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General aspects of biofilm development and implication in staphylococcal ruminant mastitis: a current literature review

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Biofilm as a survival mechanism

A characteristic of bacteria is the capacity to grow in association with a surface forming complex communities. In the recent decades, these bacterial agglomerations have been described as "biofilms". Biofilm can be defined broadly as a dynamic and well structured microbial community, attached to a solid surface and aggregated by an extracellular matrix. The ability to form biofilm is a widespread feature among prokaryotes (both in the Archaeal and Bacterial domain) and it has been found in fossil formations dating back 3.2 billion years. From an evolutionary standpoint, the formation of biofilm probably conferred an adaptive advantage by providing homeostasis against extreme conditions and fluctuations of the primitive earth (temperature, osmotic pressure, pH and exposure to UV radiation). In addition to offering protection from physical and chemical environmental factors, biofilm protects bacteria from being washed or scraped away from aquatic systems, facilitates extracellular catalytic functions (because cells remain close to each other) and promotes the concentration of nutrients on the surface (Hall-Stoodley et al., 2004). Moreover, biofilms are resistant to antimicrobial agents (e.g., antibiotics), what may be due to difficulty in penetration of the antimicrobial agent through the extracellular matrix, to the decreased growth rate of biofilm cells (β-lactam antibiotics are effective in Gram-positive cells that are actively dividing) or the existence of resistant phenotypes among a genetically heterogeneous population.

Biofilm in natural environments and its implication in infections

Biofilm formation is ubiquitous in natural environments. These types of biological structures are found at the bottom of rivers or on the surface of stagnant water; in extreme environments, from hot springs to glaciers in the Antartic; in showers or baths, favoured by the warm moist environment; inside water ducts or industrial gas and oil pipes; in symbiosis with plants, etc. Moreover, biofilm formation is implicated in the pathogenesis of many human (Hall-Stoodley et al., 2004) and veterinary (Jacques et al., 2010) infections. According to the National Institutes of Health (NIH), biofilm is involved in 80% of bacterial infections (Joo and Otto, 2012). The adhesion of *Staphylococcus* or *Streptococcus* to the proteins of the basal membrane of the damaged heart epithelium is a cause of endocarditis. In the case of cystic fibrosis patients, decreased ciliary activity of the respiratory mucosa and mucus hyperviscosity promote colonization and biofilm formation by *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. Another well known example of biofilm is the subgingival plaque of *Streptococcus mutans*. Biofilm formation has also been described in uropathogenic strains of Escherichia coli. Finally, biofilm is an important virulence factor involved in the development of implant-related infections of intravenous catheters, heart valves, prostheses, peritoneal dialysis catheters, endotracheal tubes, etc., which are mainly caused by the adhesion of *S. aureus* and *Staphylococcus epidermidis* to the surface of these implants. The contribution of biofilm to pathogenesis is attributed to its resistance to antibiotics and phagocytosis, thereby facilitating chronic infections. On the other hand, detachment of biofilm bacteria cells is a cause of septicaemia and new colonisations, while the release of endotoxins and exotoxins produce inflammation and tissue damage.

Development of *S. aureus* biofilm

Development of a bacterial biofilm can be divided into three phases, which involve specific molecular factors: (a) attachment to a surface, (b) proliferation and formation of a mature biofilm structure and (c) detachment or dispersal. The molecular determinants involved in the biofilm formation of *S. aureus* have been investigated in great detail (Joo and Otto, 2012) and are summarized below (figure 1). First (attachment), bacterial cells adhere to abiotic surfaces (such as the plastic surface of a medical implant device) by hydrophobic or electrostatic interactions determined by the nature of the bacterial and inert surfaces. Nevertheless, specific molecules, such as teichoic acids of the cell wall can participate in this stage. On the other hand, attachment to a biotic surface, such as animal tissues, requires specific interactions mediated by staphylococcal surface-anchored proteins that bind to host matrix proteins (MSCRAMMs): fibronectin binding proteins (FnBPA, FnBPB), fibrinogen binding proteins (ClfA, ClfB), collagen binding protein (Cna), bone sialoprotein binding protein (BBP) or SasX (Foster et al., 2014).

