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TESI DI DOTTORATO DI RICERCA

BIOFILM PRODUCTION UNDER STATIC AND DYNAMIC CONDITIONS BY GENETICALLY CHARACTERIZED *S. aureus* CAUSING DAIRY COW MASTITIS.

VET05

GUSTAVO GERALDO MEDINA SNEL
R09090

TUTOR: PROF.SSA. RENATA PICCININI

COORDINATORE DEL DOTTORATO: PROF. GIUSEPPE SIRONI

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1 Introduction

1.1 Bovine mastitis

Mastitis is an inflammatory process of the mammary gland. It is a physiological response to tissue injuries that can be caused by physical, chemical or, in most cases, infectious agents. Bovine mastitis is usually a result of bacterial infection: several species of both Gram positive and Gram negative bacteria are recognized as causative agents (Bramley et al., 1996).

Bovine mastitis can be either clinical, when secretion abnormalities and signs of inflammation are clearly seen, or subclinical, with no evident clinical signs. In this case, additional laboratory examination of the milk need to be carried out. Typically, the diagnosis of subclinical mastitis is performed by bacteriologic examination and by the detection of inflammatory changes in milk, most represented by somatic cell counting (SCC). Cytologic and bacteriological methods are usually combined, since low levels of bacterial growth could occur even in samples from healthy glands. Subclinical mastitis is 15 to 40 times more frequent than the clinical form and it is also economically more important. Economical loses are associated mainly with decreased milk production and quality, corresponding to roughly 75% of all costs related to subclinical mastitis (Philpot and Nickerson, 1991).

Staphylococcus aureus (*S. aureus*) is the most important causative agent of contagious mastitis, being widely distributed among herds all over the world (International Dairy Federation, 2006). It is a contagious agent, meaning that it is spread from animal to animal during milking-related procedures (Hermans et al. 2010).

1.2 *Staphylococcus aureus*

Staphylococcus aureus subsp. *aureus* (*S. aureus*) is a nonsporulating, non-motile, Gram-positive coccus, about 0.5-1.0 µm in diameter, that appears single, in pairs, short chains or clusters under microscopic examination. *S. aureus* cells divides in several planes to form irregular, and somewhat characteristic, grape-like clusters. Like other Staphylococci, members of this subspecies are

catalase-positive, mesophilic, facultative anaerobes, displaying a fermentative metabolism of carbohydrates.

A microaerophilic variant of *S. aureus*, the catalase negative *S. aureus* subsp. *anaerobius*, is also pathogenic, sharing some virulence traits with the more common subspecies. *S. aureus* subsp. *anaerobius* is genetically homogeneous and causes an specific abscess disease in sheep (De la Fuente, Suarez, Schleifer, 1985, De la Fuente et al., 2011). Most likely, *S. aureus* subsp. *anaerobius* is not relevantly implicated in any other *S. aureus* infections in human and animal species.

Typically, *S. aureus* colonies on blood agar are hemolytic, showing complete, incomplete or both types of hemolysis. At least four different hemolysins (α, β, δ and γ) are described in *S. aureus* (Götz, Bannerman, Schleifer, 2006), in spite of that, strains not expressing hemolytic activity are not so infrequent. Colonies grown under aerobic conditions are usually yellow-golden, given the production of the carotenoid, cell-wall associated pigment 4,4'-diaponeurosporene, also known as staphyloxanthin (Wieland et al., 1994). Some biochemical reactions are commonly used to differentiate *S. aureus* from other staphylococcal species: mannitol-fermentation, thermonuclease activity of a TNase encoded by the *nuc* gene and plasma coagulation by the coagulase enzyme encoded by the *coa* gene (Götz, Bannerman, Schleifer, 2006).

The genome of *S. aureus* has 33% GC content and is 2.8 Mb long, consisting of a conserved core and highly variable regions. These variable regions are constituted of genetic elements such as genome islands, integrated prophages, insertion sequences (IS), composite transposons, and integrated plasmids. These elements correspond to less than 10% of the genome, but contribute significatively to strain virulence (Gill et al., 2005). Transposons, bacteriophages and plasmids are genetic mobile elements, thus capable to promote horizontal transfer of virulence genes (Malachowa and DeLeo, 2010).

Besides its known role in bovine mastitis, *S. aureus* is the agent of a vast array of infections in several mammal species and birds. It is recognized as one of the most important infectious agents in human medicine, given the growing spread of multi-resistant strains in both hospitals and

community. Antimicrobial multi-resistance is not of great concern in bovine mastitis, since multiresistant strains are sporadically isolated and generally do not belong to the most virulent human biotypes (Botrel et al., 2010).

S. aureus virulence factors can be separated in extracellular products and cell-wall associated components. Extracellular products are mainly enzymes or highly-immunogenic proteins. Some examples of important extracellular virulence factors are: hemolysins, leucocidins (PVL, LukM), coagulase, enzymes that cleave antimicrobials (β-Lactamases), proteases (SspA, SspB and Aur) and superantigens (TSST-1 and SEA). Enterotoxins are a large family of staphylococcal superantigens responsible of a representative percentage of human cases of food poisoning. These molecules stimulate the immune system eliciting a massive specific, intestinal T-cell response, resulting in diarrhea. Enterotoxin-forming ability highlights the great importance of *S. aureus* in food microbiology and animal production, since food products of animal origin are frequently involved in staphylococcal food poisoning (Madigan et al., 2009).

Cell wall associated components playing a role as virulence factors are mostly surface proteins. Out of them, Penicillin-binding protein (PBP), protein A and Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) are probably the most studied. PBP prevents β-lactams action on the wall, thus conferring antibiotic resistance. Protein A inhibits opsonophagocytosis, binding the constant domain of IgG antibodies (Smith et al, 2011). MSCRAMMs are a group of adhesins targeting surface components of the host cells, promoting adhesion (Foster and Hook, 1998). The capsular polysaccharide (CP) is also a crucial surface-associated virulence factor. *S. aureus* strains show different capsular polysaccharide types, which classically are defined by agglutination with specific sera. Currently, 11 serotypes of capsular polysaccharides are partially or completely chemically characterized. Neverthless, some strains express nontypeable capsular types. Capsular polysaccharides can differ in some biological properties but, in general terms, help colonization and persistence by surface adhesion mechanisms and the impairment of phagocytosis (O'Riordan and Lee, 2004).

During the course of infection, *S. aureus* can be intermittently shed in milk, making its bacteriologic detection difficult. Also, depending on the strain, SCC do not always show high values (Pilla et al, 2012). Therefore, the detection of infected animals is not so easily accomplished, especially if the analysis is performed only on a single milk sample over time (Schroder and Hamann, 2005). The delay or failure in detecting infections is troublesome during control programs, because they are based on segregation, treatment and culling of *S. aureus* infected animals (Piccinini, Borromeo, Zecconi, 2010).

1.2 Bacterial biofilms

Biofilm was defined as "*a structured community of cells enclosed in a self-produced, hydrated, polymeric matrix adherent to an inert or living surface*" (Costerton, Stewart, Greenberg, 1999). Observations of environmental microorganisms showed that biofilm formation is considered more a rule than an exception in bacterial way of life. In the past 30 years, a crescent number of reports of biofilm related infections demonstrated that the same is probably true for pathogenic bacteria. Costerton Stewart and Greenberg (1999) estimated that 65-80% of all human bacterial infections are related to biofilm formation in some extent.

Biofilm formation occurs in a two step process: an attachment phase and an accumulation phase. The initial attachment is a reversible phase, with bacterial cells, usually single plaktonic cells, adhering to the surface to be colonized. Then an irreversible point is reached and the first step ends. What follows is the accumulation of extracellular polymeric substances (EPS), or matrix, as well as bacterial multiplication. The resulting multi-layered, matrix embedded structure, presents with a complex architecture. De-attachment and fluid carriage of single cells or clumps from this structure allow the colonization of substrates far from the original biofilm. Bacterial cells within the layers display significant differences in metabolic rates. Such characteristic, together with the physical protection conferred by the matrix, make biofilms intrinsically resistant to antimicrobial therapy and the immune response (Costerton, 2007, Hoiby et al., 2010).

Device (foreign-body)-related infections are well-known examples of biofilm infections. *S. aureus* and *S. epidermidis* are among the most prevalent etiological agents. These organisms originate from skin and nares of the patient, where they are present as commensals, or from other sources like healthcare workers and water (Donlan, 2011). Despite their evident epidemiological importance, these infections are not representative of the whole range of biofilm infections. When biofilms start developing directly on the affected tissue, infections are far more complex, with still several knowledge gaps needing to be filled.

