Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms

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ABSTRACT

Mastitis is the most common and detrimental infection of the mammary gland in dairy cows and has a major economic impact on the production of milk and dairy products. Bacterial mastitis is caused by several pathogens, and the most frequently isolated bacterial species are coagulase-negative staphylococci (CNS). Although CNS are considered minor mastitis pathogens, the importance of CNS has increased over the years. However, the mechanism and factors involved in CNS intramammary infection are poorly studied and defined. Biofilms have been proposed as an important component in the persistence of CNS intramammary infection. Biofilms are defined as a cluster of bacteria enclosed in a self-produced matrix. The objectives of this study were to investigate the ability of CNS to form biofilms. A total of 255 mastitis-associated CNS isolates were investigated using a standard microtiter plate biofilm assay. The biofilms of some isolates were also observed by using confocal microscopy. The presence of biofilm-associated genes *icaA*, *bap*, *aap*, *embP*, *fbe*, and *atlE* was determined by PCR in the 255 isolates. The 5 dominant species assayed were *Staphylococcus chromogenes* (n = 111), *Staphylococcus simulans* (n = 53), *Staphylococcus xylosus* (n = 25), *Staphylococcus haemolyticus* (n = 15), and *Staphylococcus epidermidis* (n = 13), and these represented 85% of the isolates. The data gathered were analyzed to identify significant links with the data deposited in the Canadian Bovine Mastitis Research Network database. Overall, *Staph. xylosus* is the species with the strongest ability to form biofilm, and *Staph. epidermidis* is the species with the lowest ability to form biofilm. Regardless of the species, the presence of *icaA*, *bap*, or the combination of multiple genes was associated with a greater ability to form biofilm. A strong relationship between the strength of

a biofilm and days in milk was also noted, and CNS isolated later in the lactation cycle appeared to have a greater ability to form biofilm than those isolated earlier in the lactation cycle. In conclusion, *Staph. xylosus* is the species with the strongest biofilm formation ability. Furthermore, days in milk and gene combinations are predicted to be the variables with the strongest effect on biofilm formation by CNS.

Key words: mastitis, coagulase-negative staphylococci, biofilm, day in milk

INTRODUCTION

Mastitis is characterized by an inflammatory response caused by the invasion of the mammary gland via the teat orifice by pathogens. Intramammary infection resulting in mastitis can manifest in subclinical (no visual symptoms) or clinical mastitis (local and systemic symptoms). For dairy cows, mastitis is the most common and detrimental disease, having a major economic impact on the production of milk and dairy products. For clinical purposes, an IMI is defined by the presence of bacteria or elevated SCC for quarter or composite samples. However, no international consensus for the definition of an IMI exists (Andersen et al., 2010). Recently, several definitions were evaluated, and it was concluded that the choice of an IMI definition is dependent on the objectives of the study or control program for which the samples are collected (Dohoo et al., 2011). Several bacterial species are considered major mastitis pathogens, including *Streptococcus uberis*, *Staphylococcus aureus*, and *Escherichia coli*.

Coagulase-negative staphylococci are the bacteria most frequently isolated from IMI (Tenhagen et al., 2006; Pyörälä and Taponen, 2009; Sampimon et al., 2009). However, CNS have traditionally been considered a minor mastitis-causing pathogen. Twelve CNS species have been isolated from bovine milk samples, but the prevalent CNS species reported in IMI are *Staphylococcus chromogenes* and *Staphylococcus simulans* (Rajala-Schultz et al., 2006; Taponen et al., 2006;

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Pyörälä and Taponen, 2009; Piessens et al., 2011; Supré et al., 2011). Mastitis associated with CNS infections is often subclinical or mild. During lactation, CNS infections may result in an increased milk SCC, which decreases the milk quality and, consequently, results in economic losses. In recent years, the prevalence of CNS-associated IMI has increased in several regions of the globe (Pyörälä and Taponen 2009). Coagulase-negative staphylococci are considered by some an emerging mastitis pathogen (Pyörälä and Taponen, 2009). With regard to a mastitis diagnosis, CNS are not usually identified at the species level and are treated as a uniform group. However, recent research has indicated that some species might be more clinically relevant than others (Supré et al., 2011; Piessens et al., 2011, 2012). Despite the emergence of CNS as pathogens, the knowledge regarding their virulence and associated mechanisms is limited, but biofilm formation has been proposed as an important virulence factor of CNS, especially with regard to the persistence of CNS IMI (Simojoki et al., 2012). Biofilm formation has also been proposed as an important virulence factor for other mastitis pathogens, including *Staph. aureus*, *E. coli*, and *Strep. uberis* (Melchior, 2011).

Biofilms are defined as structured communities of bacterial cells enclosed in a self-produced matrix attached to biotic or abiotic surfaces (Costerton et al., 1999). The ability to form biofilms is considered a universal trait of microorganisms. Furthermore, biofilms offer protection against hostile environments, such as the immune response and bactericidal concentrations of antibiotics or disinfectants. In several bacterial pathogens, biofilm formation is important for virulence; however, the role of biofilms as a virulence factor in veterinary pathogens has been poorly studied (Jacques et al., 2010). Biofilm formation in staphylococcal species is associated with several factors, such as the intracellular adhesion locus (*icaABCD*), which encodes the proteins responsible for the synthesis of poly-*N*-acetyl glucosamine (PNAG; Mack et al., 1996), the biofilm-associated protein Bap (Cucarella et al., 2004), the accumulation-associated protein Aap (Hussain et al., 1997; Rohde et al., 2005), the fibrinogen adhesin Fbe (Nilsson et al., 1998), the fibronectin adhesin EmbP (Christner et al. 2010), and the bifunctional adhesin and autolysin AtlE (Heilmann et al., 1997).

