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Coagulase-negative staphylococci species affect biofilm formation of other coagulase-negative and coagulase-positive staphylococci

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ABSTRACT

Coagulase-negative staphylococci (CNS) are considered to be commensal bacteria in humans and animals, but are now also recognized as etiological agents in several infections, including bovine mastitis. Biofilm formation appears to be an important factor in CNS pathogenicity. Furthermore, some researchers have proposed that CNS colonization of the intramammary environment has a protective effect against other pathogens. The mechanisms behind the protective effect of CNS have yet to be characterized. The aim of this study was to evaluate the effect of CNS isolates with a weak-biofilm phenotype on the biofilm formation of other staphylococcal isolates. We selected 10 CNS with a weak-biofilm phenotype and 30 staphylococcal isolates with a strong-biofilm phenotype for this study. We measured biofilm production by individual isolates using a standard polystyrene microtiter plate assay and compared the findings with biofilm produced in mixed cultures. We confirmed the results using confocal microscopy and a microfluidic system with low shear force. Four of the CNS isolates with a weak-biofilm phenotype (*Staphylococcus chromogenes* C and E and *Staphylococcus simulans* F and H) significantly reduced biofilm formation in approximately 80% of the staphylococcal species tested, including coagulase-positive *Staphylococcus aureus*. The 4 *Staph. chromogenes* and *Staph. simulans* isolates were also able to disperse pre-established biofilms, but to a lesser extent. We also performed a deferred antagonism assay and recorded the number of colony-forming units in the mixed-biofilm assays on differential or selective agar plates. Overall, CNS with a weak-biofilm phenotype did not inhibit the growth of isolates with a strong-biofilm phenotype. These results suggest that some CNS isolates can negatively affect the ability of other staphylococcal isolates and species to form biofilms via a mechanism that does not involve growth inhibition.
Key words: mastitis, coagulase-negative staphylococci, biofilm, inhibition, dispersion

INTRODUCTION

Intramammary infections trigger an inflammatory response in the udder, which may lead to mastitis in cows. Mastitis is the most common and detrimental disease in the dairy industry, and it has a major economic impact on the production and quality of milk. Coagulase-negative staphylococci are the bacteria most frequently isolated from the intramammary environment in Canada (Fry et al., 2014) and other countries (Tenhagen et al., 2006; Pyörälä and Taponen, 2009; Sampimon et al., 2009). The CNS have traditionally been considered minor pathogens causing IMI, but are increasingly being recognized as emerging mastitis pathogens (Pyörälä and Taponen, 2009).

Conflicting results about the effect of CNS on the risk of a quarter acquiring a new IMI with a major pathogen have been reported in the literature. For example, CNS IMI or teat apex colonization have long been considered to have a protective effect (Rainard and Poutrel, 1988; Matthews et al., 1991). However, a meta-analysis revealed that observational studies did not report a protective effect of pre-existing IMI with CNS (Reyher et al., 2012a). Furthermore, the presence of CNS increased the probability of a new *Staphylococcus aureus* IMI (Reyher et al., 2012b) and new IMI in the ipsilateral quarter (Reyher et al., 2013). Overall, it has been proposed that any protective or negative effect of CNS on new IMI is probably species-dependent (Reyher et al., 2012a; Vanderhaeghen et al., 2014). The mechanisms behind the positive or negative effects of CNS have yet to be characterized.

Some CNS isolated from dairy cows can produce bacteriocins with antibacterial activity against other

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mastitis pathogens, including other CNS species, *Staph. aureus*, *Streptococcus uberis*, and *Streptococcus agalactiae* (dos Santos Nascimento et al., 2005; Ceotto et al., 2010; Brito et al., 2011; Braem et al., 2014). These bacteriocins likely play an important role in interspecies competition in ecological niches such as the udder (De Vuyst and Leroy, 2007).