1.2.1 Biofilm-related infections history and the Parsek-Singh criteria

The early years of the studies of biofilm-related infections have been hampered by problems with terminology and the unavailability of sensitive methods for matrix chemical characterization. The term "slime" was used denoting capsular polysaccharide, capsule, matrix or even biofilm itself. The use of this term was intended to imply the non-rigid nature of the substance surrounding the bacteria (Flemming and Wingender, 2010). Neverthless, "biofilm" and "slime" are not interchangeable terms (Deighton, Borland, Capstick, 1996) and the use of more specific terms should be preferred when referring to biopolymers.

The lack of standard guidelines was an additional difficulty in the diagnosis of biofilm-related infections. The Parsek-Singh criteria (Parsek, Singh, 2003), a currently accepted principle, establishes four basic postulates aimed to help the identification of such infections. The original concept was recently expanded by Hall-Stoodley and Stoodley (2009). According to this updated Parsek-Singh criteria, biofilm mediated infections are:

(1) associated to tissue surfaces,

(2) present with microbial aggregates at the site of infection usually with evident, abundant EPS,

(3) confined to a particular host site;

(4) resistant to antibiotic treatment despite the demonstrated susceptibility of planktonic bacteria *in vitro*;

(5) frequently present with culture-negative results, in spite of clinically documented suspicion of infection;

(6) characterized by evidence of ineffective immune clearance, as indicated by the observation of microbial aggregates together with host inflammatory cells.

These criteria are relatively fulfilled in several human infections caused by *S. aureus*, like chronic wounds, urinary tract and cystic fibrosis associated infections (Hall-Stoodley and Stoodley, 2009).

1.3 *S. aureus* biofilm and bovine mastitis

S. aureus subclinical mastitis is often associated with chonicity/persistence and therapeutic failure when compared to other agents (Ikawati, 2010). Currently, two theories, based on the characteristics of the agent, try to explain this phenomenon. The first theory is based on *S. aureus* facultative intracellular location, the other one on biofilm formation inside the mammary gland. *S. aureus* ability to invade epithelial mammary cells and phagocytes was demonstrated by Almeida et al. (1996), Bayles et al. (1998) and Hébert et al. (2000). Intracellular survival is strain-dependent and some lineages are more adapted to mammary niche. Bovine strains harboring allele I of the accessory gene regulator (*agrI*) invaded cultivated cells more effectively when compared with *agrII* strains (Buzzola et al., 2007). However, both *agrI* and *agrII* strains are known to cause bovine mastitis (Schlotter et al., 2012) but no correlation between *agr* type and chronicity is found in the literature. Cellular invasion is poorly documented *in vivo* (Dego, van Dijk, Nederbragt, 2002) and it seems logic that intracellular maintenance and subsequent spreading should occur, in order to reactivate the infection. Nevertheless, there is still a lack of clear evidences of these processes taking place *in vivo*.

The hypothesis of biofilm-formation as a cause of bovine mastitis is widely accepted. The low therapy success rate, despite the demonstration of *in vitro* antimicrobial susceptibility of mastitis isolates, is the most important epidemiological evidence of biofilm implication (Melchior, Vaarkamp, Fink-Gremmels, 2006). Studies where *S. aureus* biofilms were developed *in vitro*,

showed that biofilm forms are, in general, more resistant when compared to their planktonic counterparts (Amorena, et al., 1999, Melchior, Fink-Gremels, Gaastra, 2007). Also, strains isolated from milk more likely formed biofilms when compared to isolates from extramammary sources (Fox, Zadoks, Gaskins, 2005). Nevertheless, both intracellular survival and biofilm formation are strongly strain-dependent and are apparently not necessarily each other related (Oliveira et al., 2011).

Other aspects of bovine mastitis caused by *S. aureus* do not fit so well in Singh-Parsek criteria. Data by both *in vitro* and *in vivo* observations do indicate that staphylococcal adhesion to epithelial cell surface is a prerequisite for the establishment of infection (Almeida et al., 1996, Hensen et al., 2000). However, bacterial biomass in affected mammary glands is barely detectable. Previous studies evidenced small numbers of bacteria in tissue in comparison with those found in milk (Hensen et al, 2000, Piccinini et al., 2012). Surely these observations can be affected by the inadequacy of slide preparation techniques, given the complex structure of the gland and the presence of milk, which is troublesome for most histopathologic techniques (Gudding, McDonald, Cheville, 1984). Hensen et al. (2000) demonstrated the presence of bacterial aggregates near the epithelial inner-surface of experimentally infected glands. The relative small size of these clumps ("*more than 30 cells*") and the lack of evident extracellular matrix around them, make it difficult to differentiate staphylococcal multiplication from biofilm formation. In the same study, the authors also observed a variable presence of inflammatory cells in the early stages of infection, with few *S. aureus* cells associated with the epithelium and the adjacent interstitial tissue. In the chronic stage, clusters of bacteria were clearly associated with inflammatory cells, but intracellular location and deeper infiltrations occurred as well and in larger extent when compared to early stages (Hensen et al., 2000).

1.3.1 *S. aureus* biofilm matrix components

Important components in *S. aureus* biofilm formation are: cell-wall associated adhesins (most importantly MSCRAMMs), teichoic acid, extracellular DNA (ecDNA) and the exopolysaccharide poly-N-acetylglucosamine (PNAG) (Otto, 2008). MSCRAMMs are very important in the initial attachment of bacteria for biofilm formation. Natural variability and functional redundancy of these proteins occur among strains (O'Riordan and Lee, 2004), while PNAG, teichoic acid and ecDNA are more likely to be constitutively expressed. Rarely, MSCRAMMs can promote biofilm accumulation even in the absence of PNAG-mediated mechanisms (O'Neill et al, 2008).

Staphylococcal teichoic acids are highly negatively charged polymers linked to peptidoglycan (cell wall teichoic acids) or the cytoplasmic membrane (lipoteichoic acids) (Götz, Bannerman, Schleifer, 2006). Bound teichoic acids are, in great part, responsible for the initial adhesion step through physicochemical interaction with surfaces (Gross et al., 2001). As showed in a strong biofilm producer *S. aureus* strains, fractions of teichoic acids can also be released from the cell wall. These extracellular teichoic acids form non-covalent complexes with other components of the extracellular matrix, thus participating in biofilm accumulation (Vinogradov et al., 2006).

The enzyme N-acetyl-glucosaminyl-transferase, is encoded by the *icaADBC* operon, and is responsible for the synthesis of PNAG. PNAG is more relevant in the accumulation phase and is by far the most studied matrix component of staphylococcal biofilms. Initially described in *S. epidermidis* biofilms, PNAG usually corresponds to 50% or more of the biofilm matrix in this species. To the contrary, in *S. aureus* biofilms, PNAG is not so abundant, changing the relative importance of other matrix constituents. The diversity in matrix composition is largely strain-dependent, evidences indicate that ecDNA is probably the main accumulation component in this species (Izano et al, 2008).

The characterization of biofilm-associated protein (Bap) in *S. aureus* allowed the discovery of a new group of proteins related to biofilm formation in several infectious bacteria, including Gram negative species (Lasa and Penades, 2006). The first description of Bap was made by Cucarella et

al. (2001) in a small proportion of bovine subclinical mastitis *S. aureus* isolates in Spain. Thereafter, no Bap-positive *S. aureus* was found, either of human origin or of animal species. (Vautor et al., 2008). On the other hand, Bap proved to be highly prevalent in coagulase negative staphylococcal species. Bap is a large cell-wall-anchored protein that mediates both initial surface adhesion and biofilm accumulation. In fact, it was demonstrated that Bap-positive mutants, lacking the *ica* locus, are still able to form biofilm *in vitro* (Cucarella et al., 2001).

In the bovine host, *S. aureus* Bap-positive strains were more able to colonize and persist in the mammary gland (Cucarella et al., 2004) even though, Bap expression seems to be strongly down-regulated or blocked in Calcium rich substrates as milk (Arrizubieta et al., 2004). Despite the host-specificity of Bap-positive *S. aureus* strains, the gene *bap* is located in a mobile pathogenicity island (SaPIbov2) and thus capable of horizontal transfer. This transfer would be possible only among *S. aureus* strains, since in other staphylococcal species, *bap* gene is not carried in mobile genetic elements (Tormo et al, 2005).

1.4 Methods to characterize biofilm formation by *S. aureus*

Characterizing the biofilm-forming ability of *S. aureus* isolates is essential to improve the knowledge on the mechanisms influencing this trait. Growth conditions can substantially change the expression of *S. aureus* biofilms. The ability to adhere to different surfaces is variable among strains and also the response to different media components (Rode et al, 2007). Molecular and phenotypic methods are available to characterize the biofilm-forming ability and the best results are reached by the combination of both methods (Vasudevan et al, 2003).

