J. Dairy Sci. 97:4838–4841
http://dx.doi.org/10.3168/jds.2014-7986

Short communication: Biofilm production characterization of *mecA* and *mecC* methicillin-resistant *Staphylococcus aureus* isolated from bovine milk in Great Britain

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ABSTRACT

Staphylococcus aureus is an important cause of contagious intramammary infection in dairy cattle, and the ability to produce biofilm is considered to be an important virulence property in the pathogenesis of mastitis. The aim of this study was to characterize the biofilm formation capacity of methicillin-resistant *Staph. aureus* (MRSA), encoding *mecA* or *mecC*, isolated from bulk tank milk in Great Britain. For this purpose, 20 MRSA isolates were grown on microtiter plates to determine the biofilm production. Moreover, the *spa*-typing and the presence of the intercellular adhesion genes *icaA* and *icaD* were analyzed by PCR. All MRSA isolates tested belonged to 9 *spa*-types and were PCR-positive for the *ica* genes; 10 of them (50%) produced biofilm in the microtiter plate assay. This is also the first demonstration of biofilm production by *mecC* MRSA.
Key words: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, biofilm

Short Communication

Staphylococcus aureus is one of the most important pathogens implicated in nosocomial infections and implanted medical device-related infections in humans. In addition, this microorganism is a common contagious bacterium responsible for bovine mastitis.

The ability to form biofilm represents an important virulence factor in both human and bovine mammary gland *Staph. aureus* infections. Bacteria enclosed in the extracellular biofilm matrix and arranged in multilayers become resistant to antimicrobial agents and to the host immune system by impairing the action of phagocytic cells (Costerton et al., 1999). Such characteristics allow *Staph. aureus* with biofilm-forming ability to adhere and colonize the mammary gland epithelium and establish persistent infections (Fox et al., 2005).

A major constituent of the staphylococcal biofilm matrix is the poly-*N*-acetyl β-1,6 glucosamine (**PNAG**) surface polysaccharide, which is involved in intercellular adhesion and synthesized by proteins encoded by the intercellular adhesion operon (*icaADBC*; Cramton et al., 1999). The *icaA* gene product is a transmembrane protein with homology to *N*-acetyl-glucosaminyltransferases, requiring the *icaD* gene product for optimal activity. *N*-Acetyl-glucosamine oligomers produced by *icaAD* reach a maximum length of 20 residues, and longer oligomer chains are synthesized when *icaAD* is co-expressed with *icaC*, which encodes a putative membrane protein. The *icaC* gene is also likely to be involved in translocation of the growing polysaccharide to the cell surface. The surface-attached protein, IcaB, is then responsible for deacetylation of the poly-*N*-acetylglucosasmine molecule (O'Gara, 2007). Conversely, *Staph. aureus* surface proteins, such as biofilm-associated protein (Bap), can mediate PNAG-independent biofilm development. A deletion of the *ica* operon in a *bap*-positive strain has no effect on the in vitro biofilm-formation capacity of the resulting mutant strain (Cucarella et al., 2004).

Recently, Bardiau et al. (2013) reported that biofilm-formation ability was present in all the methicillin-resistant *Staph. aureus* (**MRSA**) isolates analyzed from bovine mastitis in Belgium (n = 19). Resistance to β-lactam antibiotics by MRSA is mainly attributed to the acquisition of a mobile genetic element, the staphylococcal cassette chromosome (SCC*mec*), which carries either the *mecA* gene or the recently described variant *mecC* (García-Álvarez et al., 2011). The *mec* genes encode an altered penicillin-binding protein, essential for cell wall biosynthesis, which has a reduced affinity for β-lactam antibiotics.

The objective of the current study was to determine the biofilm-forming capacity of 20 MRSA isolates from bovine bulk tank milk in Great Britain, encoding either *mecA* or *mecC* genes. Moreover, all isolates

Received January 28, 2014.
Accepted April 17, 2014.
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were staphylococcal protein A (*spa*) gene typed. The *spa* gene encodes protein A, a multifunctional cell wall protein that binds immunoglobulins, thereby inhibiting opsonophagocytosis. Protein A is ubiquitous in *Staph. aureus* and is often used in strain typing on the basis of variation in the DNA sequence encoding the X region (Foster et al., 2014).