In a second step (maturation), adhered bacterial cells multiply and produce an extracellular matrix. The function of the matrix is to provide adhesion between bacterial cells, enabling the accumulation of layers that constitute the biofilm. Adhesive components of the extracellular matrix include exopolysaccharides, proteins and extracellular DNA (released as a consequence of cell lysis). The main constituent of the *S. aureus* and *S. epidermidis* extracellular matrix, responsible for the intercellular interactions, is the exopolysaccharide poly-N-acetyl-β-1,6-glucosamine (PNAG), synthesized by enzymes encoded in the *icaADBC* operon (Cramton et al., 1999). De-acetylation of PNAG polysaccharide introduces positive charges necessary to interact with other negatively charged matrix components (e.g., DNA) or cell wall components (teichoic acids), resulting in a tightly connected matrix network. Likewise, some *S. aureus* surface proteins have been described that can be involved in intercellular interaction and biofilm formation: biofilm-associated protein (Bap) (Cucarella et al., 2001), protein A (Merino et al., 2009), *S. aureus* surface proteins C and G (SasC, SasG) (Schroeder et al., 2009) (Corrigan et al., 2007), fibronectin binding proteins (FnBPA, FnBPB) (O'Neill et al., 2008), extracellular matrix binding protein (Embp) (Christner et al., 2010) and accumulation-associated protein (Aap) (Conrady et al., 2008).

In addition to the intercellular interactions that link bacteria together, biofilm maturation comprises disruptive processes that form channels in the biofilm structure in which nutrients can circulate and reach cells in deeper layers. Enzymatic degradation by proteases and nucleases may have a role in this kind of biofilm structuring. Moreover, a family of peptides with surfactant properties, the phenol-soluble modulins (PSMs), is involved in disruption of noncovalent interactions between biofilm cells and matrix molecules.

Disruptive processes also ultimately cause the detachment of cell clusters from a biofilm, which controls biofilm expansion and has important consequences for *in vivo* biofilm infection, as it may lead to systemic dissemination.

Expression of all PSMs is under control by the Quorum-sensing system (QS), a regulatory mechanism mediated by the AgrA DNA binding protein that controls gene expression in a cell-density-dependent manner. In general, Agr up-regulates PSMs, toxins and other acute virulence factors and down-regulates surface proteins such as MSCRAMMs that are expressed only during the initial attachment phase. According to the experimental observations, a model was developed in which *agr* expression in mostly outer layers of a biofilm leads to detachment, while some level of expression in deeper layers is required for the efficient formation of channels (Periasamy et al., 2012).

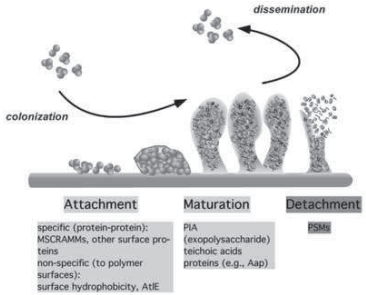

Figure 1. Phases of biofilm development in staphylococci (Edited from Otto, 2008).

Implication of biofilm in ruminant mastitis caused by *S. aureus*

In an experimental intramammary challenge in sheep, a biofilm producing *S. aureus* strain showed a higher colonization capacity than the non-biofilm producer variant of the same strain (Baselga et al., 1993). Moreover, Fox et al. (2005) observed that *S. aureus* associated with milk are more likely to produce biofilm as compared to extramammary sources. These studies suggest that biofilm production is a risk factor for mammary gland infection. From this point of view, a model for the implication of biofilm in bovine and ovine mastitis caused by *staphylococci* can be proposed, in which bacterial cells attach to the epithelial cells of the mammary gland and grow into colonies surrounded by an extracellular matrix, thereby forming the biofilm. Because of its size, biofilm is not capable of being phagocytised by polymorphonuclear neutrophils or macrophages and, moreover, it confers resistance to antibiotics, thereby promoting the chronicity of infection.

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Various studies demonstrate the presence of the *icaADBC* operon, which encodes the enzymes responsible for the biosynthesis of the PNAG exopolysaccharide, the main component of the extracellular matrix of the biofilm, in 94.36% (Cucarella et al., 2004) or 100% (Vasudevan et al., 2003) of *S. aureus* isolated from bovine mastitis. Apart from this genetic capacity, a number of studies have also demonstrated the ability of bovine mastitis isolates to form biofilm *in vitro*. In this regard, Vasudevan et al. (2003) found that 91% of isolates of *S. aureus* from bovine mastitis had the ability to form biofilm *in vitro* by determination of colonial morphology on agar plates with Congo red, whereas 69% showed adhesion in a microplate assay. In another study, Oliveira et al. (2007) characterized 80.8% of isolates of *S. aureus* and 75.9% of isolates of *S. epidermidis* in bovine mastitis as in vitro producers of biofilm. Dhanawade et al. (2010) found that 48.03% of the strains of *S. aureus* isolated from bovine mastitis had the ability to form biofilm *in vitro* by the culture test on agar plates with Congo red.

