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Interaction between bovine-associated coagulase-negative staphylococci species and strains and bovine mammary epithelial cells reflects differences in ecology and epidemiological behavior

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ABSTRACT

Bacteria adherence seems to be an essential first stage for the internalization of bacteria into the cytoplasm of the host cell, which is considered an important virulence strategy enabling bacteria to occupy a microenvironment separated from host defense mechanisms. Thus, this study aimed to explore the difference in the capacity of 4 bovine-associated staphylococci species or strains to adhere to and internalize into bovine mammary epithelial cells (MEC). Three different isolates of coagulase-negative staphylococci (CNS) were used: one strain of *Staphylococcus fleurettii* isolated from sawdust and considered an environmental opportunistic bacterium, and 2 dissimilar *Staphylococcus chromogenes* isolates, one cultured from a heifer's teat apex (*Staph. chromogenes* TA) and the other originating from a chronic intramammary infection (*Staph. chromogenes* IM). Also, one well-characterized strain of *Staphylococcus aureus* (Newbould 305) was used for comparison with a major mastitis pathogen. The CNS species and strains adhered to and internalized into MEC slower than did *Staph. aureus*. Still, we observed high variation in adhesion and internalization capacity among the different CNS, with *Staph. chromogenes* IM showing a greater ability to adhere to and internalize into MEC than the 2 CNS strains isolated from extramammary habitats. In conclusion, the 3 well-characterized bovine-associated CNS species and strains originating from distinct habitats showed clear differences in their capacity to adhere to and internalize into MEC. The observed differences might be related to their diversity in ecology and epidemiological behavior.

Key words: coagulase-negative staphylococci, *Staphylococcus aureus*, mastitis, dairy cow

INTRODUCTION

Mastitis is one of the most common and detrimental diseases that dairy cows can experience. Moreover, mastitis threatens the income of farmers as well as the image of the entire dairy sector because of animal welfare issues and issues related to milk quality and public health due to an increased risk of antimicrobial residues and the emergence of resistant bacteria (Andrew et al., 2009; Huijps et al., 2009). Staphylococcal bacteria remain an important cause of bovine mastitis. The genus is divided into the coagulase-positive staphylococci, with *Staphylococcus aureus* remaining the most significant mastitis pathogen among the staphylococci, and the heterogeneous group of the CNS, which have become the most commonly isolated bacteria from milk samples of dairy cows and heifers (Fox, 2009; Piepers et al., 2009; De Vliegher et al., 2012), as well as small ruminants (Souza et al., 2012), in many regions and countries around the world. Despite their high prevalence as a cause of IMI, we have only started to learn about differences between species in epidemiological behavior, virulence, and interactions with the host.

Some authors have associated CNS with chronic IMI (Taponen et al., 2007; Thorberg et al., 2009; Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014) and an increase in milk SCC (Supré et al., 2011; Fry et al., 2014; Tomazi et al., 2015), although their clinical relevance is still under debate (Schukken et al., 2009; Piepers et al., 2010, 2013). Nevertheless, conflicting

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results as to the importance of CNS as mastitis-causing agents is likely due to the failure to acknowledge variations within and between species (Fry et al., 2014). The development and validation of molecular identification techniques allow accurate speciation and strain-typing of bovine-associated CNS (Piessens et al., 2011, 2012a; Supré et al., 2011; De Visscher et al., 2014), identifying variations among species in effect on SCC, and different traits such as persistence of infection, antimicrobial resistance, virulence, and epidemiological behavior (Taponen et al., 2007; Piessens et al., 2011, 2012a,b; Supré et al., 2011; Avall-Jääskeläinen et al., 2013; De Visscher et al., 2014; Fry et al., 2014; Vanderhaeghen et al., 2014, 2015; Breyne et al., 2015; Tomazi et al., 2015). Bacterial adherence seems to be an essential first stage for the internalization of bacteria into the cytoplasm of the host cell, whereas internalization into host cells is considered an important virulence strategy. It allows bacteria to occupy a micro-environment protected from the host defense mechanisms operable at the mucosal surface (Almeida and Oliver, 2001; Peton et al., 2014). Apart from 3 studies (Burriel, 1999; Almeida and Oliver, 2001; Hyvönen et al., 2009), no information is available on the interaction between different staphylococci species and mammary epithelial cells (**MEC**). Also, none of the latter studies investigated the potential link between the adherence and internalization capacity, on the one hand, and the ecology and epidemiological behavior of CNS, on the other hand.

