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

Coralie Goetz,*† Yannick D. N. Tremblay,*† Daphnée Lamarche,* Andréanne Blondeau,* Annie M. Gaudreau,* Josée Labrie,* François Malouin,†‡ and Mario Jacques*†¹

*Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, Québec, Canada J2S 2M2

†Canadian Bovine Mastitis and Milk Quality Research Network, St-Hyacinthe, Québec, Canada J2S 2M2

‡Department of Biology, Faculty of Science, Université de Sherbrooke, Sherbrooke, Québec, Canada J1K 2R1

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 mastitis pathogens, including other CNS species, *Staph. aureus*, *Streptococcus uberis*, and *Streptococcus agalactiae* (dos Santos Nascimento et al., 2005; Ceotto et al.,

Received January 23, 2017.

Accepted April 26, 2017.

¹Corresponding author: mario.jacques@umontreal.ca

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 xylosum*, *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 Vliegheer (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.

Single-Species Biofilm Assay

Biofilms from CNS and *Staph. aureus* were cultured in BHI supplemented with glucose (BHIG; 0.25% wt/

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)
<i>Staphylococcus chromogenes</i>		
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
<i>Staphylococcus simulans</i>		
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

¹The ability of a CNS isolate to form a biofilm was classified as negative (absorbance at 490 nm, A₄₉₀ <0.110), weak (A₄₉₀ 0.110–0.500), or moderate (A₄₉₀ 0.500–1.500; Tremblay et al., 2013).

²From Tremblay et al. (2013).

vol) in 96-well microtiter plates (Corning Costar #3595; Corning Inc., Corning, NY) as described previously (Tremblay et al., 2013). Briefly, colonies from BHI agar were suspended in BHIG to a 0.5 McFarland standard, and 200 μ L was aliquoted in 3 wells. The plate was then incubated for 24 h at 37°C. After incubation, cells were removed by washing the wells 3 times with PBS. The biofilms were air-dried and stained with 0.1% (wt/vol) safranin for 10 min. The stain was then released with 200 μ L of the destaining solution [50% (vol/vol) ethanol, 50% (vol/vol) glacial acetic acid] and quantified by measuring the absorbance at 490 nm (A_{490}). Each bacterial isolate was tested in 3 wells on 3 independent days.

Table 2. Bacterial isolates (symbols correlate to those in Figure 1) with a strong-biofilm phenotype¹ used in this study

Bacterial species and isolates	Biofilm (A_{490})
<i>Staphylococcus chromogenes</i>	
101 (●)	1.686 ²
102 (■)	3.500 ²
103 (▲)	3.466 ²
104 (◆)	3.334 ²
105 (▼)	2.823 ²
<i>Staphylococcus simulans</i>	
106 (●)	3.124 ²
107 (■)	3.559 ²
108 (▲)	2.759 ³
109 (◆)	1.906 ²
110 (▼)	3.464 ²
<i>Staphylococcus xylosus</i>	
111 (●)	2.349 ²
112 (■)	2.195 ²
113 (▲)	3.500 ²
114 (◆)	3.060 ²
115 (▼)	3.537 ²
<i>Staphylococcus epidermidis</i>	
116 (●)	2.096 ³
117 (■)	1.420 ³
118 (▲)	2.400 ³
119 (◆)	1.916 ³
120 (▼)	1.960 ³
<i>Staphylococcus haemolyticus</i>	
121 (●)	3.278 ²
122 (■)	2.620 ²
123 (▲)	2.683 ²
124 (◆)	3.192 ²
125 (▼)	1.180 ²
<i>Staphylococcus aureus</i>	
126 (t13401) (●)	2.000 ³
127 (t13401) (■)	1.000 ³
128 (t13401) (▲)	1.400 ³
129 (t605) (◆)	2.810 ³
130 (MRSA) ⁴ (▼)	2.060 ³

¹The 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), moderate (A_{490} 0.500–1.500), or strong (A_{490} >1.500); Tremblay et al. (2013).

²From Tremblay et al. (2013).

³This study.

⁴Methicillin-resistant *Staphylococcus aureus*.