The Canadian Bovine Mastitis Research Network (CBMRN) is an organization that spearheaded a data collection platform for a single national-level longitudinal cohort for mastitis research. The platform consists of a National Cohort of Dairy Farms (NCDF), Mastitis Laboratory Network, and Mastitis Pathogen Culture Collection (MPCC). The characteristics of the herds and the data collection process

were described previously (Reyher et al., 2011). Briefly, health data, production data, and milk samples were collected from the NCDF, milk samples were processed for SCC and bacteriology purposes by the Mastitis Laboratory Network, and bacteria isolated from milk samples were conserved in the MPCC. Furthermore, a small sample of the CNS of the MPCC were speciated based on their *rpoB* gene sequence. Almost 50% of the CNS were *Staph. chromogenes* and the other CNS were *Staph. simulans* (24.0%), *Staphylococcus xylosus* (8.8%), *Staphylococcus haemolyticus* (4.9%), and 16 other species (Dufour et al., 2012; J. R. Middleton, University of Missouri, Columbia, personal communication).

The objectives of this study were to investigate the biofilm-forming ability of CNS isolates from the MPCC, determine the frequency of biofilm-associated genes among these CNS, and, using the data from the NCDF, determine whether biofilm formation is associated with any factors such as SCC, DIM, and lactation number.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Coagulase-negative staphylococci isolates were obtained from the MPCC, which is managed by the CBMRN (St-Hyacinthe, Québec, Canada; Reyher et al., 2011). A total of 255 isolates were randomly and blindly selected from 1,049 CNS isolates that were identified at the species level. Coagulase-negative staphylococci were routinely cultured on brain-heart infusion broth (BHI) agar and incubated for 24 h at 37°C.

Microtiter Biofilm Assay

Biofilms were cultured in 96-well microtiter plates as described by Mitchell et al. (2010), with some modifications. Briefly, colonies from a 16-h BHI agar plate were suspended into fresh BHI supplemented with glucose (0.25% wt/vol) to a 0.5 McFarland standard. A volume (200 µL) of this inoculum was transferred into 3 wells, and the microtiter plate (Corning Costar no. 3595) was incubated at 37°C. After a 24-h incubation, the liquid medium was removed using a vacuum, and the planktonic cells were removed by washing the wells three times with PBS. The biofilms were air-dried and then stained with 0.1% (wt/vol) safranin for 10 min. The biofilms were washed once with distilled water and then dried at 37°C for 15 min. The stain was then released with 200 µL of the destaining solution [50% (vol/vol) ethanol, 50% (vol/vol) glacial acetic acid], and the amount of released stain was quantified by measuring the absorbance at 490 nm (A_{490}) with a microplate reader. *Staphylococcus epidermidis* strain ATCC 35984

Table 1. Polymerase chain reaction primers used in this study

Name ¹	Primer sequence	Description	Length of amplicon (bp)	Reference
icaFSe	5'-CTGTTTCATGGAACTCC-3'	<i>icaA</i>	200	Rohde et al. (2007)
icaRSe	5'-TCGATGCGATTTGTTCAAACA T-3'			
aapF2	5'-GAAGCACCGAATGTTCCAATATC-3'	<i>aap</i>	289	Rohde et al. (2005)
aapR1	5'-AGTTGGCGGTATATCTATTGTA-3'			
applongF	5'-GATTTAGATGGTGCA ACATTGACAT-3'	Variable region of <i>aap</i>	Variable	Rohde et al. (2005)
applongR	5'-TTGACGATT TTCACCTGTATCAGGT-3'			
bapF-6m	5'-ACTTAYTRCCHTATATCGAARTAG-3'	<i>bap</i>	900	Cucarella et al. (2004)
sasp-7c	5'-GCTGTTGAAGTTAATACTGTACCTGC-3'			
fbeF	5'-CTACAAGTTCAGGTCAAGGACAAGG-3'	<i>fbe</i>	273	Rohde et al. (2007)
fbeR	5'-GCGTCGGCGTATATCCTTCAG-3'			
atlEF	5'-CAACTGCTCAACCGAGAACA-3'	<i>atlE</i>	682	Rohde et al. (2007)
atlER	5'-TTTGTAGATGTTGTGCCCA-3'			
embPF	5'-AGCGGTACAAATGTCAAT-3'	<i>embP</i>	455	Rohde et al. (2007)
embPR	5'-AGAAGTGCTCTAGCATCATCC-3'			

¹F = forward; R = reverse.

was used as a strong biofilm control (Gill et al., 2005), and *Staph. epidermidis* strain ATCC 12228 was used as a weak biofilm control (Zhang et al., 2003). Each CNS isolate was tested on 3 independent days. Additionally, inocula were incubated at 37°C for 24 h and their growth was assessed visually. After 24 h, growth appeared to be equal for all isolates and no slow-growing isolates were identified.

DNA Isolation

Genomic DNA was isolated as described by Arciola et al. (2001), with some modifications. Briefly, bacteria from 1.5 mL of overnight culture were harvested by centrifugation. The pellets were resuspended in a lysostaphin solution [0.1 mg/mL (Sigma, St. Louis, MO) in Milli-Q water (EMD Millipore, Billerica, MA)] and incubated for 30 min at 37°C. A volume (150 µL) of proteinase K solution (0.1 mg/mL of proteinase K (Sigma) in 0.1 M Tris-HCl pH 7.5, 1 mM CaCl₂) was added and the mixture was incubated for 10 min at 37°C. The samples were then heated for 5 min at 100°C. Cells and insoluble debris were settled by centrifugation and supernatants were transferred to new tubes.