Recent work suggests that some bovine-associated CNS species, such as *Staphylococcus fleurettii*, are typically present in dairy cows' environment and rarely cause IMI; in contrast, others, such as *Staphylococcus chromogenes*, colonize the teat apices, are commonly isolated from milk causing IMI, yet are seldom found in dairy cows' environment (Piessens et al., 2011; De Visscher et al., 2014; Vanderhaeghen et al., 2014, 2015). In that respect, we recently demonstrated that epidemiologically different CNS species and strains induce a differential host innate immune response in the murine mammary gland (Breyne et al., 2015).

We hypothesized that differences in epidemiological behavior and ecology of different CNS species and strains are reflected in their interaction (adherence and internalization) with MEC.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Three different isolates of CNS belonging to 2 species were used, the same isolates as used in a recently published study (Breyne et al., 2015): 1 strain of *Staph. fleurettii* isolated from sawdust and considered to represent *Staph. fleurettii* as an environmental or opportunistic species, and 2 dissimilar *Staph. chromogenes* isolates. The first *Staph. chromogenes* isolate was cultured from a heifer's teat apex (**TA**) and has in vitro protective effects against major pathogens such as *Staph. aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* (De Vliegher et al., 2004; Breyne et al., 2015). The other strain of *Staph. chromogenes* originated from a chronic IMI (**IM**; Supré et al., 2011) and is considered to behave as an udder-adapted bacterium (Piessens et al., 2011, 2012a; Breyne et al., 2015). Also, one well-characterized and host-adapted strain of *Staph. aureus* (Newbould 305) associated with mild and chronic bovine mastitis (Peton et al., 2014) was included as a positive control, being a major mastitis pathogen.

The isolates were stored at −80°C and thawed at 37°C. First, the strains were grown on Columbia sheep blood agar plates (Oxoid, Wesel, Germany). Then, fresh colonies of each bacteria were grown overnight in brain heart infusion (**BHI**) broth at 37°C. Subsequently, all strains were diluted at 1:1,000 in fresh BHI broth and incubated until they reached their respective late-exponential growth phase. After bacterial growth, the bacterial broth was centrifuged at 2,500 × *g* for 15 min and washed twice with 1× Dulbecco's phosphate buffered saline (**DPBS**; cat. no. 14190185, Gibco, Paisley, UK). Then, the bacteria were resuspended in Dulbecco's modified Eagle's medium (**DMEM**, cat. no. 42430-025, Gibco) supplemented with 10% fetal bovine serum, 5 μg/mL insulin (cat. no. I3536, Sigma Aldrich, St. Louis, MO), and 1 μg/mL hydrocortisone (cat. no. H0888, Sigma Aldrich). The inoculum was adjusted to 3×10^5 cfu/mL (Bonnefont et al., 2012) and stored at −80°C until further processing.

The bacterial count for the assay was determined using spectrophotometry at absorbance 600 nm. The inoculum suspension was also cultured on trypticase soy agar in dilution series, and colonies were counted to confirm the final inoculum dose.

Bovine Mammary Epithelial Cells

A clonal bovine mammary epithelial cell line originating from primary bovine alveolar cells (**MAC-T**; Huynh et al., 1991) were cultured using MAC-T medium containing DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL Fungizone (cat. no. 15240-096, Gibco), 5 μg/mL insulin, and 1 μg/mL hydrocortisone in 6-well plates, and incubated in a humidified incubator with 5% CO_2 at 37°C. To obtain a confluent monolayer, the cell line was treated with 0.25% trypsin (cat. no. 154000-054, Gibco), resuspended in fresh MAC-T