Dual-Species Biofilm Assay

For the CNS–CNS and CNS–*Staph. aureus* dual-species biofilms, the weak-biofilm CNS suspensions and the strong-biofilm CNS or *Staph. aureus* suspensions were prepared as above, and 100 μ L from each suspension was added to a microtiter plate for a total volume of 200 μ L in 3 wells. The plate was incubated for 24 h at 37°C and treated as described above. In each experiment, single-species biofilms and wells with sterile BHIG acted as controls. Each assay was repeated on 3 independent days, with 3 technical replicates on each day.

Dispersion of Biofilms by CNS

Single-species biofilms were grown as described above. After incubation for 24 h, the liquid above the biofilm was removed and 200 μ L of a weak-biofilm CNS suspension in BHIG was added to 3 wells with pre-established biofilms. For control purposes, bacterial suspensions were also added to wells without biofilms, and untreated biofilms were incubated with 200 μ L of BHIG. After another 24 h of incubation, the biofilms were stained as described above. Each assay was repeated on 3 independent days, with 3 technical replicates on each day.

Deferred Antagonism Assay

The deferred antagonism assay was adapted from De Vlieghe et al. (2004). Briefly, CNS weak-producer colonies from BHI agar were suspended in BHIG to a 0.5 McFarland standard and streaked with a sterile swab at the center of a BHI agar Petri dish. After 24 h of incubation at 37°C, the agar was turned upside down and swabbed with a 0.5 McFarland standard of CNS strong-producer colonies to achieve full coverage. After another 24 h of incubation, the presence of a growth inhibition zone was investigated. We used *Staph. chromogenes* isolate 2, a characterized bacteriocin producer obtained from S. De Vlieghe, as a control strain.

Colony-Forming Units on Differential and Selective Media

Dual-species biofilms were prepared and, after a 24-h incubation, the biofilm and planktonic cells from 3 different wells were recovered by scraping the surface with sterile pipette tips, vigorously shaken and serially diluted. The dilutions were plated on BBL CHROMagar *Staph. aureus* and on BBL Mannitol Salt Agar (BD, Mississauga, ON, Canada) to differentiate CNS spe-

cies from other CNS and from *Staph. aureus*. Plates were incubated at 37°C for a minimum of 16 h and a maximum of 48 h. Each assay was repeated on 2 independent days.

Confocal Laser Scanning Microscopy

Single- and dual-species biofilms were prepared as described above and stained with SYTO 9 green fluorescent nucleic acid stain (Invitrogen, Waltham, MA) as recommended by the manufacturer (<https://tools.thermofisher.com/content/sfs/manuals/mp10316.pdf>). The stained biofilms were visualized by confocal laser scanning microscopy (FV1000 IX81; Olympus, Markham, ON, Canada) and acquired images using Fluoview software (Olympus).

Biofilm Formation in a Microfluidic System

Dynamic biofilm growth was evaluated using the BioFlux 200 device (Fluxion Biosciences, South San Francisco, CA). The method used to observe growing biofilms was adapted from Moormeier et al. (2013). Briefly, colonies of CNS were suspended in 4 mL of fresh, warmed (37°C) BHIG to an optical density at 600 nm of approximately 1. The microfluidic channels were dampened with prewarmed BHIG and inoculated by injecting the bacterial suspension into the output reservoir for 20 s at 0.5 dyn/cm². The microfluidic plate was incubated for 1 h at 37°C to allow bacterial adhesion to the surface. Fresh, prewarmed, diluted BHIG (5%) was added into the input reservoir, and the flow of fresh medium was initiated at 0.5 dyn/cm² for 23 h. Then, biofilms were washed by injecting PBS from the input reservoir for 20 min at 0.5 dyn/cm². Each assay was repeated on 2 independent days. Images of biofilms were obtained using a microscope (CKX41; Olympus) equipped with a 40× objective, a digital camera (Retiga EX; QImaging, Surrey, BC, Canada) and the software provided with the BioFlux 200 device.

Statistical Analysis

For the microtiter plate assay, we used a 1-way ANOVA followed by a Dunnett post-test (GraphPad Prism version 5.03 software; GraphPad Software Inc., La Jolla, CA) to compare biofilm production by a bacterial isolate with a strong-biofilm phenotype in the absence of treatment to biofilm production in the presence of CNS isolates with a weak-biofilm phenotype. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Inhibition of Biofilm Formation by *Staph. chromogenes* and *Staph. simulans* Under Static Conditions

Preliminary results revealed that *Staph. simulans* isolate I, which had a weak-biofilm phenotype, reduced the biofilm formation of *Staph. chromogenes*, *Staph. xylosus*, *Staph. epidermidis*, and *Staph. haemolyticus* with a strong-biofilm phenotype (data not shown). We made similar observations for *Staph. chromogenes* isolate A, which had a weak-biofilm phenotype, but not with *Staph. xylosus*, *Staph. haemolyticus*, or *Staph. epidermidis* isolates (data not shown).