Detection of *icaA*, *aap*, *bap*, *fbe*, *embP*, and *atlE* by PCR

The presence of *icaA*, *aap*, *bap*, *fbe*, *embP*, and *atlE* were detected with primers (Table 1) described previously (Cucarella et al. 2004; Rohde et al. 2005, 2007). Briefly, the typical PCR mixture (50 µL) was composed of 200 ng of genomic DNA, 0.2 mM deoxynucleotide 5'-triphosphate, 1.5 mM MgCl₂, 0.5 µM forward primer, 0.5 µM reverse primer, 5 µL of 10× PCR reaction buffer, 1.25 U of Taq DNA polymerase (GE Healthcare,

Waukesha, WI), and water (to 50 µL). The PCR mixtures were subjected to 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 54°C for *aap*, 57°C for *icaA* and *bap* or 47°C for *fbe*, *embP*, and *atlE*, and 30 s at 72°C, and a final 1 min at 72°C using a thermocycler. Genomic DNA from *Staph. epidermidis* strain ATCC 35984 was a positive control for *icaA*, *aap*, *fbe*, *embP*, and *atlE*. For *bap*, genomic DNA from *Staph. aureus* strain V329 (Cucarella et al., 2004) was used as the positive control. For a negative control, sterile water was used instead of DNA. Samples were considered PCR positive when the amplicon size was equal to the positive control. Additionally, some PCR products were sent for sequencing to verify the identity of the amplicon.

Amplification of the Variable Region of *aap*

The length of the variable region of *aap* was measured as described before (Rohde et al., 2005). Briefly, the typical PCR mixture (50 µL) was composed of 200 ng of genomic DNA, 0.2 mM deoxynucleotide 5'-triphosphate, 3.0 mM MgCl₂, 0.5 µM of forward primer (applongF; Table 1), 0.5 µM of reverse primer (applongR), 10 µL of 5× Phusion GC buffer (New England Biolabs, Ipswich, MA), 1.25 U of Phusion High Fidelity DNA polymerase (New England Biolabs), and water (to 50 µL). The PCR mixtures were subjected to 5 min at 98°C, 30 cycles of 10 s at 98°C, 10 s at 60°C and 2 min at 72°C, and a final 5 min at 72°C using a thermocycler. Genomic DNA from *Staph. epidermidis* strains ATCC 35984 and ATCC 12228 were used as positive controls. For a negative control, sterile water was used.

Dispersion of Biofilms by Enzymatic Treatments

A biofilm dispersion assay was performed as described previously (Izano et al., 2007). A total of 30

isolates representing each species and genotype were selected for this assay. Briefly, biofilms were grown as described in the Microtiter Biofilm Assay section, and after the 24-h incubation, 50 μ L of DNase I (500 μ g/mL in 150 mM NaCl, 1 mM CaCl₂), 50 μ L of dispersin B (100 μ g/mL in PBS; Kane Biotech Inc., Winnipeg, MB, Canada), or 50 μ L of proteinase K (500 μ g/mL in 50 mM Tris-HCl pH 7.5, 1 mM CaCl₂) was added directly to the biofilms. Control wells were treated with 50 μ L of the buffer without the enzyme. Wells treated with dispersin B were incubated for 5 min at 37°C, and those treated with proteinase K or DNase I were incubated for 1 h at 37°C. After the treatments, the biofilms were treated as described in the Microtiter Biofilm Assay section.

Confocal Laser Scanning Microscopy

Biofilms were prepared as described in the Microtiter Biofilm Assay section but were stained with the FilmTracer FM 1-43 (Invitrogen, Eugene, OR), the FilmTracer LIVE/DEAD Biofilm Viability Kit (Invitrogen), wheat-germ agglutinin (WGA) conjugated with Oregon green (Invitrogen), BOBO-3 (Invitrogen), or the FilmTracer SYPRO Ruby Biofilm Matrix Stain (Invitrogen), as prescribed by the manufacturer. According to the manufacturer, WGA will bind to *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid residues, BOBO-3 is a cell-impermeable DNA stain, and SYPRO Ruby stain labels most classes of proteins. The stained biofilms were visualized by confocal laser scanning microscopy (CLSM; FV1000 IX81; Olympus, Markham, ON, Canada) and images were acquired using Fluoview software (Olympus).

Statistical Analysis

After a normality check (and eventually data transformation), a linear mixed model was used to investigate biofilm absorbance according to the CNS species. Because samples could be repeated within cows that were clustered within herd, a multilevel hierarchical model was implemented in R (R Foundation for Statistical Computing, Vienna, Austria) with the package lme4 (Bates et al., 2011) and languageR (Baayen et al., 2008). The models with random effects for udder quarter, cow and herd, or only herd were compared between each other with the likelihood ratio test and the parametric bootstrap, together with improvement in the Akaike information criterion. A second set of models were developed by adding covariates to the previous model. The covariates added were genes (*icaA*, *bap*, *aap*, *embP*, *fbe*, *atlE*), the interactions between these 6 genes, DIM, lactation number, and the SCC

(on a log scale). Backward stepwise covariate selection was made by comparison of the full model with the nested model in the same fashion as above, beginning with the interaction term. Species and genes were kept in the model whatever their significance. Multiple comparisons between species were run with the Tukey test (multcomp package; Hothorn et al., 2008).

RESULTS

Biofilm-Formation Potential Among CNS Isolates

The microtiter plate-based assay is a good tool to determine the potential of an isolate to form a biofilm. Based on the amount of safranin bound by the biofilm (A_{490}), the ability of a CNS isolate to form a biofilm was classified as negative ($A_{490} < 0.110$), weak ($0.110 < A_{490} < 0.500$), moderate ($0.500 < A_{490} < 1.500$), or strong ($A_{490} > 1.500$). The majority (85.1%) of the isolates were able to form a biofilm, whereas a minority (14.9%) were considered biofilm-negative isolates (Table 2). Among the biofilm-positive isolates, 36.4, 25.5, and 23.1% were weak, moderate, and strong biofilm formers, respectively. Most *Staph. xylosus* isolates (84%) were moderate to strong biofilm formers, whereas most *Staph. epidermidis* isolates (84.6%) were biofilm negative or weak biofilm formers. This suggested that *Staph. xylosus* isolates have the highest ability to form biofilms and *Staph. epidermidis* isolates have the lowest ability to form biofilms.