1.4.1 Genotypic characterization

The PCR detection of the *ica* operon in *S. epidermidis* from device-related infections was initially conceived to help the identification of clinically-relevant isolates. This approach proved to be useful in the diagnosis of CNS infections (Arciola, Baldassari, Montanaro, 2001) and a good correlation

was expected also in infections caused by *S. aureus*. To the contrary, in this bacterial species the presence of *ica* was not sufficient to determine biofilm formation, since *ica* is detected in the majority of *S. aureus* isolates (Rohde et al., 2001). Moreover, other genes of biofilm-associated components (e.g.: MSCRAMMs, capsular polysaccharide) act in similar way. The presence of genetic markers seems an inefficient way to characterize biofilm formation, since genetic profile only corresponds to the biofilm-forming potential. Therefore, gene expression seems to be the key factor determining biofilm formation. Gene expression of biofilm components is controlled by complex regulatory systems. Environmental triggers, nutrient availability, temperature, osmolarity and cell density activate or block systems that coordinate genes encoding biofilm components and enzymes catalyzing the process. Examples of regulatory systems implicated in *S. aureus* biofilm formation are *sigB*, the quorum-sensing system encoded by the accessory gene regulator (*agr*) and *sarA* (Rachid et al., 2000, Vuong et al., 2000, Beenken, Blevins, Smeltzer, 2009). Regulation occurs in other points beyond the establishment of biofilm. Biofilm maturation and the de-attachment of cells is controlled by the late expression of proteases, modulation of endonuclease activity and autolysis mechanisms (Lauderdale et al, 2009).

1.4.2 Phenotypic characterization

The first implemented and diffused method for biofilm characterization was the tube method also known as Christensen's method. This semi-quantitative test is based on the staining of biofilm adherent to the walls of a glass tube after overnight incubation in liquid media without shaking (Christensen et al., 1982).

The Congo Red Agar (CRA) is also a qualitative characterization method, proposed as an alternative to the tube test. The medium was developed to detect slime-forming strains of *S. epidermidis*, that appear as black-rough colonies in contrast to red-smooth colonies of slime-negative strains. Despite the problems regarding the definition of "slime", as mentioned before, CRA has obvious advantages, since it is easy to perform and more reproducible, when compared to

the tube test (Freeman, Falkiner, Keane, 1989). Presumably, only clinically significant isolates from catheter infections would form slime on CRA (Arciola et al., 2002). Since *S. aureus* is also an important catheter-related infection agent, the method was soon adapted to screen slime-production in this species as well. Only a small correlation (3.8%) between CRA and a quantitave methods was observed by Knobloch et al. (2002), indicating that the test is not adequate for biofilm characterization of *S. aureus* strains from human infections. Characterization of bovine mastitis strains using CRA showed variable results as well as concordance with other methods described in the available literature (Vasudevan et al., 2003, Oliveira et al., 2006, Delgado et al., 2011).

The microtiter plate test, also developed by Christensen et al. (1985), is based on the same principle of the tube test, but plastic (polystyrene) multi-well plates are used for biofilm development and the stained biomass is measured by spectrophotometry, rendering it quantitative. The test was originally designed to characterize *S. epidermidis* biofilms, but was later adapted to several other bacterial and yeast species. Variations of this method are currently the most used tests to measure biofilm-formation. The advantages of this type of characterization method is its low cost, the possibility to screen several strains and to set different conditions in the same microtiter plate (Coenye and Nelis, 2010). Following the recommendations of Kennedy and O'Gara (2004), only hydrophilic, negatively charged polystyrene should be used to test *S. aureus* strains, given the physical-chemical properties of its cell wall. These characteristics are commonly found in tissue culture treated plates and for that reason some authors refer to this test as tissue culture plate method or TCP (Vasudevan et al., 2003, Dhanawade et al., 2009).

Currently, more refined biofilm characterization methods are based on dynamic systems. The general principle of dynamic systems is to allow biofilm formation inside chambers fed with medium flow (flow chambers or flow cells). The constant medium flow circunvents the interference of accumulated signaling molecules and metabolic residues, which occurs in static/closed systems, like the microtiter plate test (Coenye and Nelis, 2010). The flow itself is an important factor for biofilm formation: fluid shear forces induce rolling migration of bacterial aggregates and trigger

stronger adhesion to the surfaces (Rupp, Fux, Stoodley, 2005). These methods represent a more realistic approach, emulating some basic aspects of the environments where biofilms occur. Disadvantages are that these systems are expensive, time consuming and do not allow a large number of samples to be characterized simultaneously.

2 Aims of the study

Biofilm formation has been pointed out as an important factor in the establishment of *S. aureus* bovine mastitis, particularly in its chronic form. Most authors used static systems and matrices other than milk to characterize biofilm formation by strains isolated from bovine mastitis. Since *in vitro* biofilm formation can be highly affected by medium composition and experimental conditions, characterization results obtained in static systems using broth are probably not adequate to access the potential of this bacterial trait in mastitis. The objectives of the study were: (1) to investigate the occurrence of *ica* and/or *bap*-positive *S. aureus* strains in Italian herds; (2) to characterize biofilm of bovine mastitis isolates using a standard static method and to compare the results with a flow based assay (3) to look for genetic markers to identify strains capable of biofilm formation in a dynamic system.

3 Materials and methods

3.1 Strains

Bacterial isolates have been collected from quarter milk samples of dairy cow mastitis in 85 different Italian herds, which were participating in a control program for contagious pathogens. Quarter milk (10 μL) was plated on blood agar plate (5% bovine blood; Oxoid, Cambridge, UK) and incubated overnight at 37 °C. Milk somatic cells (SCC) of all samples were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska MN, USA) and recorded. Colonies of growth were isolated and identified as *S. aureus* by cultural and biochemical tests (Hogan et al., 1999). A real time PCR, targeting constitutive genes *nuc* and *Sa442* (Pilla et al., 2012), was carried out to confirm the initial identification. Strains were maintained at −80 °C in Microbank Bacterial Preservation System (Thermo Fisher Scientific Inc., Waltham MA, USA). Overall 112 S. aureus strains were tested in the present study. One strain per herd was selected, except when phenotypically different *S. aureus* isolates were found in the same herd. In these cases, additional strains representing each phenotype were also selected.

3.2 DNA extraction for PCR

S. aureus strains were thawed, plated onto blood agar plates (5% bovine blood; Oxoid), incubated for 24h at 37° C and checked for purity. Three colonies were suspended in 500 μL of PCR-grade water (GIBCO, Grand Island, NY, USA) and homogenized by vortexing for ten seconds. Bacterial genomic DNA was extracted using a commercial kit (QIAamp DNA mini kit QIAGEN, USA) following the manufacturer’s instructions with the addition of lysostaphin (5mg/mL; Sigma-Aldrich, MO, USA) to enhance bacterial lysis. DNA amount and purity were checked with a ND-100 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Extracted DNA was used in the following reactions: real time PCR for the detection of *nuc* and *Sa422*; conventional PCR for the detection of *bap*, *icaA* and *icaD*.

3.3 Experiment 1 – *bap* detection in Italian dairy herds

3.3.1 *icaA and icaD* PCR

The protocols to amplify *icaA* and *icaD* were those described by Arciola , Baldassari, Montanaro, (2001) with minor modifications. The reactions were performed in 25 μL volumes containing 1 μM of each primer, 10mM buffer, 2.5 mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphates, 1.5 U of Taq DNA polymerase (Promega, USA) and approximately 50 ng of DNA template. The same thermal cycle was used for both *icaA* and *icaD* amplifications with the following steps: initial denaturation at 94°C for 2 min, followed by 50 cycles at 94° C for 15 s, 49° C for 20 s and 72° C for 15 s, with a final extension step at 72° C for 2 min. PCR products were analysed by electrophoresis on 1.5% agarose gel containing 0.5 μg/mL ethidium bromide in TAE buffer (Sigma, USA). Amplification bands were visualized on an UV transilluminator. Expected product sizes were 188 bp and 198 bp, respectively.

3.3.2 *bap* PCR

PCR to detect *bap* was performed using primers and conditions described by Cucarella et al. (2004) with minor modifications. Briefly, each 25 μL amplification reaction mixture contained 5 μM of each primer (Fw sasp-6m: 5′ CCCTATATCGAAGGTGTAGAATTGCAC 3′, Rw sasp-7c: 5′ GCTGTTGAAGTTAATACTGTACCTGC 3′), 200 mM deoxynucleoside triphosphates (dNTPs), Green GoTaq Flexi Buffer (Promega, WI, USA), 2 mM $MgCl_2$, 1 U of GoTaq DNA polymerase (Promega, WI, USA) and approximately 50 ng of DNA template. The thermal protocol consisted of an initial step at 94° C for 2 min, followed by 40 cycles including 94° C for 20 s, 50° C for 20 s and 72° C for 50 s, with a final extension at 72° C for 5 min. Expected amplicon size was 971 bp. PCR products were analyzed by electrophoresis on 0.8% agarose gel containing ethidium bromide (0.5 μg/mL) in TAE buffer. As positive or negative controls, one *S. aureus bap*-positive reference strain (strain V329, kindly provided by Dr. José Penadés) and one *S. aureus bap*-negative strain (ATCC 23213) were used. PCR products, compatible with the expected *bap* amplicon, were excised from

the gel, purified with a commercial kit (Wizard SV Gel and PCR Clean-Up System, Promega, USA) and submitted to sequencing in an external facility laboratory.