Because we found biofilm-inhibition activity mainly in *Staph. chromogenes* and *Staph. simulans*, we conducted further evaluations of 5 isolates with a weak-biofilm phenotype for each of these 2 species. After selecting these isolates, we performed dual-species biofilm assays and observed different profiles of inhibition (Figure 1, Supplemental Table S1; <https://doi.org/10.3168/jds.2017-12629>). All 5 *Staph. chromogenes* isolates with a weak-biofilm phenotype significantly ($P < 0.05$ to $P < 0.001$) inhibited biofilm formation by the 5 isolates of *Staph. chromogenes*, *Staph. xylosus*, and *Staph. haemolyticus* with a strong-biofilm phenotype; the effect was smaller for the 5 *Staph. simulans* isolates with a weak-biofilm phenotype (Figures 1A, 1C, and 1D). We also observed a reduction in biofilm formation for some *Staph. epidermidis* and *Staph. aureus* isolates with a strong-biofilm phenotype when grown in the presence of a weak-biofilm producer (Figure 1E and 1F). Overall, *Staph. chromogenes* isolates with a weak-biofilm phenotype had stronger biofilm-inhibition activity against each species tested, except for some *Staph. simulans* isolates. These isolates appeared to be less sensitive to biofilm inhibitory activity (Figure 1B). Furthermore, *Staph. simulans* isolate 107, with a strong-biofilm phenotype, was not inhibited by any of the CNS isolates with a weak-biofilm phenotype (Figure 1B).

We confirmed biofilm inhibitory activity by using a confocal microscope to visualize the biofilm of an isolate with a strong-biofilm phenotype (*Staph. chromogenes* 104) grown in the presence of 1 of the 4 CNS with the greatest ability to inhibit biofilm formation (*Staph. chromogenes* C). As expected, the single-species biofilm of the *Staph. chromogenes* 104 ($A_{490} = 2.515$) appeared as a complex structure and had a thickness of approximately 120 μm (Figure 2A). The single-species biofilm of the *Staph. chromogenes* C ($A_{490} = 0.394$) was very thin, with a thickness of 10 μm (Figure 2B). When both isolates were mixed and grown together, the mixed-species biofilm ($A_{490} = 0.13$) was very thin,

Figure 1. Biofilm inhibition by 10 isolates of *Staphylococcus chromogenes* and *Staphylococcus simulans* with a weak-biofilm phenotype (x-axis) against 5 isolates with a strong-biofilm phenotype representing 6 major species: (A) *Staph. chromogenes*, (B) *Staph. simulans*, (C) *Staphylococcus xylosus*, (D) *Staphylococcus haemolyticus*, (E) *Staphylococcus epidermidis*, and (F) *Staphylococcus aureus*. Biofilm formation is expressed as the percentage of the remaining biofilm compared with a single-species biofilm. Each point represents the mean of the 3 tests from 3 independent days for each isolate. The symbols (see Table 2) represent the 5 isolates with a strong-biofilm ability phenotype from each species. The bars represent the median values for each group.

with a thickness of approximately 12 μm (Figure 2C). This finding confirmed that the strong-biofilm producer *Staph. chromogenes* 104 was unable to form its thick biofilm layer in the presence of the weak-biofilm producer *Staph. chromogenes* C (Figure 2C).