The median value of all isolates of each species combined was also used to evaluate the ability of CNS to form biofilms. As with the categories above, *Staph. xylosus* had the highest median value for its isolates and *Staph. haemolyticus* has the second highest median value (Figure 1). This suggests that both *Staph. xylosus* and *Staph. haemolyticus* had the greatest ability to form biofilms. *Staphylococcus epidermidis* had the lowest median value (Figure 1). Again, this suggests that *Staph. epidermidis* isolates had the lowest ability to form biofilms.

Distribution of Biofilm-Associated Genes Among CNS Isolates

The majority (224, 87.8%) of the isolates were positive for at least 1 of the 6 genes, and only 31 isolates (12.2%) were negative for all 6 genes (Table 2).

***icaA*.** In total, 134 isolates (52.5%) were positive for *icaA*, and the distribution of *icaA* among species appeared to occur at similar frequencies (~50%). However, the presence of *icaA* might occur at a lower frequency in *Staph. epidermidis* (30.8%).

Table 2. Biofilm-formation potential and distribution of biofilm-associated genes among CNS species

Item	CNS species						Total, number (%)
	<i>Staph. chromogenes</i>	<i>Staph. simulans</i>	<i>Staph. xylosus</i>	<i>Staph. haemolyticus</i>	<i>Staph. epidermidis</i>	Others	
Number (%) of isolates	111 (43.5)	53 (20.8)	25 (9.8)	15 (5.9)	13 (5.1)	38 (14.9)	255
Biofilm potential ¹							
Negative ($A_{490} < 0.110$)	17	8	2	1	4	6	38 (14.9)
Weak ($0.110 < A_{490} < 0.500$)	34	27	2	5	7	18	93 (36.5)
Moderate ($0.500 < A_{490} < 1.500$)	32	13	9	3	1	7	65 (25.5)
Strong ($A_{490} > 1.500$)	28	5	12	6	1	7	59 (23.1)
Biofilm-associated gene							
None	20	7	0	3	0	1	31 (12.2)
<i>icaA</i>	61	25	12	8	4	24	134 (52.5)
<i>bap</i>	2	1	23	2	4	6	37 (14.5)
<i>aap</i>	75	32	19	10	6	26	168 (65.8)
<i>embP</i>	4	1	3	1	10	6	25 (9.8)
<i>fbe</i>	13	12	5	2	11	16	59 (23.1)
<i>atlE</i>	12	2	3	11	11	3	42 (16.5)

¹ A_{490} = absorbance at 490 nm.

bap. Only 37 isolates (14.5%) were *bap* positive, and the distribution of *bap* among CNS species varied considerably. The presence of *bap* in *Staph. chromogenes* and *Staph. simulans* isolates was rare. For *Staph. haemolyticus* and *Staph. epidermidis*, 13.3 and 30.8% of the isolates, respectively, were *bap* positive. The presence of *bap* was detected in almost every *Staph. xylosus* isolate (92%).

embP. The presence of *embP* among CNS isolates was rare, and only 25 (9.8%) isolates were positive. This gene was almost exclusively detected in *Staph. epidermidis*: 76.9% of these isolates were positive.

atlE. Among CNS isolates, 42 isolates (16.5%) were positive for *atlE*. This gene was mostly detected in 2 species, *Staph. epidermidis* (84.6%) and *Staph. haemolyticus* (73.3%).

fbe. Compared with *embP*, *bap*, and *atlE*, *fbe* was more frequently detected in isolates (23.1%). Again, *fbe* was almost exclusively in *Staph. epidermidis*, with 84.6% of these isolates being positive.

aap. A total of 168 isolates, which is a majority (65.8%), were *aap* positive, and the distribution of *aap* among species appeared to occur at similar frequencies (~65%). However, the presence of *aap* might occur at a lower frequency in *Staph. epidermidis* (46.2%). Attempts to amplify the variable region of *aap* failed for most of the *aap*-positive isolates (89.9%) and appeared to be successful for 17 isolates. When amplification was successful, most isolates appeared to have 1, 2, or 3 repeats in the variable region, and only 1 isolate appeared to have 8 repeats. No trends were noted between the length of the *aap* repeats and biofilm formation.

Gene Combination. Among isolates that were positive for 1 of the 6 genes, 71 isolates (27.8%) had only 1 of the 6 genes (Supplemental Tables 1 and 2). Unlike *icaA*, *bap*, *aap*, and *fbe*, the genes *embP* and *atlE* were never detected alone and were always observed in combination with another gene. Most isolates (60%) had at least 2 genes, and 84 of those isolates (32.9%) had a 2-gene combination, 55 (21.6%) had a 3-gene combination, 9 (3.5%) had a 4-gene combination, 4 (1.6%) had a 5-gene combination, and 1 isolate of *Staph. epidermidis* (0.4%) had all 6 genes (Supplemental Tables 1 and 2). *Staphylococcus simulans* isolates were the only species in which a 3-gene combination was the largest combination observed. The combination most frequently observed was *icaA-aap* (86 isolates), and 55 of those were not combined with another gene. The most common triplet was the *icaA-aap-atlE* combination, and it was detected in 18 isolates. This triplet was almost exclusively found in 2 species, *Staph. chromogenes* (9