Strains were collected as part of prevalence surveys of MRSA in dairy bulk tank milk in Great Britain (Paterson et al., 2012b; 2014b). A PCR for *mecA* and *mecC* typing was performed, as previously described (Paterson et al., 2012a). The MRSA isolates were *spa* typed as described by Shopsin et al. (1999).

To determine the genetic basis of biofilm formation, the intercellular adhesion genes *icaA* and *icaD* were amplified using PCR according to Zmantar et al. (2008) and sequenced to confirm the identity of the products. A quantitative determination of the biofilm production was performed in polystyrene microtiter plates (Stepanović et al., 2007). Overnight cultures (18 h) were diluted 1:100 in tryptic soy broth with 2% glucose, and 200 μL of this cell suspension was used to inoculate 96 flat-well microtiter plates. After 24 h of incubation at 37°C, the content of the treated plates was discarded. Wells were washed 3 times with PBS (pH 7.2) and air-dried. Cells were heat-fixed by exposing them to hot air at 60°C for 60 min. The adherent biofilm was stained with 0.1% safranin for 1 min and excess stain was removed by rinsing with tap water. After drying, the optical density of the biofilm was measured with an ELISA microplate reader at a wavelength of 492 nm. Each assay was performed 4 times on different days and using 8 wells in each plate for the culture of one strain. The optical density values for 32 wells were obtained for each isolate and used to determine the mean value. The ATCC 29213 human isolate (referred to as SA12H in this paper) was used in each assay as a weak biofilm producer, as described by Prenafeta et al. (2010); the SA13H strain was used as a positive control in each microtiter plate (strong biofilm producer according to Prenafeta et al., 2010). Wells with uninoculated medium were used as a negative control. Strains were classified as nonbiofilm producers when they did not show a significant difference to the optical density mean of uninoculated wells ($P > 0.05$), weak producers when the optical density mean was significantly higher than that of uninoculated wells ($P < 0.05$), and moderate- to strong-biofilm producers when the optical density mean was significantly higher than the SA12H strain ($P < 0.01$) and similar to the SA13H positive control strain.

Differences in biofilm production between isolates in the microtiter plate assay were evaluated by one-way ANOVA using SPSS 11.5 (IBM Corp., Armonk, NY).

The *spa* type and *mec* gene, as well as the biofilm production attributes for the 20 MRSA isolates analyzed in the current study, are summarized in Table 1. Nine *spa* types were identified with different prevalences: t6292 (25%), t011 (20%), t843 (15 %), t742 (10 %), t6300 (10%), t1328 (5%), t2346 (5%), t4184 (5%), and t015 (5%). Among the most prevalent *spa* types observed in our study, t6292 is associated with the *mecC* genotype, whereas t011 has been detected only in the *mecA* genotype. The *spa* type t011 was also identified previously in 91 (Vanderhaeghen et al., 2010) and 16% (Bardiau et al., 2013) of the *mecA* MRSA strains isolated from bovine mastitis in Belgium. All the MRSA strains were observed to be PCR-positive for both *icaA* and *icaD* genes. The culture of the 20 MRSA strains in microtiter plates revealed that 3 isolates were moderate- to strong-biofilm producers, 7 isolates were weak-biofilm producers, and 10 isolates did not show the ability to form biofilm.

Biofilm formation is an important property in *Staph. aureus* virulence, and we investigated biofilm production of MRSA from bulk tank milk. The *icaA* and *icaD* genes were detected in all MRSA isolates analyzed, whereas Dhanawade et al. (2010) indicated a prevalence of these genes in 35.29% of *Staph. aureus* from subclinical bovine mastitis in India. Nevertheless, our results agree with earlier studies that reported a high prevalence of the *ica* genes among *Staph. aureus* mastitis isolates (Cucarella et al., 2001; Vasudevan et al. 2003).