Bacterial Growth in Dual-Species Biofilms

Next, we performed qualitative (deferred antagonism assay) and quantitative (cfu) assays to exclude the possibility that a bactericidal effect was responsible for

the observed biofilm inhibition. The CNS isolates that had a significant effect on biofilm formation (*Staph. chromogenes* C and *Staph. simulans* F) did not show a clear zone of inhibition against CNS with a strong-biofilm phenotype compared with the control isolate (*Staph. chromogenes* isolate 2 with the ability to produce a bacteriocin). Then we added *Staph. chromogenes* C and *Staph. simulans* F in a dual-species biofilm assay with *Staph. xylosus* 113, which had a strong-biofilm phenotype. In the dual-species assay, the number of *Staph. xylosus* decreased by 1.0 $\log_{10}$ in the presence of

Figure 2. Biofilm formation by (A) an isolate with a strong-biofilm phenotype (*Staphylococcus chromogenes* 104); (B) an isolate with a weak-biofilm phenotype (*Staph. chromogenes* C); or (C) dual-species biofilms visualized by confocal laser scanning microscopy. The absorbance (at 490 nm, A_{490}) measurements of the microtiter-plate biofilms are displayed for reference purposes. One-way ANOVA analysis was used to obtain statistical data; *** $P < 0.001$. OD = optical density. Error bars represent the standard deviation. Color version available online.

Figure 3. Microscope images of biofilms formed by (A) an isolate with a strong-biofilm phenotype (*Staphylococcus chromogenes* 104); (B) an isolate with a weak-biofilm phenotype (*Staph. chromogenes* C); or (C) dual-species biofilms. Images were obtained after 24 h of growth in a flow chamber of the BioFlux 200 microfluidic system (Fluxion Biosciences, South San Francisco, CA).

Staph. chromogenes C and by $2.02 \log_{10}$ in the presence of *Staph. simulans* F, compared with a monospecies biofilm. We observed similar results with *Staph. haemolyticus*, *Staph. epidermidis*, and *Staph. aureus* isolates that had a strong-biofilm phenotype (data not shown). As a control, we grew the *Staph. chromogenes* isolate 2 (DeVliegher et al., 2004) with strong-biofilm producers, and this resulted in complete growth inhibition of the strong-biofilm producers; the reduction in count (cfu) was equal to $6.32 \log_{10}$. Because the CNS with a weak-biofilm phenotype did not markedly inhibit the growth of *Staphylococcus* isolates with a strong-biofilm phenotype and because the reduction in count (cfu) was not important compared with the control, the observed biofilm inhibition may not have been due to antibacterial activity.

Inhibition of Biofilm Formation by *Staph. chromogenes* and *Staph. simulans* Under Dynamic Conditions

Microtiter plates used to evaluate biofilm formation under static conditions are closed systems that often lead to the accumulation of bacterial metabolic products. Furthermore, shear forces were lacking in this model, and shear forces are present at several infection sites in the host or produced by instruments used for milking. The BioFlux device is a microfluidic system with low shear force that may be used to resemble in vivo conditions (Benoit et al., 2010). Therefore, we tested the same isolate combination (*Staph. chromogenes* 104 and *Staph. chromogenes* C) in a dynamic system. The *Staph. chromogenes* isolate 104, which had a strong-biofilm phenotype under static conditions, also formed a homogeneous and dense biofilm in a flow system (Figure 3A). The *Staph. chromogenes* C isolate, which had a weak-biofilm phenotype under static conditions, formed a non-continuous biofilm characterized by

scattered microcolonies in a flow system (Figure 3B). When both isolates were mixed and grown together, the resulting biofilm resembled the one observed for the single-species culture of *Staph. chromogenes* C (Figure 3C). The strong-biofilm producer *Staph. chromogenes* 104 was unable to form its typical robust biofilm in the presence of the weak-biofilm producer *Staph. chromogenes* C. We observed similar results with other isolate combinations (Figure 4).

Dispersion of Established Biofilms by *Staph. chromogenes* and *Staph. simulans*

Our previous results revealed that some CNS isolates with a weak-biofilm phenotype significantly ($P < 0.05$ to $P < 0.001$) reduced the biofilm formation of several staphylococcal species with a strong-biofilm phenotype (Figures 1, 2, 3, and 4). The next step was to investigate whether weak-biofilm producers could disperse pre-established biofilms. We added suspensions of the weak-biofilm producers to pre-established biofilms of strong-biofilm producers, and both species interacted for 24 h. The effect of the weak-biofilm producer on the dispersion of biofilm formed by staphylococcal isolates with a strong-biofilm phenotype varied greatly and was species- and isolate-dependent (Figure 5, Supplemental Table S2; <https://doi.org/10.3168/jds.2017-12629>). Every *Staph. chromogenes* with a weak-biofilm phenotype and some *Staph. simulans* with a weak-biofilm phenotype could significantly ($P < 0.05$ to $P < 0.001$) disperse the biofilm of certain *Staph. simulans*, *Staph. xylosus*, *Staph. epidermidis*, *Staph. haemolyticus*, and *Staph. aureus* isolates. The 5 *Staph. simulans* isolates with a weak-biofilm phenotype were unable to disperse the biofilms formed by *Staph. chromogenes* isolates with a strong-biofilm phenotype (Figure 5A). Some CNS isolates with a weak-biofilm phenotype could also disperse the biofilms formed by other staphylococcal isolates.