3.3.3 Second sampling in one herd with *bap*-positive *S. aureus* isolates

Further bacteriological analyses of milk were performed, six months apart from the original screening, in one herd with a *bap*-positive *S. aureus* isolate. Quarter milk samples were collected from all lactating cows (n = 182) check the frequency of *bap*-positive strains.

3.4 Experiment 2 – Biofilm characterization of *S. aureus* mastitis strains

3.4.1 Congo Red agar (CRA)

Twenty six *S. aureus* isolates were randomly selected to be tested by Congo Red Agar (CRA) method. A *Staphylococcus pseudintermedius* from bovine mastitis was also tested. Such isolate had been genetically characterized by *rpoB* gene sequencing according to Drancourt and Raoult (2002), Three reference strains were also tested: *S. aureus* ATCC23213, *S. aureus* ATCC25923, and *S. epidermidis* ATCC12128. All strains were inoculated in BHI broth (Oxoid, UK) and incubated at 37° C overnight. Bacterial cultures were then vortexed, diluted 1:250 in sterile physiological saline and plated onto Congo Red Agar plates. Colonies were checked for colour and surface texture after 24 h incubation at 37° C and after additional 48 h at room temperature (Arciola et al., 2002). Black/rough colonies were considered as slime producing, while red/smooth colonies were considered as slime negative. Combinations of black/smooth, brown/smooth, bordeaux/smooth, red/rough or black/rough colonies with red centres were classified as undetermined. Two different CRA batches were used to test the reproducibility of medium preparation. The test was repeated three times on different days.

3.4.2 Tissue culture plate assay (TCP)

Overnight TSB cultures were vortexed, diluted 1:40 in Tryptic Soy Broth (Oxoid, UK) supplemented with 0.75% Glucose (TSBglu1%) and inoculated (200 µL per well), in triplicate, in a 96 well flat bottom polystyrene tissue culture plate (Life Technologies, USA). After 18-hour incubation at 37° C in a humid chamber, well content was removed by carefully inverting and tapping the plate. Wells were washed with 250 µL of PBS and then with 250 µL of distilled water. The plate was inverted and air dried at room temperature. The wells were stained with 0.1% Safranin for 5 min, the dye was poured off and the excess was removed by washing twice with distilled water, then the plate was inverted onto clean absorbent paper and air dried. Finally, 200 µL of acid alcohol (ethanol absolute/12mM hydrochloric acid) was added to each well and the optical density was measured at 492 nm (OD_{492nm}) on a spectrophotometer (Spectramax, Molecular Devices, USA). Three uninoculated wells containing TSBglu1% served as blank. To each plate, a positive (*S. aureus* ATCC 25923) and a negative control (*ica*-negative strain *S. aureus* MB543) were also added in triplicate. All strains were tested three times on different days. The cut-off value to discriminate biofilm-forming from non biofilm-forming strains was defined using the classical method described by Christensen et al. (1985) and by Cluster analysis.

3.4.3 Flow cell assay

A qualitative flow cell assay, based on the gravimetric system described by Bos et al. (1994), was used to characterize the biofilm formation, mimicking basic intramammary environment conditions. A simplified scheme of our "once through" flow system is depicted in Fig. 1. To ensure that the flow system would allow biofilm formation without interfering with the process, the flow system was initially tested using TBSglu1% as culture medium. 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC, Sigma, D) was added to a final concentrarion of 0.05% (w/v), to facilitate biofilm visualization. TTC was used as a cellular marker, since its reduced form, formazan, accumulates only inside metabolic active cells. Such procedure avoids the manipulation of the delicate, recently

formed biofilms for staining purposes. As controls, four TCP tested strains, were selected: two negatives, one positive and one strong positive. Thereafter, UHT milk was used as culture media. All strains tested by the dynamic system were previously checked for their ability to reduce colourless TTC to red formazan in both TSB and milk. Finally, 30 strains out of 112 tested by TCP method, were chosen to be characterized by a flow system using UHT milk with TTC as substrate (MFC method). Each strain was characterized by a different level of biofilm formation, accordingly to OD_{492nm} values. Both TCP controls were also tested by MFC.

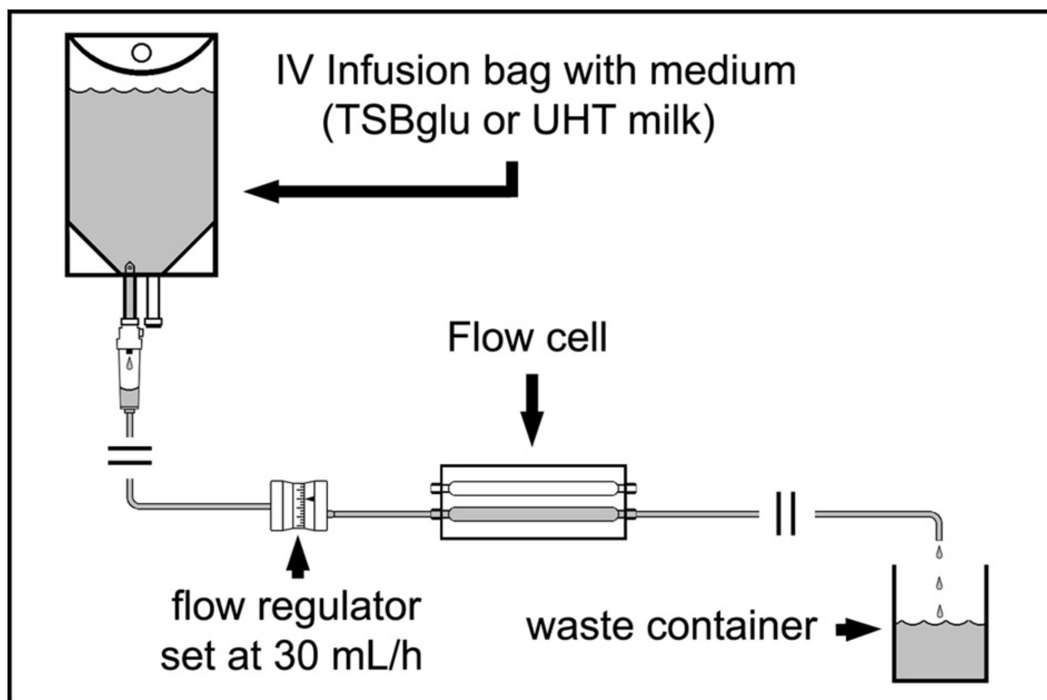

Fig. 1: schematic representation of the flow system for biofilm characterization

3.4.3.1 Flow system assembly

The general system consisted of a drilled polycarbonate flow cell with two grooves (65 x 5 x 4 mm, volume: 1.3 mL each) fed by an intravenous infusion bag (Galenica Senese, Italy). The flow cell was custom-manufactured following the design recommendations of Palmer et al. (2006). A cell culture-treated slide (Sarstedt, Germany) was fixed onto the chamber using silicone sealant to close

the flow cell. Flow velocity was set using a sterile flow regulator line (Ferrari, Italy) and all remaining connections were made with 5 mm internal diameter silicone tubes.

3.4.3.2 Sterilization

The system was sterilized using 200 mL of a freshly prepared 0.5% sodium hypochlorite solution flowing through the system for 4 h. The lines were then emptied and washed two times with 100 mL of sterile distilled water to remove any hypochlorite residue. The flow cell was then briefly washed with the medium and both the inlet and outlet tubing were clamped with haemostatic forceps.

3.4.3.3 Inoculation and incubation

Inoculum concentration was determined by spectrophotometric analysis (OD_{600nm}) of bacterial suspensions, using a Spectramax instrument (Molecular Devices, USA), followed by standard colony counting on plate count agar (Oxoid, UK).

After removal of the outlet clamping, approximately 1.0 x 10^7 *S. aureus* CFU was injected directly inside the medium filled channel through the inlet tube, using sterile needle and syringe (Terumo Corp., UK). The outlet tube was once again clamped and the flow cell was incubated at 37° C with the polystyrene slide facing downward to permit bacterial adhesion. After 1 h, the chamber was turned upward and the medium flow was initiated and maintained at 30 mL/h for 18 h. Aliquots (25 μL) of both inlet and outlet medium were plated onto 5% bovine blood agar plates (Oxoid, UK) and incubated overnight at 37°C, to check medium sterility and strain purity. Finally, the system was washed with 30 mL of sterile distilled water for 1 h, to remove any residual unbound bacteria. Strains were considered as biofilm-positive when a red-stained layer of bacterial growth was clearly visible along the slide surface inside the chamber (Fig. 2).

