}
tCC97	CC97	22	*cap5 agrI bap*$^{-}$ *ica*$^{+}$ *bla*$^{+}$	FnBP^{+} SpA^{+}
ST136	ST136	14	*cap5 agrIII bap*$^{-}$ *ica*$^{+}$ *bla*$^{-}$	FnBP^{+} SpA^{+}
CC151	CC151	42	*cap8 agrII bap*$^{-}$ *ica*$^{+}$ *bla*$^{-}$	FnBP^{-} SpA^{-}