Biofilm production in microtiter plates was confirmed in 10 of the 20 MRSA isolates. Our study indicates that the ability to form biofilm is a virulence property present in 50% of the MRSA isolated from bovine milk in Great Britain, not as prevalent as Bardiau et al. (2013) observed in mastitis MRSA isolates from Belgium (100%). This discrepancy could be due to the criteria used by Bardiau et al. (2013) to determine biofilm formation ability in a microtiter assay, based on a cut-off defined as 3 standard deviations above the mean optical density of the uninoculated wells (according to Stepanović et al., 2007); whereas, in our study, we used a statistical analysis to compare the mean optical density differences between the MRSA strains and the uninoculated wells or a weak-biofilm-producing *Staph. aureus* strain. If we use the recommendations of Stepanović et al. (2007) for the quantitative classification of biofilm production, based on the optical density cut-off, the 20 MRSA isolates analyzed in our study are determined as biofilm producers. Even though all the analyzed MRSA isolates possess the *icaA* and *icaD* genes, only 50% of the strains are considered biofilm producers according to our specifications, which could possibly be due to point mutations in the *ica* locus or

Table 1. The *spa* type, *mec* gene, *icaA* and *icaD* presence, and biofilm producer phenotype among bovine methicillin-resistant *Staphylococcus aureus* (MRSA) isolates[1]

Isolate	*spa* type	*mec* gene	*icaA*/*icaD*	OD_{492} (SD)	Biofilm production
6H	t6292	*mecC*	+/+	0.344 (0.170)[b]	+
6T	t6292	*mecC*	+/+	0.316 (0.187)[b]	+
13P	t843	*mecC*	+/+	0.692 (0.535)[a,b]	++
13x	t742	*mecC*	+/+	0.862 (0.486)[a,b]	++
15AL	t6300	*mecC*	+/+	0.425 (0.503)[b]	+
18–31	t843	*mecC*	+/+	0.445 (0.436)[b]	+
40–25	t6300	*mecC*	+/+	0.361 (0.349)[b]	+
25–73	t1328	*mecA*	+/+	0.191 (0.143)	–
25–26	t011	*mecA*	+/+	0.129 (0.073)	–
22–79	t011	*mecA*	+/+	0.141 (0.055)	–
18–67	t843	*mecC*	+/+	0.125 (0.070)	–
30–59	t2346	*mecA*	+/+	0.146 (0.100)	–
31–7	t011	*mecA*	+/+	0.100 (0.037)	–
33–6	t6292	*mecC*	+/+	0.264 (0.261)[b]	+
34–121	t6292	*mecC*	+/+	0.235 (0.161)	–
34–122	t6292	*mecC*	+/+	0.258 (0.183)	–
37–55	t41844	*mecA*	+/+	0.391 (0.317)[b]	+
42–57	t011	*mecA*	+/+	0.649 (0.186)[a,b]	++
45–164	t015	*mecA*	+/+	0.116 (0.058)	–
27–28	t742	*mecC*	+/+	0.082 (0.037)	–
SA13H	ND[2]	ND	+/+	0.845 (0.458)[a,b]	++
SA12H	ND	ND	ND	0.244 (0.07)[b]	+

[a]Denotes significant difference compared with the weak biofilm producer SA12H strain ($P < 0.01$).

[b]Denotes significant difference ($P < 0.05$) compared with the uninoculated wells (mean optical density = 0.045; SD = 0.012).

[1]Biofilm formation is indicated by the mean optical density (OD_{492}) in the microtiter plates and the standard deviation of the mean. The isolates were classified as nonproducers (–), weak producers (+), and moderate to strong producers (++)

[2]ND = not determined.

other yet-unidentified factors that negatively regulate PNAG polysaccharide synthesis or influence biofilm formation, as have been reported by Cramton et al. (1999).

The present study is the first to characterize biofilm production of bovine milk MRSA isolates from Great Britain and the first to demonstrate biofilm production by *mecC* MRSA, which is an emerging human and animal pathogen (Paterson et al., 2014a). The emergence of MRSA in cattle could imply a reduction of effective antibiotic treatments. Moreover, a high prevalence of biofilm-producing MRSA isolates could promote the chronicity of bovine mastitis, with the consequence of persistent bacterial infection and increased shedding and spread from infected animals, including potential zoonotic transmission.

ACKNOWLEDGMENTS

We thank Mireia Muntada and Anna Corominas (Hipra Scientific, Amer, Spain) for their excellent technical assistance in the biofilm characterization analysis.

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