Slides were also observed under light microscopy with 1000x magnification and classified as follows: positive, in the presence of multilayered cell growth and abundant extracellular matrix, or negative, when rare cocci or small clusters were attached in monolayer (Fig. 3).

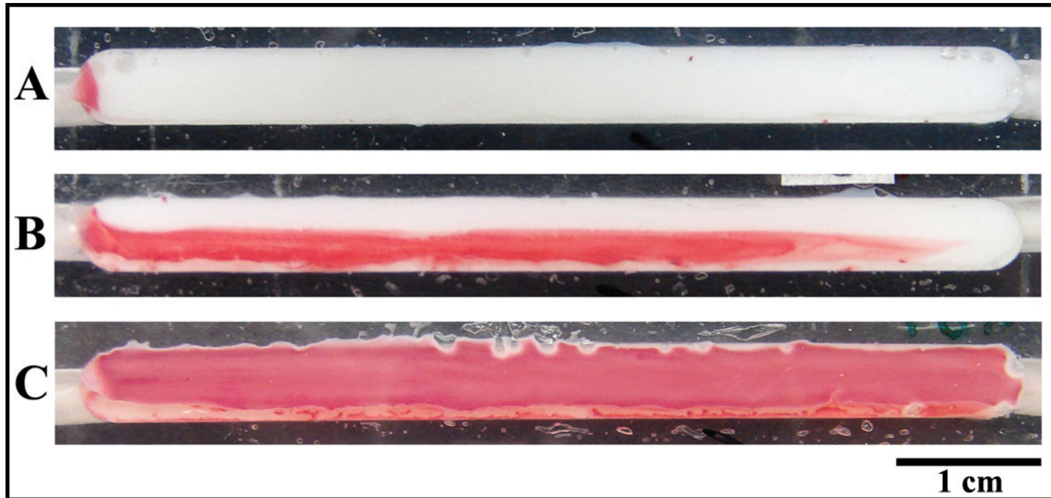

Fig. 2: macroscopic appearance of MFC flow chambers, A) non-biofilm forming strain, B and C) biofilm-forming strains.

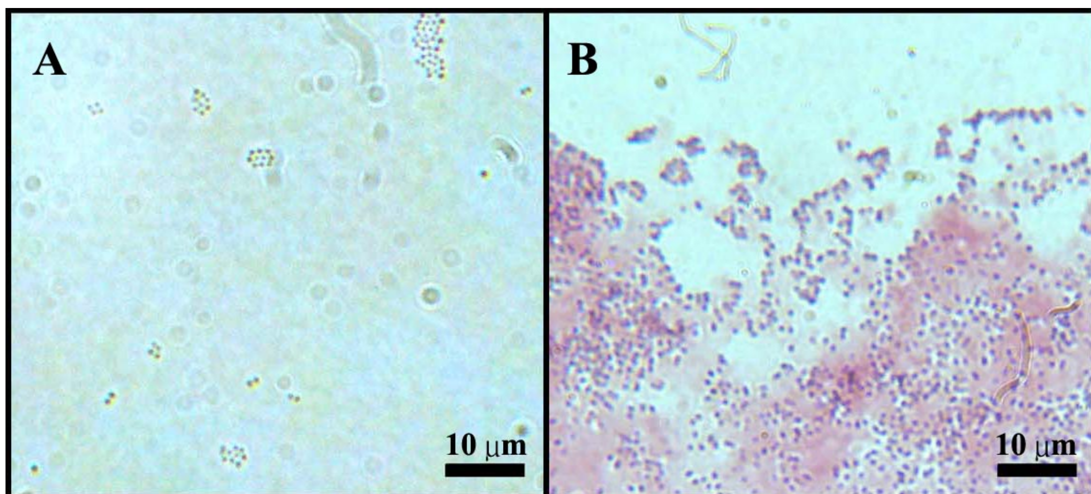

Fig. 3: microscopic inspection of MFC slides (1000x), A) non-biofilm forming strain, B) biofilm-forming strain.

3.4.4 DNA Microarray

All MFC-tested strains were genetically characterized using a DNA microarray, which detects a total of 329 different sequences, including accessory gene regulator (agr) alleles, genes coding for virulence factors (toxins, enterotoxins, putative toxins, haemolysins, proteases, and biofilm formation factors) and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsule type-specific genes, and numerous antimicrobial resistance genes (Supplementary table 1). The genotyping service was performed at Alere Technologies (Jena, Germany).

3.5 Statistical analysis

All statistical analyses were performed using SPSS Statistics 17.0 software (SPSS Inc., USA).

The determination of the cut-off value for the classification of the strains tested as biofilm-forming or non-biofilm forming in TCP assay, was performed by Cluster Analysis using Ward's hierarchical method. The optimal number of categories was defined following the considerations of Hair et al. (2009) for aggregative processes.

The association between the ability to form biofilm in the flow model and the strain genetic characteristics was analyzed by means of multiple regression analysis.

The agreement between TCP and MFC results was estimated by Cohen's kappa test for binary variables.

4 Results

4.1 Experiment 1 – *icaA/D* and *bap* detection in Italian dairy herds

4.1.1 *icaA* and *icaD* PCR

Out of 112 strains tested by PCR, 73 (65.18%) carried *icaA* and *icaD*, 2 (1.79%) only *icaA*, 15 (13.39%) only *icaD* and 22 (19.64%) were negative for both genes.

4.1.2 *bap* PCR

In seven (6.25%) *S. aureus* strains, out of 112 tested, a fragment compatible with *bap* was amplified; amplicons were further analysed by sequencing and confirmed as the constant part of *bap* gene. The result showed 98% to 99% homology with the reference *bap* gene (NCBI accession AY220730.1) using Genbank BLAST® analysis (http://blast.ncbi.nlm.nih.gov/). The strains were from four herds located in different Italian regions. In three herds (A, B and D), additional *bap*-negative *S. aureus* strains were also isolated. Overall, milk samples infected by *bap*-positive strains showed lower SCC and higher bacterial counts, when compared to the other milk samples (Table 1). Phenotypically, all strains produced macroscopically visible clumps in broth culture (Fig. 4), similarly to what observed with the *bap*-positive control strain *S. aureus* V329. Also, all strains showed a strong biofilm formation in the tissue culture plate assay, with higher OD_{492nm} values than the positive control *S. aureus* ATCC 25923 (data not shown).

	***Bap*-positive strains**		***Bap*-negative strains**	
Herd	SCC/mL	*S. aureus* count (UFC/mL)	SCC/mL	*S. aureus* count (UFC/mL)
A	$3x10^5$	> 2.000	$6.8x10^5$	< 400
B	$1x10^6$	> 2.000	$2x10^6$	< 400
D	$1.3x10^5$	>2000	$5.7x10^5$	<400

Table 1. Milk somatic cell and bacterial counts in milk samples from herd A, B and D where *bap*-positive and *bap*-negative strains were detected.

4.1.3 Second sampling in one herd with *bap*-positive *S. aureus* isolates

S. aureus was isolated from 31 quarter milk samples from 23 animals in the second sampling in herd B. Differently from the previous sampling, only *bap*-positive *S. aureus* were isolated. *S. aureus* counts in milk were higher than 2.0 x 10^3 CFU/mL in 70% of samples. Following the SCC categorization scheme proposed by Cucarella et al. (2004), 48% of samples SCC were classified as very low (<2.0 x 10^5 cells/mL), and in 22% as medium-low (2.0 x 10^5 - 7.5 x 10^5 cells/mL).