Figure 1. Biofilm strength for the dominant CNS species [*Staphylococcus chromogenes* (n = 99), *Staphylococcus epidermidis* (n = 11), *Staphylococcus haemolyticus* (n = 14), *Staphylococcus simulans* (n = 50), *Staphylococcus xylosus* (n = 24)]. Points outside the box and whiskers are considered outliers.

isolates) and *Staph. haemolyticus* (6 isolates). The combination *icaA-bap-aap* was the second most common triplet (17 isolates), but it appeared almost exclusively in *Staph. xylosus* (9 isolates). The third most common triplet, with 16 isolates, was the *icaA-aap-fbe* combination, and it was mostly observed in *Staph. chromogenes* (6 isolates). The *embP-fbe-atlE* combination was the fourth most common triplet (14 isolates), but it was almost exclusively observed in *Staph. epidermidis* (10 isolates). Half of the *embP-fbe-atlE*-positive *Staph. epidermidis* isolates had 1, 2, or 3 of the other genes (*bap*, *ica*, or *aap*).

Biofilm Matrix Composition of Selected CNS Isolates

To study the biofilm matrix composition of the CNS isolates, 30 isolates representing different biofilm phenotypes and genotypes were selected for enzymatic digestion of the matrix. Among the selected strains, some isolates were dispersed by dispersin B or DNase I, and most were dispersed by proteinase K (data not shown). This suggested most CNS isolates have a protein-based matrix and a few have a PNAG-based matrix.

To further characterize the biofilm matrix, 1 strong biofilm former and 1 weak biofilm former of each dominant species were selected for more tests. When the biofilms of the strong biofilm formers were treated with enzymes, none of the isolates was sensitive to the dispersin B treatment (Figure 2A), all isolates were sensitive to the proteinase K treatment (Figure 2B), and 2 of the 5 isolates were sensitive to the DNase treatment (less than 75% of the biofilm remained after treatment; Figure 2C). Only the biofilms of *Staph. chromogenes* and *Staph. xylosus* were sensitive to the DNase I treatment. For the weak biofilm formers, 3 of the 5 isolates were sensitive to the dispersin B treatment, 4 of the 5 isolates were sensitive to the proteinase K treatment, and all isolates were sensitive to the DNase I treatment (data not shown). *Staphylococcus simulans* and *Staph. epidermidis* were resistant to the dispersin B treatment, whereas *Staph. haemolyticus* was resistant to the proteinase K treatment. Again, this suggested that proteins and extracellular DNA (eDNA) play a larger role than PNAG in CNS biofilm formation.

To further study the composition of the matrix, CLSM was performed with several fluorescent stains that have different purposes, such as evaluating the amount of bacteria in the biofilm. The stain FM 1-43 integrates into the membrane of bacteria, and this helps in evaluating the number of cells and the general morphology of the biofilm. With the exception of the *Staph. epidermidis* isolate, biofilm morphology and the amount of bacteria were fairly similar (Figure 3). The biofilm of *Staph. epidermidis* appeared to be composed of several

clusters or microcolonies (Figure 3). The LIVE/DEAD kit is composed of 2 stains, 1 (SYTO-9) that binds DNA in cells with intact cell membranes (viable) and 1 (propidium iodide) that binds DNA in cells with damaged cell membranes (dead). According to these stains, the *Staph. simulans*, *Staph. epidermidis*, and *Staph. haemolyticus* biofilms had a larger number of dead cells than did the *Staph. chromogenes* and *Staph. xylosus* biofilms (Figure 3). Wheat-germ agglutinin binds to *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid residues, and WGA is used to indicate the presence of PNAG in the biofilm matrix. The other stains used are specific for biofilm matrix components, such as proteins (SYPRO Ruby) and eDNA (BOBO-3). The biofilms of all isolates were bound by WGA, BOBO-3, and SYPRO Ruby, which suggests that the biofilm matrix contains PNAG, eDNA, and proteins (Figure 3). For *Staph. simulans*, *Staph. xylosus*, and *Staph. haemolyticus*, the amounts of *N*-acetyl-glucosamine polymer in their biofilms appeared to be similar; *Staph. chromogenes* appeared to have the lowest amount of PNAG, whereas *Staph. epidermidis* appeared to have the largest amount of PNAG (Figure 3). With regard to the amount of protein, the *Staph. simulans* and *Staph. epidermidis* isolates appeared to have the largest amounts of proteins in their biofilm matrix (Figure 3). Although BOBO-3 is a membrane-impermeable DNA stain and does not penetrate bacterial cells with intact cell membranes, it stains eDNA and may stain DNA inside cells with a compromised cell membrane. For eDNA, the relative number of dead cells stained with propidium iodide must be taken into account when evaluating the quantity of eDNA (Seper et al., 2011). At first glance, the amount of eDNA appeared to be similar in all species; however, the *Staph. simulans*, *Staph. haemolyticus*, and *Staph. epidermidis* biofilms had large populations of damaged or dead cells. This suggests that the eDNA BOBO-3 stain could also bind DNA from damaged cells. On the basis of this interpretation, the *Staph. chromogenes* and *Staph. xylosus* isolates would have the largest amounts of eDNA. This correlates well with the DNase treatment because the biofilms of *Staph. chromogenes* and *Staph. xylosus* were the only isolates sensitive to the treatment (Figure 2C).

Similar CLSM experiments were done for some negative, weak, and moderate biofilm formers to confirm observations from the microtiter plate assay. When the stain FM 1-43 was used, moderate biofilm formers had fewer cells than the strong biofilm formers but had substantially more cells than the weak or negative biofilm formers (data not shown). For the negative biofilm formers, scattered individual cells were observed, which suggests minimum binding to the substrate (data not shown). When WGA staining was positive, the stain

was mainly focused around individual cells in the case of negative and weak biofilm formers (data not shown). For moderate biofilm formers, the biofilm morphology revealed by the stain was similar to the morphology of the strong biofilm formers, but the amount of stain was lower (data not shown). With regard to protein staining (SYPRO Ruby) and eDNA staining (BOBO-3), the stains were mostly focused around individual cells in the case of negative and weak biofilm formers, as observed with WGA staining (data not shown). Moderate biofilm formers were not stained with SYPRO Ruby and BOBO-3.