DISCUSSION

The effect of pre-existing CNS colonization on new infections is a major topic of discussion (Reyher et al., 2012a; Vanderhaeghen et al., 2014), and conflicting results have been reported on this effect. The exact nature of the observed effect with CNS is still unknown. Interspecies interaction in bacteria is complex, and multiple factors influence the outcomes, including biofilm formation. Biofilm formation may be an important factor for the persistence of CNS in the intramammary environment (Tremblay et al., 2013). The objective of our study was to investigate the interactions between CNS and *Staph. aureus* in the context of biofilm formation, specifically, the effect of CNS with a weak-biofilm phenotype on the biofilm formation of other staphylococci.

We observed that *Staph. chromogenes* and *Staph. simulans* isolates with a weak-biofilm phenotype had the ability to inhibit biofilm formation by other *Staphylococcus* isolates. We observed this inhibition for isolates from each of the 5 major CNS species and for *Staph. aureus*. We also observed that the inhibition occurred under static and dynamic biofilm conditions. For example, the strong-biofilm producer *Staph. chromogenes* 104 was unable to form its thick and robust biofilm layer in the presence of the weak-biofilm producer *Staph. chromogenes* C (Figures 2C and 3C). The results observed under dynamic conditions (Figures 3 and 4) confirmed the biofilm-inhibition properties of some CNS isolates and suggested that the inhibitory activity was not solely dependent on bacterial metabolic products accumulat-

ing in a closed system. To the best of our knowledge, this was the first study to show *Staph. chromogenes*, *Staph. haemolyticus*, and *Staph. xylosus* biofilm formation in a BioFlux system. Importantly, we identified 4 CNS isolates (*Staph. chromogenes* C and E and *Staph. simulans* F and H) that could significantly ($P < 0.05$ to $P < 0.001$) inhibit biofilm formation by at least 50% in 76 to 83% of the staphylococcal isolates tested (Figure 1). Overall, the *Staph. chromogenes* isolates appeared to have stronger biofilm-inhibition activity than the *Staph. simulans* isolates. Furthermore, the *Staph. haemolyticus* and *Staph. xylosus* isolates seemed to be more sensitive to this biofilm-inhibition effect. The variation we found in inhibitory effects among species was in agreement with the latest literature, which indicates that the beneficial or harmful effect of CNS IMI on secondary infections appears to be species-dependent (Vanderhaeghen et al., 2014). Furthermore, we showed that the biofilm inhibitory effect appeared to depend on the isolate or combination, suggesting that more than 1 mechanism might be involved.

The degree of biofilm dispersion in assays for the effect of pre-established biofilms was not as important as that observed for biofilm inhibition. Indeed, the 4 CNS isolates that had a significant effect on biofilm formation (*Staph. chromogenes* C and E, and *Staph. simulans* F and H) were able to significantly ($P < 0.05$ to $P < 0.001$) disperse at least 50% of the biofilm preformed by 23 to 43% of the staphylococcal isolates tested (Figure 5). This finding could be explained by the fact that different factors and mechanisms are involved in staphylococci biofilm formation and dispersion (Otto,

Figure 4. Microscope images of biofilms formed by isolates with a strong-biofilm phenotype: (A) *Staphylococcus epidermidis* 120, (D) *Staphylococcus xylosus* 113, and (G) *Staphylococcus haemolyticus* 122; or a weak-biofilm phenotype: (B) *Staphylococcus chromogenes* E, (E) *Staphylococcus simulans* F, and (H) *Staph. simulans* H grown as single species (A, B, D, E, G, and H) or dual-species biofilms (C, F, and I). Images were obtained after 24 h of growth in a flow chamber of the BioFlux 200 microfluidic system (Fluxion Biosciences, South San Francisco, CA).