Recently, Bardiau et al. (2013) published that biofilm formation ability was present in all the methicillin-resistant *S. aureus* (MRSA) isolates analyzed from bovine mastitis in Belgium. Moreover, the biofilm formation capacity of MRSA, encoding *mecA* or *mecC*, isolated from bulk tank milk in Great Britain was characterized and we observed that all the strains analyzed were PCR positive for the ica genes and 50% produced biofilm in the microtiter plate assay. This is also the first demonstration of biofilm production by *mecC* MRSA (Prenafeta et al., 2014). 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.

While the genetic capacity and *in vitro* biofilm production in *S. aureus* isolates from bovine mastitis seems clear, there is some evidence demonstrating the *in vivo* biofilm production of *S. aureus* in the mammary gland. Watson et al. (1989) observed by electron microscopy the production of a polysaccharide extracellular matrix (called pseudocapsule by the authors) in *S. aureus* cells isolated directly from the milk of sheep and cows with clinical mastitis. Shortly afterwards, Baselga et al. (1993) demonstrated the production of an exopolysaccharide matrix in *S. aureus* cells by immunohistochemical analysis of mammary gland parenchymal tissue samples from sheep experimentally infected with *S. aureus* by intramammary route. The in vivo exopolysaccharide expression has also been shown indirectly by observing the production of specific antibodies against PNAG and against Slime Associated Antigenic Complex (SAAC) in sheep and cows, respectively, experimentally infected with *S. aureus* by intramammary route (Perez et al., 2009; Prenafeta et al., 2010).

Vaccines against the biofilm of *S. aureus* to combat mastitis in ruminants

Given that biofilm formation is an important virulence factor of *S. aureus* in the pathogenesis of mastitis in sheep and cows, the efficacy of different experimental vaccines has been tested, with various levels of protection demonstrated. Watson et al. (1993) and Nordhaug et al. (1994) used vaccines based on whole *S. aureus* inactivated cells embedded in their own extracellular matrix called pseudocapsule. The experimental vaccines in a study by Amorena et al. (1994) consisted of a mixture of slime (biofilm exopolysaccharide matrix) in liposomes, toxoid and various inactivated *S. aureus* isolates. More recently, knowing that the PNAG exopolysaccharide is the major component of the extracellular matrix of the *S. aureus* biofilm. Perez et al. (2009), conducted an efficacy trial against an intrammamary challenge with a virulent *S. aureus* strain in sheep, using bacterins (whole and inactivated bacterial cells), crude extract, or purified PNAG, with different adjuvants as a vaccines. The results of this study showed that bacterins from strong biofilm-producing bacteria induced the highest titres of specific antibodies to PNAG and conferred the greatest protection against an intramammary challenge, compared to vaccines containing bacterins from weak biofilm-producing bacteria, crude extract or purified PNAG. The study by Prenafeta et al. (2010) clarifies the role of SAAC-specific antibodies in protecting against the mastitis caused by *S. aureus* in an experimental infection in cows. SAAC is an isolated cell fraction from *S. aureus* strains that produce biofilm. The presence of this extracellular component has been determined for all isolates of *S. aureus* characterized as slime producers in agar plates with Congo red (Figure 2) and its production is directly related to the in vitro biofilm formation (Table 1). The chemical and immunological characterization showed that the SAAC is comprised of deacetylated forms of PNAG. It is noteworthy that antibodies to deacetylated forms of PNAG are those with the greatest capacity for opsonization (specific antibody binding to antigen) and protection against infection by *S. aureus* (Cerca et al., 2007).

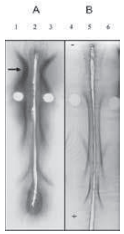

Figure 2. Analysis by immunoelectrophoresis in agarose gel for bacterial extracts from a strain of an *S. aureus* biofilm producer (A: wells 1 and 3) and non-producing strain (B: wells 4 and 6), using a polyclonal serum against whole bacteria (lines 2 and 5). The arrow shows the line of immunoprecipitation for the SAAC antigen, which is present only in strains characterized as exopolysaccharide producers in agar plates with Congo red.