Statistical Analysis

To find relevant links between the data gathered by this study and data accumulated in the CBMRN database, statistical analyses were performed. For the purpose of statistical analysis, isolates identified as *Staph. chromogenes*, *Staph. simulans*, *Staph. xylosus*, *Staph. haemolyticus*, and *Staph. epidermidis* with the strongest confidence level were kept. After this round of elimination, 209 isolates were left for the analysis. The sample size was further restricted by eliminating isolates that came from composite samples when the isolate from the same cow was present in a quarter sample and the sampling was done on the same day. After this second round, 9 composite samples that were already sampled as quarter samples were removed and 198 isolates were used for our statistical analyses. Taking multiple comparisons into account, *Staph. xylosus* had a greater ability to form biofilm than did *Staph. epidermidis* and *Staph. simulans*, as well as *Staph. chromogenes* versus *Staph. simulans*, *Staph. epidermidis* versus *Staph. haemolyticus*, and *Staph. haemolyticus* versus *Staph. simulans* (Table 3). However, the effect was confounded by the genes harbored by each species (Table 4). The presence of *icaA*, *bap*, or *fbe* was predicted to have a positive effect on biofilm formation, whereas the presence of *embP* was predicted to have a negative effect on biofilm formation (Table 4). The presence of *aap* or *atlE* was predicted to have no significant effect on biofilm formation (Table 4). The combination of multiple genes was associated with a greater ability to form a biofilm, with the triplet *icaA-bap-aap* and the pair *icaA-aap* being strong predictors of biofilm formation. Furthermore, biofilm formation was significantly increased when 4 or more genes were present. This suggests that a doselike response occurred with respect to the number of genes and biofilm formation (data not shown).

It is interesting that a strong relationship between the strength of a biofilm and the DIM was noted (Table 4), and it appears that CNS isolated later in the lacta-

Figure 2. Dispersion of the CNS biofilms by (A) dispersin B (Kane Biotech Inc., Winnipeg, MB, Canada), (B) proteinase K, and (C) DNase I. The bar represents the results from 1 isolate. Genotype of the strain used for the dispersion assay is indicated under the graph. *Staphylococcus epidermidis* strain ATCC 35984 was used as a control because its biofilm is dispersed by all 3 enzymes.

Figure 3. Confocal laser scanning microscopy of CNS biofilms stained with FM 1-43, BOBO-3, wheat-germ agglutinin (WGA)-Oregon green, SYPRO Ruby, SYTO, and propidium iodide (all from Invitrogen, Eugene, OR). PNAG = poly-*N*-acetyl glucosamine; eDNA = extracellular DNA. Color version available in the online PDF.

tion cycle had a greater ability to form biofilms than did those isolated earlier in the lactation cycle. However, species were not evenly distributed across DIM. Additionally, no association between biofilm and SCC or parity was observed in the statistical model (Table 4). Overall, DIM and gene combinations are predicted

to be the variables with the strongest effect on biofilm formation.

DISCUSSION

Biofilm formation is critical for the virulence of several pathogens and has also been proposed as an impor-

Table 3. Multiple comparison of log(biofilm production + 1) between species in the first model

Item	Estimate	SE	t-value	P-value
<i>Staphylococcus chromogenes</i> vs.				
<i>Staphylococcus epidermidis</i>	-0.389	0.145	-2.676	0.053
<i>Staphylococcus haemolyticus</i>	0.138	0.121	1.145	0.77
<i>Staphylococcus simulans</i>	-0.222	0.078	-2.860	0.031
<i>Staphylococcus xylosus</i>	0.260	0.099	2.632	0.059
<i>Staph. epidermidis</i> vs.				
<i>Staph. haemolyticus</i>	0.527	0.179	2.942	0.024
<i>Staph. simulans</i>	0.167	0.153	1.094	0.80
<i>Staph. xylosus</i>	0.648	0.164	3.942	0.001
<i>Staph. haemolyticus</i> vs.				
<i>Staph. simulans</i>	-0.360	0.130	-2.764	0.041
<i>Staph. xylosus</i>	0.121	0.142	0.851	0.91
<i>Staph. simulans</i> vs.				
<i>Staph. xylosus</i>	0.481	0.108	4.475	<0.001

tant virulence factor for the mastitis pathogens *Staph. aureus*, *E. coli*, and *Strep. uberis* (Melchior, 2011). In the case of CNS, it has been established that biofilms play an important role in the virulence of *Staph. epidermidis*, especially for infections associated with indwelling medical devices (Otto, 2008). With the exception of *Staph. epidermidis*, information on the biofilm formation of other CNS species has been very limited and, in the case mastitis-associated CNS, is limited to 2 studies (Tormo et al., 2005; Simojoki et al., 2012). The objective of our study was to characterize the ability of CNS associated with mastitis to form biofilms.