Figure 5. Biofilm dispersion by 10 isolates of *Staphylococcus chromogenes* and *Staphylococcus simulans* with a weak-biofilm phenotype (x-axis) against 5 isolates with a strong-biofilm phenotype representing the 6 major species: (A) *Staph. chromogenes*, (B) *Staph. simulans*, (C) *Staphylococcus xyloso*, (D) *Staphylococcus haemolyticus*, (E) *Staphylococcus epidermidis*, and (F) *Staphylococcus aureus*. Biofilm formation is expressed as the percentage of the remaining biofilm compared with a single-species biofilm. Each point represents the mean of the 3 tests from 3 independent days for each isolate. The symbols (see Table 2) represent the 5 isolates with a strong-biofilm ability phenotype from each species. The bars represent the median values of each group.

2013). Indeed, the inhibition of biofilm formation can be achieved by preventing attachment of the bacteria to the surface. For example, DNase I will cleave extracellular DNA (eDNA) in the biofilm matrix of *Staph. aureus* and *Pseudomonas aeruginosa* and prevent biofilm formation on abiotic surfaces (Mann and al, 2009). Additionally, quorum-sensing inhibition by hamamelitannin will prevent intracellular signals by repressing RNA III, a quorum-sensing regulator in *Staph. aureus* and *Staph. epidermidis* (Kiran and al., 2008). Biofilm dispersion can be achieved by using matrix-degrading enzymes such as dispersin B, which depolymerize poly-N-acetyl-glucosamine, a polysaccharide found in some staphylococcal biofilms (Kaplan and al., 2004).

Some CNS isolated from dairy cows are known to produce bacteriocins that have antibacterial activity against other mastitis pathogens, including other CNS species and *Staph. aureus* (dos Santos Nascimento et al., 2005; Ceotto et al., 2010; Brito et al., 2011; Braem et al., 2014). We observed no growth inhibition using the deferred antagonism test on BHI agar plates. Furthermore, when we determined cfu in our mixed-biofilm assays, we observed no marked growth inhibition when isolates were grown with the 4 CNS isolates that had the strongest biofilm-inhibition activity. These CNS isolates did not seem to produce bacteriocins under the conditions tested, and the inhibition of biofilm formation did not seem to be due to bactericidal activity.

Some CNS isolates with a weak-biofilm phenotype can negatively affect the biofilm-forming ability of other staphylococci, including *Staph. aureus*, one of the most prevalent pathogens causing IMI in dairy cattle (Barnouin et al., 2005; Piepers et al., 2007; Sato et al., 2008; Petrovski et al., 2009; Olde Riekerink et al., 2010). These findings were in contrast with most studies, which have focused on the cooperative interactions and advantages provided by living in a biofilm community (e.g., antibiotic resistance and protection against immune defenses; Harriott and Noverr, 2009; Peters and al., 2010; Elias and Banin, 2012; Burmølle et al., 2014). Recent studies have, however, highlighted the importance of competitive interactions in biofilm communities (Rendueles and Ghigo, 2012). For example, *Streptococcus pneumoniae* can eradicate a preformed *Staph. aureus* biofilm using physical contact (Khan et al., 2016). Another study observed that spatial competition between *Lactococcus lactis* and *Listeria monocytogenes* in mixed-species biofilms resulted in the inhibition of *L. monocytogenes* (Habimana et al., 2011).

The mechanisms behind the biofilm-inhibition and dispersion effect of CNS have yet to be characterized, and their effect on other important mastitis pathogens, including *Streptococcus* spp., *Klebsiella* spp., and *Esch-*

erichia coli should also be evaluated to determine the spectrum of this activity.

ACKNOWLEDGMENTS

This research was supported by a grant from the Dairy Research Cluster II (Dairy Farmers of Canada, Agriculture and Agri-Food Canada, Canadian Dairy Network, Canadian Dairy Commission) through the Canadian Bovine Mastitis and Milk Quality Research Network. CG and AMG are the recipients of a NSERC-CREATE scholarship in milk quality. CG is also the recipient of a FRQNT Op+Lait Strategic Cluster scholarship. We thank Sarne De Vliegheer (Ghent University, Belgium) for the generous gift of the *Staph. chromogenes* 2 isolate. We also thank Frederic Berthiaume for his help with the confocal laser scanning microscopy, and Marguerite Cameron and Greg Keefe (University of Prince Edward Island, Canada) for their help with CNS species confirmation by MALDI-ToF.

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