Isolate *S. aureus*	OD in the biofilm test (SD[1])	Production of SAAC (SD[1])
SA1H	1.444 (0.04)	54.0 (0.012)
SA2H	1.597 (0.02)	63.3 (0.015)
SA3H	0.385 (0.03)	20.8 (0.011)
SA4H	1.499 (0.04)	60.5 (0.012)
SA5H	1.521 (0.03)	27.6 (0.015)
SA6H	0.088 (0.01)	2.2 (0.011)
SA7H	1.030 (0.02)	26.5 (0.012)
SA8H	0.388 (0.06)	Nd[2]
SA9H	0.200 (0.02)	Nd[2]
SA10H	0.145 (0.01)	0.1 (0.010)
SA11H	0.130 (0.01)	Nd[2]
SA12H	0.235 (0.01)	Nd[2]
SA13H	0.632 (0.02)	6.9 (0.013)

[1] SD: standard deviation of the mean.
[2] Nd.: Not detected

Table 1. Determination of the biofilm formation capacity in microplate (OD of the biofilm in the test) and production of SAAC (mg SAAC/mg total protein) in isolates of *S. aureus*. The correlation between the production of SAAC and the ability to form biofilm in microplate is significant (R = 0.882).

One of the advantages of using PNAG or the SAAC component as vaccine antigens, unlike capsular antigens, is that no serotypes have been reported among isolates of *S. aureus*. Therefore, the antibodies induced by vaccination with these antigens confer cross-protection regardless of the capsular type of *S. aureus*.

STARTVAC® (HIPRA) is the first vaccine against bovine mastitis registered throughout the European Union via the EMA (European Medicines Agency). This vaccine contains inactivated cells of a high biofilm-producing *S. aureus* strain with a high content of cell-associated SAAC. Clinical trials carried out with STARTVAC® showed that vaccination significantly reduced the incidence of mastitis caused by *S. aureus* and increased the spontaneous cure rate (Schukken et al., 2014). Moreover, this vaccine proved to be effective in reducing the incidence of sub-clinical and clinical mastitis due to coagulase-negative staphylococci in vaccinated versus control heifers (Noguera et al., 2011).

Perspectives of biofilm in mastitis

The ability to form biofilm is an important virulence factor of *S. aureus* involved in bovine and ovine mastitis. Although other virulence factors may be involved in the pathogenesis of mastitis, the PNAG or SAAC-specific antibodies may prevent the establishment of infection of *S. aureus* in the mammary gland by binding to the exopolysaccharide extracellular matrix (before the establishment of the biofilm), thereby facilitating polymorphonuclear neutrophil-mediated phagocytosis and elimination of infection.

References

Amorena, B., R. Baselga and I. Albizu, 1994. Use of liposome-immunopotentiated exopolysaccharide as a component of an ovine mastitis staphylococcal vaccine. Vaccine. 2:243-249.

Bardiau, M., K. Yamazaki, J.N. Duprez, B. Taminiau, J.G. Mainil and I. Ote, 2013. Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk of bovine mastitis. Lett. Appl. Microbiol. 57:181-186.

Baselga, R., I. Albizu, M. De La Cruz, E. Del Cacho, M. Barberan and B. Amorena, 1993. Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. Infect. Immun. 61:4857-4862.

Cerca, N., K.K. Jefferson, T. Maira-Litrán, D.B. Pier, C. Kelly-Quintos, D.A. Goldmann, J. Azeredo and G.B. Pier, 2007. Molecular basis for preferential protective efficacy of antibodies directed to the poorly acetylated form of staphylococcal poly-N-acetyl- -(1-6)-glucosamine. Infect. Immun. 75:3406-3413.

Christner, M., G.C. Franke, N.N. Schommer, et al. 2010. The giant extracellular matrix-binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin. Mol. Microbiol. 75:187-207.

Conrady, D.G., C.C. Brescia, K. Horii, A.A. Weiss, D.J. Hassett and A.B. Herr, 2008. A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. Proc. Natl. Acad. Sci. USA. 105:19456-19461.

Corrigan, R.M., D. Rigby, P Handley and T.J. Foster, 2007. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. Microbiology. 153:2435-2446.

Cramton, S.E., C. Gerke, N.F Schnell, W.W. Nichols and F. Götz, 1999. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. 67:5427-5433.

Cucarella, C., M.A. Tormo, C. Úbeda, M.P Trotonda, M. Monzón, C. Peris, B. Amorena, I. Lasa and J.R. Penadés, 2004. Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. Infect. Immun. 72:2177-2185.

Dhanawade, N.B., D.R. Kalorey, R. Srinivasan, S.B. Barbuddhe and N.V. Kurkure, 2010. Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. Vet. Res. Commun. 34:81-9.

Foster, T.J., J.A. Geoghegan, V.K. Ganesh and M. Höök. 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat. Rev. Microbiol. 12:49-62.
Fox, L. K., R. N. Zadoks, and C. T. Gaskins. 2005. Biofilm production by *Staphylococcus aureus* associated with intramammary infection. Vet. Microbiol. 107:295-299.