The majority (85.1%) of the isolates tested were biofilm positive, and a minority (14.9%) were biofilm negative. Using a similar assay, Simojoki et al. (2012) observed the opposite: most mastitis-associated CNS were biofilm negative (68.7%) and a minority of isolates were biofilm positive (31.3%). The distribution of CNS species in both studies was similar, with the exception that *Staph. xylosus* was not reported as a separate group by Simojoki et al. (2012). In our study, we ob-

served that the ability to form biofilms varied among CNS species and that *Staph. haemolyticus* and *Staph. xylosus* were strong biofilm formers and *Staph. simulans* and *Staph. epidermidis* were weak biofilm formers. Previous studies with human isolates demonstrated that most *Staph. haemolyticus* (53/72) and *Staph. xylosus* (11/12) strains were able to form biofilms (Planchon et al. 2006; Fredheim et al., 2009). This supports our observation that *Staph. haemolyticus* and *Staph. xylosus* have strong biofilm-formation potential. Therefore, the distribution of CNS species in the sample tested likely influenced the percentage of biofilm formers and may offer an explanation for the difference observed between our study and that by Simojoki et al. (2012). Another potential reason for the discrepancy between our study and the study by Simojoki et al. (2012) is the media used (BHI vs. tryptic soy broth). Minor differences in media composition and source, such as the manufacturer, have an effect on biofilm formation, and this was previously observed for *Staph. haemolyticus* (Fredheim et al., 2009) and in our laboratory for the

Table 4. Effect of genes and selected covariates on log(biofilm + 1) in the linear mixed model

Gene and covariate	Estimate	SE	t-value	MCMC ¹ P-value
Intercept	0.624	0.254	2.459	0.042
Species				
<i>Staphylococcus chromogenes</i>	Reference	—	—	—
<i>Staphylococcus epidermidis</i>	-0.149	0.176	-0.846	0.46
<i>Staphylococcus haemolyticus</i>	0.175	0.127	1.378	0.10
<i>Staphylococcus simulans</i>	-0.136	0.079	-1.730	0.072
<i>Staphylococcus xylosus</i>	-0.072	0.138	-0.524	0.42
Presence of <i>icaA</i>	0.173	0.054	3.188	0.003
Presence of <i>bap</i>	0.333	0.125	2.666	0.003
Presence of <i>aap</i>	0.063	0.061	1.030	0.56
Presence of <i>embP</i>	-0.306	0.133	-2.298	0.016
Presence of <i>fbe</i>	0.119	0.089	1.337	0.043
Presence of <i>atlE</i>	-0.127	0.089	-1.436	0.073
DIM	0.001	0.000	2.966	0.001
SCC (log)	-0.023	0.019	-1.261	0.46

¹MCMC = Markov chain Monte Carlo.

swine pathogen *Actinobacillus pleuropneumoniae* (Labrie et al., 2010).

In this study, we screened mastitis-associated CNS for genes associated with biofilm formation of *Staph. epidermidis* (Rohde et al., 2007). In the study by Simojoki et al. (2012), mastitis-associated CNS were screened for MSCRAMM (microbial surface components recognizing adhesive matrix molecules), which are traditionally associated with the adhesion and invasion of host cells and biofilm formation of *Staph. aureus* (Rohde et al., 2007; Otto, 2008; Heilmann, 2011). The common genes in both studies were *icaA* and *bap*. In our studies, 52.5 and 14.5% of the isolates were positive for *icaA* and *bap*, respectively. Simojoki et al. (2012) did not detect *icaA* or *bap* in their isolates. The difference between the amount of *bap* reported in this study and that by Simojoki et al. (2012) is likely linked to the CNS species included in both studies. In the study by Simojoki et al. (2012), *Staph. xylosus* was not reported as a separate group, and in the current study, the *bap* gene was predominantly detected in *Staph. xylosus* (23/25) and was rarely detected in the other CNS species (14/230). In support of our observation, Planchon et al. (2006) also observed a high percentage of *Staph. xylosus* isolated from human skin that was positive for *bap* by PCR (9/12) or Southern blot (12/12). Another reason for the discrepancy might be the primers used to amplify *bap*. Although the source for the primer design and the reverse primer was identical, the design for the forward primer was optimized by creating a degenerate primer and slightly shifting the position of the primer. To redesign the forward primer, the *bap* sequence from various CNS, which are deposited in GenBank (Tormo et al., 2005), were aligned with the primer *sasp-6m* (Cucarella et al., 2004) and a new primer was designed to decrease the number of mismatches. The source of the discrepancy between the number of *icaA* reported in this study and those reported by Simojoki et al. (2012) is probably the primers used to amplify *icaA*. Simojoki et al. (2012) used primers that were specific for *Staph. aureus icaA*, whereas we used primers specific for *Staph. epidermidis icaA*. The use of primers specific for *Staph. epidermidis icaA* is probably more suitable than primers based on *Staph. aureus icaA* for the detection of *icaA* in CNS. However, the *icaA* primers and PCR conditions used in our study may lack sensitivity. With CLSM, WGA detected PNAG in the biofilms of *icaA*-negative isolates and, based on our experience, the relationship is good between WGA staining of a biofilm and PNAG production by bacteria. Therefore, WGA staining of *icaA*-negative isolates suggests that these isolates are either *icaA* positive or produce a structure containing *N*-acetyl-glucosamine. Such a phenomenon has also been observed in *Staph. haemolyticus*, an *ica*-

negative human isolate that was stained with WGA (Fredheim et al., 2009).

The lack of sensitivity may not be limited to the *icaA* PCR and may extend to *embP*, *atlE*, and *fbe* PCR. The primers used in those PCR are based on *Staph. epidermidis*, and those genes were detected in a larger proportion of *Staph. epidermidis*. However, *embP*, *atlE*, and *fbe* were detected in every species, suggesting that the PCR had good specificity and acceptable sensitivity. Furthermore, bifunctional autolysin can be used to differentiate staphylococci species (Albrecht et al., 2012) and subtype *Staph. epidermidis* (Sivadon et al., 2006). Therefore, a negative *atlE* gene may indicate that an isolate possesses a different autolysin type. However, the sensitivity and specificity could not be fully assessed given the lack of genetic sequences (e.g., genome and genes) for CNS, with the exception of *Staph. epidermidis*. Therefore, strains with known sequence that may act as controls were lacking, and information on genomes of minor CNS would thus help improve the sensitivity of the PCR screens.