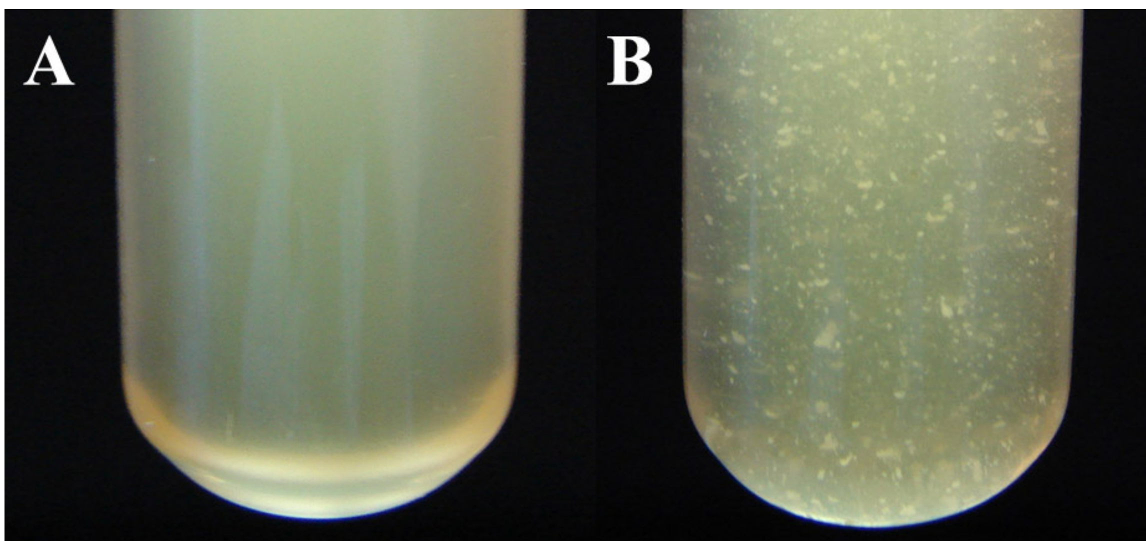

Fig. 4: Presence of clumps in broth culture of a *bap* positive strain (B) and absence in a *bap* negative strain.

4.2 Experiment 2 – Biofilm characterization of *S. aureus* mastitis strains

4.2.1 Congo Red agar (CRA)

Out of 27 mastitis isolates tested, 6 (22.2%) grew as black/rough colonies ("slime-producing") and 20 (74%) were classified as undetermined. *S. aureus* reference strain ATCC 25923 displayed classic "slime-producing" black/rough colonies while *S. aureus* ATCC 23213 displayed black/smooth colonies and *S. epidermidis* ATCC12128 bordeaux/smooth colonies, therefore were classified as undetermined. The aspect of isolated colonies was very conserved, no differences were observed

between repetitions and medium batch. *S. pseudintermedius* was the single strain showing red/smooth colonies.

4.2.2 Tissue culture plate assay (TCP)

The results of TCP assay were analysed by cluster analysis. The data were naturally grouped in two clusters with an optimal OD_{492}nm cut-off value of 0.95: cluster one comprised the negative control and samples with low OD_{492nm} values (mean 0.40; SD 0.27) while cluster two grouped the positive control and samples with high OD_{492nm} values (mean 1.75; SD 0.52). Therefore, 88 (78.57%) strains with an OD_{492nm} value lower than 0.95 were classified as non-biofilm forming while 24 (21.43%) with OD_{492nm} higher than 0.95 were considered as biofilm forming.

According to the classification proposed by Christensen et al (1985), 85 (75.89%) out of 112 strains were classified as non biofilm-forming and the remaining 27 (24.11%) as biofilm-forming. Using such scheme, the cut-off used for average OD_{492nm} values was 0.83.

4.2.3 Flow cell assay

4.2.3.1 TSB1%glu flow assay

The results of the flow system using TSB were equivalent to TCP results: both TCP negative strains produced no macroscopically visible biofilm while the two TCP positive strains formed a confluent growth along the slide surface. No clear difference between the two positives was observed.

4.2.3.2 Raw milk flow assay

While raw milk would provide even more realistic conditions, attempts to use raw milk collected with aseptic technique were unsuccessful in our system. The problems were attributed to the high milk fat content and contaminant bacteria.

4.2.3.3 UHT milk flow assay (MFC)

Twelve biofilm-forming and 18 non biofilm-forming strains (n=30), with average ODs ranging from 0.17 to 2.56, were selected out of the 112 TCP tested strains. Out of these 30 field strains, 6 (20%) formed biofilm in milk under flow conditions. Of these 6 MFC-positives, 4 where previously classified as non biofilm-forming by TCP. Also, both TCP positive and negative controls were not able to produce detectable (macro and microscopically) biofilms in the flow cell using milk as substrate.

According to the kappa coefficient test, no agreement was found between the two methods with any apparent concordance being attributed to chance (kappa = -0.037).

Sterility controls were run in all MFC experiments, plating aliquots from the inlet medium in bovine blood agar 5% (Oxoid, UK) Overall, apparent bacterial and/or fungal contamination could be observed. Also, all strains used were recovered in purity and in high concentrations from the outlet medium at the end of the 18h incubation period, independently of biofilm formation status.

4.2.3.4 DNA Microarray characterization and correlation with biofilm formation on MFC.

MFC-tested strains were assigned to 12 different clonal complexes (CC), according to microarray results. Strains classified as biofilm-forming by MFC grouped in CC479 (2 strains), or in CC705 (2 strains), or in CC97, or in an undefined *agrII*/cap5 ST of animal origin. Out of 30 tested strains, the most frequent clonal complex was CC8 (11 strains), which included only MFC biofilm negatives and TCP-positives or -negatives.

Microarray results showed genetic differences among MFC-tested strains, but only a limited number of genes (n= 14) was statistically related to strain ability to form biofilm in milk.

A list of genes significantly related to MFC-positivity is summarized in table 2. The relative distribution of such genes among MFC-tested and MFC-positive strains are also reported. Only 1 out of 32 MFC-tested strains, did not carried the *icaADBC* operon (*icaA*, *icaC* and *icaD* genes). Such strain was used as TCP negative control and did not form biofilm in MFC.

Eight adhesion factors/MSCRAMMs (*clfA*, *clfB*, *ebpS*, *eno*, *fnbA*, *map*, *sdrC* and *vwb*) out of 15 tested in the microarray were present in all MFC-tested strains. The remaining 7 factors (*bbp*, *cna*, *ebh*, *fib*, *fnbB*, *sasG* and *sdrD*) were distributed among the strains in 12 distinct profiles. No single adhesion/MSCRAMM or MSCRAMM profile was associated with biofilm formation in milk, since the 6 MFC positive strains showed 5 different profiles.

Table 2: Distribution and statistical significance of bacterial genes associated with biofilm production using the MFC method.

Genes		Distribution Overall (n=32)	MFC-positive (n=6)	Significance (p-value<0.05)
agrI	type-I accessory gene regulator	16 (50%)	1 (16.67%)	-
agrII	type-II accessory gene regulator	14 (48%)	5 (83.33%)	0.022
agrIII	type-III accessory gene regulator	1 (3.12%)	none	-
agrIV	type-IV accessory gene regulator	1 (3.12%)	none	-
cap5	capsular polysaccharide (CP) type 5	23 (71.9%)	2 (33.33%)	-
cap8	capsular polysaccharide (CP) type 8	9 (28.1%)	4 (66.66%)	0.024
bla	β-lactamase operon (*blaZ, blaI* and *blaR*)	17 (53.13%)	1 (16.67%)	0.036
fosB	metallothiol transferase	21 (65.63%)	1 (16.67%)	0.006
lmrP (RF122)	multidrug transporter (efflux pump)	8 (25%)	4 (66.67%)	0.011
lmrP (other than RF122)		24 (75%)	2 (33.33%)	-
lukM/*lukF-PV* (P-83)	bicomponent leukocidin	7 (21.88%)	4 (66.67%)	0.004
sec	enterotoxin C (*sec*, *sel* and *tst*1)	2 (7%)	2 (33.33%)	0.003
sel	enterotoxin-like L	2 (7%)	2 (33.33%)	0.003
tst1	toxic shock syndrome toxin 1	2 (7%)	2 (33.33%)	0.003
ssl7 (absence)	staphylococcal superantigen-like protein 7	28 (87.5%)	2 (33.32%)	0.003
ssl8 (absence)	staphylococcal superantigen-like protein 8	28 (87.5%)	3 (50%)	0.033
ssl9 (absence)	staphylococcal superantigen-like protein 9	26 (81.25%)	2 (33.33%)	0,000
Q2FXC0 (absence)	hypothetical protein, located next to serine protease operon	12 (37.5%)	none	0.030
Q7A4X2	hypothetical protein, located next to enterotoxin gene cluster (egc)	8 (25%)	5 (83.33%)	0.022

5 Discussion

5.1 Biofilm characterization

5.1.1 CRA

Our first characterization of *S. aureus* isolates from bovine mastitis was carried out using the qualitative CRA method. Growth on CRA is reported in the literature as an easy to perform technique of good reproducibility, being indicated as a cost-effective screening test. Our preliminary results confirmed that the method is indeed reproducible, but important variations of phenotypes were observed among strains. Therefore, the results are not easily interpreted and the classification becomes a subjective task. Later on, it was clear that correlations between phenotype on CRA and the presence of *ica* locus or bap gene with TCP or MFC results were inexistent (data not shown), in accordance with some published studies (Knobloch et al., 2002, Rode et al., 2007). Even though not all isolates tested on CRA were tested also by the MFC method, at least two MFC positive isolates and one MFC negative shared the exact same appearance, displaying smooth/black colonies on CRA.