In addition to bacteriocins, biofilms formed by commensal bacteria at the surface of epithelial cells may prevent colonization by certain pathogens (Rickard et al., 2003; Kuboniwa et al., 2006). Biofilms are a universal trait of microorganisms; they are structured communities of bacterial cells enclosed in a self-produced matrix attached to surfaces (Costerton et al., 1999; Jacques et al., 2010). The thickness and the composition of the biofilm have an important role in its functionality. Specifically, the polymer matrix acts as a barrier and reduces or blocks the diffusion of antibiotics; a thicker biofilm may make it more difficult for antibiotics to penetrate the barrier and reach bacterial cells. Furthermore, electrostatic charges at the surface of the polymeric matrix will bind charged microbial agents and prevent them from reaching their effective concentration. As well, the protective barrier of the biofilms might not be limited to antimicrobials. For example, the ability to form biofilms might protect bacteria against host inflammatory cells in the mammary gland. Nutrient and oxygen gradients also develop during growth in biofilm, resulting in the presence of slow-growing or metabolically inactive bacterial cells that are less affected by antibiotics (Hathroubi et al., 2017). Although in vitro studies have focused mainly on single-species biofilms, multispecies biofilms are predominant in the context of host colonization and environmental conditions. Furthermore, chronic and biofilm-related infections may be facilitated by the persistence of polymicrobial biofilms (Burmølle et al., 2014; Miquel et al., 2016). Consequently, researchers are now focusing their efforts on understanding the complexity and interactions of multispecies biofilms (Burmølle et al., 2014). Isolates of CNS recovered from dairy cows have been reported to form biofilms (Piessens et al., 2012; Simojoki et al., 2012; Tremblay et al., 2013), but this finding was not associated with an increase in SCC (Simojoki et al., 2012; Tremblay et al., 2013). Still, biofilms might also facilitate the environmental transmission of CNS and support their persistence (Tremblay et al., 2013). Specifically, the production of strong biofilms by CNS isolates was associated with later stages of the lactation cycle (Tremblay et al., 2013). Furthermore, biofilm formation decreases CNS susceptibility toward commonly used antibiotics on dairy farms (Tremblay et al., 2014). In the context of biofilm formation, interactions between CNS and other mastitis pathogens has yet to be investigated. The objective of this study was to investigate the effect of CNS with a weak-biofilm phenotype on the biofilm formation of staphylococci associated with bovine mastitis.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Coagulase-negative staphylococci and *Staph. aureus* isolates were obtained from the Mastitis Pathogen Culture Collection, managed by the Canadian Bovine Mastitis and Milk Quality Research Network (St-Hyacinthe, QC, Canada; Reyher et al., 2011). Isolates of CNS were first selected from the 5 main species found on Canadian farms (i.e., *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus haemolyticus,* and *Staphylococcus epidermidis*; Fry et al., 2014; Tables 1 and 2). The 5 *Staph. aureus* isolates that were previously described as strong-biofilm producers were also selected from the Mastitis Pathogen Culture Collection to represent the predominant spa types found in Canada, plus 1 methicillin-resistant *Staph. aureus* (Veh et al., 2015; Table 2). The isolates were assigned to a species by amplifying and sequencing the staphylococcal *rpoB* gene (Fry et al., 2014). This identification was then confirmed using matrix-assisted laser desorption ionization-time of flight (**MALDI-ToF**) mass spectrometry analysis (Cameron et al., 2017). We obtained *Staph. chromogenes* isolate 2, a characterized bacteriocin producer, from Sarne De Vliegher (Ghent University, Belgium). Isolates of CNS and *Staph. aureus* were cultured on brain heart infusion (**BHI**) agar and incubated for 16 h at 37°C.

Table 1. Bacterial isolates with a negative-, weak-, or moderate-biofilm phenotype used in this study

Bacterial species and isolate	Biofilm phenotype[1]	Biofilm (A_{490})
Staphylococcus chromogenes		
A	Weak	0.300[2]
B	Negative	0.083[2]
C	Negative	0.074[2]
D	Weak	0.152[2]
E	Negative	0.089[2]
Staphylococcus simulans		
F	Weak	0.139[2]
G	Moderate	0.652[2]
H	Weak	0.156[2]
I	Weak	0.172[2]
J	Weak	0.183[2]

[1]The ability of a CNS isolate to form a biofilm was classified as negative (absorbance at 490 nm, A_{490} <0.110), weak (A_{490} 0.110–0.500), or moderate (A_{490} 0.500–1.500; Tremblay et al., 2013).

[2]From Tremblay et al. (2013).