Hall-Stoodley, L., J.W. Costerton and P. Stoodley, 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. 2: 95-108.

Jacques, M., V. Aragon and Y.D.N. Tremblay, 2010. Biofilm formation in bacterial pathogens of veterinary importance. Anim Health Res Rev. 11:97-121.

Joo, H. and M. Otto. 2012. Molecular basis of in-vivo biofilm formation by bacterial pathogens. Chem Biol. 19: 1503-1513.

Merino, N., A. Toledo-Arana, M. Vergara-Irigaray, J. Valle, C. Solano, E. Calvo, A.J. López, T.J. Foster, J.R. Penadés and I. Lasa, 2009. Protein A-mediated multicellular behaviour in *Staphylococcus aureus*. J. Bacteriol. 191:832-843.

Noguera, M., R. March, R. Guix, A. Prenafeta and A. Foix, 2011. Evaluation of the efficacy of a new vaccine against bovine mastitis caused by CNS: field trial results. Pages 187-188 in Proc. NMC Annual Meeting, Arlington, VA. Natl. Mastitis Counc., Madison, WI.

Nordhaug, M.L., L.L. Nesse, N.L. Norcross and R. Gudding, 1994. A field trial with an experimental vaccine against *Staphylococcus aureus* mastitis in cattle. 1. Clinical parameters. J. Dairy Sci. 77:1267-1275.

Oliveira, M., S.F. Nunes, C. Carneiro, R. Bexiga, F. Bernardo and C.L. Vilela, 2007. Time course of biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. Vet. Microbiol. 124:187-191.

O'Neill, E., C. Pozzi, P Houston, D. Smyth, H. Humphreys, D.A. Robinson, A. Loughman, T. J. Foster and J.P O'Gara, 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J. Bacteriol. 190:3835-50.

Otto, M., 2008. Staphylococcal biofilms. Curr. Top. Microbiol. Immunol. 322:207-228.

Pérez, M.M., A. Prenafeta, J. Valle, J. Penadés, C. Rota, C. Solano, J. Marco, M.J. Grilló, I. Lasa, J.M. Irache, T. Maira-Litran, J. Jiménez-Barbero, L. Costa, G.B. Pier, D. de Andrés and B. Amorena, 2009. Protection from *Staphylococcus aureus* mastitis associated with poly-*N*-acetyl -1,6 glucosamine specific antibody production using biofilm-embedded bacteria. Vaccine. 27, 2379-2386.

Periasamy, S., H.J. Joo, A.C. Duong, T.L. Bach, V.Y. Tan, S.S. Chatterjee, G.Y.C. Cheung and M. Otto. 2012. How *Staphylococcus aureus* biofilms develop their characteristic structure. PNAS 109:1281-1286.

Prenafeta, A., R. March, A. Foix, I. Casals and L. Costa, 2010. Study of the humoral immunological response after vaccination with a *Staphylococcus aureus* biofilm-embedded bacterin in dairy cows: possible role of the exopolysaccharide specific antibody production in the protection from *Staphylococcus aureus* induced mastitis. Vet. Immun. Immunopathol. 134:208-217.

Prenafeta, A., M. Sitjà, M.A. Holmes and G.K. Paterson, 2014. Biofilm production characterization in methicillin-resistant *Staphylococcus aureus*. J. Dairy Sci. (in press).

Schroeder, K., M. Jularic, S.M. Horsburgh, N. Hirschhausen, C. Neumann, A. Bertling, A. Schulte, S. Foster, B.E. Kehrel, G. Peters and C. Heilmann, 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. PLoS ONE. 4(10):7567.

Y.H. Schukken, Y.H.,V. Bronzo, C. Locatelli, C. Pollera, N. Rota, A. Casula, F. Testa, L. Scaccabarozzi, R. March, D. Zalduendo, R. Guix and P Moroni, 2014. Efficacy of vaccination on *Staphylococcus aureus* and Coagulase Negative Staphylococci intramammary infection dynamics in two dairy herds. J. Dairy Sci. (in press).

Vasudevan, P, M.K.M. Nair, T. Annamalai and K.S. Venkitanarayanan, 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet. Microbiol. 92:179-185.

Watson, D.L. and Watson, N.A., 1989. Expression of a pseudocapsule by *Staphylococcus aureus*: influence of cultural conditions and relevance to mastitis. Research in Veterinary Science. 47:152-157.

Watson, D.L. and H.I. Davies, 1993. Influence of adjuvants on the immune response of sheep to a novel *Staphylococcus aureus* vaccine. Vet. Microbiol. 34:139-153.