5.1.2 TCP

The TCP method was originally conceived to characterize the biofilm forming ability of *S. epidermidis* from human infections. Thereafter, *S. epidermidis* has been serving as a model to study biofilm formation in Staphylococci, although, this species differs greatly from *S. aureus* in the mechanisms used for biofilm formation (Rode et al., 2007). The classical setup of TCP method includes inoculation of a diluted bacterial suspension in TSB supplemented with glucose, since Staphylococci generally enhance matrix exopolysaccharide expression in the presence of increased concentrations of glucose (Götz, 2002). Glucose is a monosaccharide highly available in the host organism, particularly in blood. In the mammary gland compartment it is found mainly as part of the milk disaccharide lactose, and thus it is not readily available. The lack of correlation between TCP and MFC results indicates that biofilm characterization in closed/static systems, especially

those applying glucose medium, should not be used to draw general conclusions about the biofilm-forming potential.

During the standardization phase of TCP, some important technical and interpretative limitations of this method could be noted, which possibly affect its performance. The first issue regards how to selecte control strains, particularly the negative one. Fox, Zadoks and Gaskins (2005) used the *ica*-defective *S. epidermidis* strain ATCC 12228 as the negative control strain to characterize *S. aureus* biofilms. This reference strain was also tested during our study and displayed an overall reduced growth, measured by broth turbidity and OD_{600nm} after the incubation period (data not shown) when compared to *S. aureus* strains. We also had only access to non biofilm-forming *S. aureus* reference strains from human origin. For this reasons, we used one of our non biofilm-forming *S. aureus* field isolates, MB543, which is an *ica*-defective strain. The problem is that the average negative control OD_{492nm} is used in the cut-off point calculation following Christensen et al (1985). In this particular example, the measured OD_{492nm} for *S. aureus* MB543 was 0.5 (SE = 0.11) against 0.27 (SD = 0.11) for *S. epidermidis* ATCC 12228, determining cut-off values of 0.83 and 0.57, respectively. As a consequence, the selection of controls itself represents a bias for the classification of strains as biofilm positive or negative.

The differences in OD_{492nm} values observed in the non-biofilm forming group raised some doubts about the biological relevance of the test. Statistically, the use of the negative control OD_{492nm} plus three times the standard deviation (Christensen et al., 1985) it is absolutely reasonable. The curious fact is that the OD_{492nm} values of non-biofilm forming strains do not converge around any overall similar value. Such observation would be expected, since similar concentrations were employed in the inocula and the final growth density of the majority of our strains is relatively uniform (data not shown). As observed by graph plotting, data of the non-biofilm forming strains are clearly continuous, similar to what observed in the biofilm-forming group. The variation between non biofilm-forming strains could be explained by diversely expressed adhesion factors among strains.

Each strain adhesin profile may promote different levels of adhesion of bacterial cells to the bottom of the well, even in the absence of matrix production.

Another important issue is related to the concentration of the TCP inoculum. Some authors used 1:100 (Melchior et al., 2009), while others used 1:40 dilution of overnight broth cultures of *S. aureus* (Cucarella et al., 2001, Vasudevan et al., 2003). We tested both dilutions using a reduced number of strains during the setup phase. Keeping unaltered other conditions like time of incubation, media and temperature, variations in the resulting OD_{492nm} were observed for several, but not all strain tested (data not shown). The use of 1:40 dilution proved to be more reliable, resulting in more consistent OD_{492nm} values between wells and plates, as verified by reduction of standard variation values. For this reason, we applied the 1:40 dilution factor.

The washing step was also considered an important factor capable to significantly impact TCP results. While some authors wash away unbound bacteria without mentioning any particular concern (Vasudevan et al., 2003), others suggest that this step should be performed in very delicate manner (Cucarella et al., 2001, Melchior et al., 2009). In our experience, inadequate washing such applying the fluid with micropipettes directly over the bacterial pelicle or even at well walls, may produce "holes" and biofilm de-attachments. These procedures showed to easily remove not only unbound bacterial cells, but also clumps of matrix-embedded bacteria (as observed by light microscopy) giving incongruent results.

5.1.3 MFC

Few papers used milk as medium for *S. aureus* culture to characterize biofilm formation of mastitis isolates. Milk degradation by staphylococcal enzymes observed in the traditional TCP assay is linked to space limitation and static condition causing an artificial aggregation of milk residues to the matrix (Amorena et al., 1999). Enzymatic expression and activity can be highly variable among *S. aureus* strains, therefore the use of milk in static systems seems to provide only limited or unreliable information about the biofilm-forming ability of strains. Amorena et al. (1999) and

Melchior et al. (2007) used conventional media or skimmed UHT to establish biofilms of *S. aureus* mastitis isolates and noted striking differences in biofilm structure and functional characteristics.

We used a dynamic method in order to avoid milk coagulation that occurs in closed systems. Milk is continuously produced inside the mammary gland; liquid flow is variable over time and is heterogeneous in the different gland compartments. Laminar milk flow is more likely to occur in the upper parts of the mammary gland, characterized by ducts of smaller diameter, draining the milk just released inside the alveoli. Laminar flow was used in our system mainly to allow reproducible experimental conditions, since milk flow removes the excess of metabolites, unattached cells and supplies bacteria with fresh medium, provided by the continuously produced milk in the gland environment.

Characteristics of milk flow inside the mammary gland, such as velocity and sense, are hard to investigate *in vivo*. Such information is mostly unknown and also expected to be variable among animals. We could not find in the literature studies regarding flow systems based on milk to study *S. aureus* biofilms. Therefore, time of adhesion and flow velocity were initially tested with settings from published papers, that used different systems, but showing at least some similarities with our conditions. Flow velocity was set at 30 mL/h according to Beenken, Melvins and Smeltzer (2003). The initial setting of 30 min for cell adhesion (Jaglic et al., 2011) was changed, after experimental tests, to 1 hour. This modification was made to provide a highly favorable environment to biofilm formation with more time to adhere.

To ensure that the flow system would allow biofilm formation comparable to that observed in TCP assay, the flow system was initially tested using the same culture medium. Four control strains, previously tested by TCP, were selected for this purpose: two negatives, one positive and one strongly positive strain. The results obtained were similar to TCP results: both TCP negative strains produced no macroscopically visible biofilm under flow conditions, while the two TCP positive strains formed a confluent growth along the slide surface.

Later on, to better reproduce intra-mammary conditions, we tried to use aseptically collected raw milk with very low SCC counts (<1000/mL), as culture medium. Our attempts proved unsuccessful because of flow obstructions related to high fat content and unspecific TTC reduction by contaminant bacteria. Thereafter, skimmed UHT milk (<0.05% fat) was used, since it is a sterile alternative to raw milk and spray dried powdered milk.

The small number of biofilm-forming strains in milk (n = 6, 20%) was an unexpected result, since 37.5% of MFC-tested strains were considered biofilm-producers by the TCP assay. Overall, only 2 strains were positive by both methods, while 4 MFC-positives were classified as TCP-negatives. Therefore, no statistical correlation could be found between the two methods. In the present study, a high and statistically determined cut-off value was used in order to identify the TCP strong positive strains. Even if the less severe and commonly used cut-off point were applied, no statistic association between static and dynamic system could be established (data not shown). These data strongly suggest that no useful information about the relation between biofilm formation and mastitis can be obtained from the standard characterization method using broth instead of milk.

The array characterization assigned MFC-positive strains to CC705, CC479, CC97 and to an undefined *agrII*/caps 5 animal strain. Following Holmes and Zadoks (2011), CC705 is associated with the bovine host and CC479 and CC97 are among the most common genotypes associated with bovine mastitis across herds and countries. The prevalent group among MFC-tested strains was CC8 with 11 strains, none of these formed biofilm in milk. CC8 is one of the 10 major human lineages worldwide, but strains belonging to this group were also isolated from bovine mastitis, showing a lower degree of host-association.

With the exception of two strains, those tested in MFC assay belonged either to *agrI* or to *agrII* allele types in similar proportion, but a clear preponderance of *agrII* strains (5/6) was observed among MFC positives, in accordance with the results of Melchior et al. (2009). A possible explanation could be that *agrI* strains better survive in the intracellular environment, while *agrII* strains evolved and adapted to the extracellular environment (Buzzola et al., 2007). Also, only one

penicillin-resistant strain was detected in the MFC-positive group. This finding confirms above considerations, since penicillin-resistance was reported as negatively correlated with *agrII* mastitis isolates (Melchior et al., 2009).