The *icaA* gene was present in roughly 50% of the isolates tested, and the biofilms of most CNS, including *icaA*-positive isolates, were not detached by dispersin B. The biofilms of some *icaA*-negative isolates were also stained with WGA. This suggests that CNS associated with mastitis likely forms PNAG-independent biofilms and that PNAG plays a complementary role as a matrix component. Poly-*N*-acetyl glucosamine-independent biofilm formation has been observed in other staphylococci (Toledo-Arana et al., 2005; Frank and Patel, 2007; Qin et al. 2007). Given that PNAG does not appear to be a major component of the biofilm matrix, other molecules, such as eDNA and proteins, likely play a more important role. Extracellular DNA may act as cell-to-surface or cell-to-cell adhesins, or both (Whitchurch et al., 2002). Fluorescent staining and DNase treatment of CNS biofilms indicated eDNA is present in the biofilm matrix of CNS associated with mastitis. This has also been reported for human isolates of *Staph. haemolyticus* (Fredheim et al., 2009). Given that dispersion by DNase never exceeded 50%, eDNA may play only a secondary role as a component of the biofilm matrix. The most significant dispersion was observed with proteinase K-induced biofilm dispersion; 50 to 100% of the biofilm could be dispersed using this enzyme. Several proteins have been reported to play a role in *Staph. epidermidis* biofilms, including Bap, Aap, EmbP, Fbe, and AtlE. The Aap mediates intercellular adhesion and biofilm formation in *Staph. epidermidis* and it can act independently or cooperatively with the *ica* operon (Rohde et al., 2005). Additionally, Bap can mediate biofilm formation in the absence of PNAG in several *Staphylococcus* species (Tormo et al., 2005). It is likely, based on

the distribution of *bap*, that biofilm formation in *Staph. xylosus* is Bap dependent. Furthermore, the presence of genes that would help mediate a protein-based biofilm was detected in very few isolates, with the exception of *aap*. Our statistical analysis indicates that some of the genes, such as *bap*, *icaA*, and *aap*, may interact to increase biofilm formation, thus indicating that polysaccharides and proteins are required for a strong biofilm despite the proteinaceous nature of the biofilm. However, some combinations were restricted to one species and, in some rare cases, the absence of the 6 genes tested was noted in strong biofilm formers. Therefore, uncharacterized proteins must mediate biofilm formation in most CNS. Proteinaceous biofilms formed with unknown proteins have also been reported for *Staph. epidermidis* (Qin et al., 2007) and *Staph. haemolyticus* (Fredheim et al., 2009). Furthermore, the protein-based biofilm formed by mastitis-associated CNS explains the poor relationship between biofilm formation and slime production observed previously for mastitis-associated CNS (Simojoki et al., 2012).

Biofilms are often associated with chronic and persistent infections (Costerton et al., 1999). It has been hypothesized that biofilms are involved in mastitis because 50% of the CNS IMI are persistent (Taponen et al., 2006). Previously, Simojoki et al. (2012) did not observe an association between biofilm formation and CNS IMI persistence and severity. We did not observe a relationship between biofilms and SCC; therefore, biofilms do not appear to affect CNS IMI severity. Unfortunately, the data available in the CBMRN database do not differentiate all CNS isolates at the species level; thus, a solid statistical model cannot be established to test whether an association exists between biofilm formation and persistent IMI. However, our statistical analysis did reveal a positive relationship between DIM and biofilm strength. This suggests that strong biofilm formers are selected for during lactation. The reason for such selection is unclear, but it is likely that biofilms increase the ability of CNS to persist in the intramammary environment. This indirectly indicates a relationship between biofilm formation and persistent IMI. Furthermore, the presence of biofilm-positive CNS at the onset of the dry-off period might have an effect on blanket dry-cow therapy, given that a biofilm increases bacterial resistance to antibiotics. A biofilm-positive isolate might survive blanket dry-cow therapy, and this could increase the probability of a recurrent CNS IMI in the lactation period. This hypothesis has yet to be tested.

Alternatively, the ability to form biofilms might facilitate the environmental transmission of CNS at a later stage of lactation. Piessens et al. (2011) consid-

ered, based on a reservoir study, that *Staph. xylosus* is an environmental bacterium, *Staph. chromogenes* and *Staph. epidermidis* are host-adapted bacteria, and *Staph. haemolyticus* and *Staph. simulans* are pathogens that persist in the environment. In our sampling, *Staph. xylosus* was associated with the highest DIM values and was 1 of the 2 strongest biofilm formers among CNS species. Therefore, biofilms might help *Staph. xylosus* survive in the environment and facilitate its transmission from the farm environment to the intramammary environment when the cow is lactating. This hypothesis can also be applied to *Staph. haemolyticus*, given that it is the other strong biofilm former and that it can persist in the environment. Environmental biofilms have been shown to be important in the persistence of other bacterial pathogens, such as *E. coli*, *Vibrio cholerae*, and *Legionella pneumophila* (Cooper and Hanlon, 2010; Shikuma and Hadfield, 2010). The presence of environmental biofilms would require on-farm management to reduce the risk of infection. Recently, it has been demonstrated that the management of the cow's environment, such as bedding conditions or pasture access, has a positive outcome on CNS IMI incidence (Dufour et al., 2012).

CONCLUSIONS

Most CNS isolates from subclinical mastitis have the ability to form protein-based biofilms. Biofilm formation ability varied among CNS species, with *Staph. xylosus* and *Staph. haemolyticus* having the highest ability to form biofilms. Biofilm formation was associated with the presence of specific gene combinations (*icaA-bap-aap* and *icaA-aap*). Additionally, a strong positive association existed between DIM and biofilm strength.

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