Capsular polysaccharide (CP) serotypes 5 and 8 are reported as the most prevalent ones in bovine mastitis isolates. The proportion of each serotype is geographically variable and seems to be unrelated to any form of mastitis (Tollersrud et al., 2000). The 32 strains tested by MFC showed mainly capsular type 5, but a significant 66.6% of biofilm-positive strains was CP8. Both CP5 and CP8 mastitis strains are known to enhance capsular polysaccharide expression when grown in milk and in vivo (Sutra et al. 1990, Lee et al. 1993), but CP8 strains are less aggressive in a murine model and more sensitive to intracellular killing compared to CP5 strains (Watts et al., 2005). These characteristics could indicate that biofilm formation in mastitis is not related to intracellular survival, in accordance with *agr* findings.

Statistical correlation was evidenced between carriage of leukocidin genes lukM/lukF-PV (P83) and biofilm formation in MFC, since 4 out of 6 MFC positive strains carried both subunits. Such bicomponent toxin is the most active leukotoxin against bovine neutrophils, but it is rarely found in human isolates (Rainard et al., 2003).

Positivity in the dynamic system was also significantly associated with the LmrP multidrug transporter, but only with the RF122 homologous sequence. Interestingly, the 2 MFC-positive strains lacking lukM/lukF-PV harboured a different lmrP gene and were the only two showing CP5. It is important to note that statistical correlation was found only with RF122 and P83, which are bovine mastitis isolates, the former was the first whole sequenced strain, the latter was originally isolated from clinical mastitis (Kaneko et al., 1997, Fitzgerald et al., 2001).

The significant correlation between biofilm formation under milk flow and absence of superantigen-like proteins genes ssl7, ssl8 and ssl9 could not be biologically explained. Similarly, the function of the hypothetical proteins encoded by the sequences Q2FXC0 and Q7A4X2 is still not known, therefore the possible implication in biofilm production under MFC conditions is not clear.

Also, MSCRAMMs could not explain biofilm formation, neither as individual determinants nor in associations. Such result was expected, as a consequence of the natural variability and functional redundancy of MSCRAMMs in *S. aureus* strains (O'Riordan and Lee, 2004).

5.2 Bap

The large proportion of *ica* operon carriage among the MFC tested strains (96.88%) is in accordance with the literature (Vasudevan et al., 2003). The carriage of *ica* was not correlated with biofilm forming activity, either in TCP or in MFC assay. Interestingly, 9 strains were identified as *ica* positive only by microarray, being misidentified as *ica* negative by PCR. The inadequacy of primers could explain this difference found in our strains and also the great variance of *ica* carriage in other published articles that used PCR to inquiry the *ica* prevalence among *S. aureus* isolates (Arciola, Baldassari, Montanaro, 2001, Rhode et al., 2001).

The low prevalence of *bap* recorded in the present study is in agreement with Cucarella et al. (2001). Also, all *bap*-positive *S. aureus* strains were from subclinical bovine mastitis and SCC were likely to be lower in milk samples harbouring those strains, in accordance with the limited literature on the subject (Cucarella et al., 2004).

Differently from the first sampling, only *bap*-positive *S. aureus* were isolated in the second sampling in herd B. This replacement is probably due the low SCC promoted by bap-harboring strains, what delays mastitis detection. Cucarella et al. (2004) suggested that biofilm formation and long term tissue adhesion of *bap*-positive strains could determine persistent infections. *In vitro* observation showed that Bap is not expressed in medium with Calcium (Ca^{2+}) concentrations similar to those found in milk (Arrizubieta et al., 2004) Therefore, the involvement of extracellular biofilm formation in the mammary gland could be ruled out. On the other hand, Bap could also be expressed in the intracellular environment. Biofilm components were shown to be expressed intracellularly, as it was demonstrated in experimental urinary tract infections caused by *Escherichia coli* (Anderson et al, 2003). Nevertheless, *S. aureus* use of such strategy, mediated by

Bap seems improbable, since eukaryotic cells contain high concentration of Ca^{2+} in intracellular stores (Arrizubieta et al., 2004). In addition, recent proteomic results showed that few proteins are up-regulated (around 10%) in *in vitro* intracellular *S. aureus*. Up-regulated proteins are mainly those associated with countermeasure systems against oxidative stress (Schmidt et al., 2010), which is not the case of the majority of biofilm-related proteins. Besides that, Bap was shown to negatively affect MSCRAMM activity (Cucarella et al., 2002), and at the same time reduces cell internalization through binding to host cell receptors (Valle et al., 2012).

The results of the present study corroborate with the observations of Arrizubieta et al. (2004). None of our *bap*-positive strains (n = 3), which were all *ica* locus positive and strongly biofilm-positive by TCP, was able to produce a detectable biofilm in milk under flow conditions.

6 Conclusion

The biofilm forming ability of *S. aureus* strains from bovine mastitis showed different results when tested by the classical static assay, or by a dynamic system using milk as a culture medium. Therefore, *S. aureus* biofilm-forming potential of these strains should be critically evaluated and tested applying conditions similar to mammary environment. The strains sharing some of the genes most commonly detected in dairy cows isolates were more able to form biofilm in the dynamic system.

From a pathogenetic point of view, the results of the present study could not give clear explanation of the role of the genes involved in biofilm formation with the establishment and/or development of dairy cow mastitis. While the longitudinal study, carried out in one herd with both *bap*-negative and *bap*-positive strains, showed a complete substitution of the first ones with the others, on the other hand no correlation was found between *bap*/*icaAD* and biofilm formation in the milk dynamic system. Therefore, further studies are necessary to determine whether the ability to form biofilm in MFC is correlated with increased virulence, facilitating the establishment of chronic infections in the mammary gland. Also, longitudinal studies in herds with bap-positive *S. aureus* strains will help understand their epidemiological behavior in dairy herds. While such strains are present both in Spanish and Italian herds in low prevalence, they seem well adapted to the bovine host and could potentially become the dominant strain inside a herd.

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	Species marker	39	*kdpD-SCC*	76	*tet(K)*
1	*rrnD1* (*S. aureus*)	40	*kdpE-SCC*	77	*tet(M)*
2	*gapA*	41	*mecI*	78	*cat* (total)
3	*katA*	42	*mecR*	79	*cat* (pC221)
4	*coA*	43	*xylR*	80	*cat* (pc223)
5	*nuc1*	44	*ccrA-3*	81	*cat* (pMC524/pC194)
6	*spa*	45	*ccrB-3*	82	*cat* (pSBK203R)
7	*sbi*	46	*merA*	83	*cfr*
	Regulatory genes	47	*merB*	84	*fexA*
8	*sarA*	48	*ccrAA* (MRSAZH47)_probe 1	85	*fosB*
9	*saeS*	49	*ccrAA* (MRSAZH47)_probe 2	86	*fosB* (plasmid)
10	*vraS*	50	*ccrC (85-2082)*		**Resistance: Efflux systems**
11	*agrI* (total)	51	*ccrA-4*	87	*qacA*
12	*agrB-I*	52	*ccrB-4*	88	*qacC* (total)
13	*agrC-I*		**Penicillinase**	89	*qacC* (*consensus*)
14	*agrD-I*	53	*blaZ*	90	*qacC* (equine)
15	*agrII* (total)	54	*blaI*	91	*qacC* (SA5)
16	*agrB-II*	55	*blaR*	92	*qacC* (*Ssap*)
17	*agrC-II*		**Resistance: MLS-Antibiotics**	93	*qacC* (ST94)
18	*agrD-II*	56	*erm(A)*	94	*sdrM*
19	*agrIII* (total)	57	*erm(B)*		**Resistance: Glycopeptides**
20	*agrB-III*	58	*erm(C)*	95	*vanA*
21	*agrC-III*	59	*lnu(A)*	96	*vanB*
22	*agrD-III*	60	*msr(A)*	97	*vanZ*
23	*agrIV* (total)	61	*mef(A)*		**Toxic shock syndrome toxin**
24	*agrB-IV*	62	*mph(C)*	98	*tst1* (*consensus*)
25	*agrC-IV*	63	*vat(A)*	99	*tst1* ("human" allele)
26	*hld*	64	*vat(B)*	100	*tst1* ("bovine" allele)
	Methicillin resistance and	65	*vga(A)*		**Enterotoxins**
	SCCmec typing	66	*vga(A)* (BM 3327)	101	*sea*
27	*mecA*	67	*vgB(A)*	102	*sea* (320E)
28	*delta_mecR*		**Resistance: Aminoglycosides**	103	*sea* (N315)/*sep*
29	*ugpQ*	68	*aacA-aphD*	104	*seb*
30	*ccrA-1*	69	*aadD*	105	*sec*
31	*ccrB-1*	70	*aphA3*	106	*sed*
32	*plsSCC* (COL)		**Resistance: Miscellaneous**	107	*see*
33	*Q9XB68-dcs*		**genes**	108	*seg*
34	*ccrA-2*	71	*sat*	109	*seh*
35	*ccrB-2*	72	*dfrS1*	110	*sei*
36	*kdpA-SCC*	73	*far1*	111	*sej*
37	*kdpB-SCC*	74	*fusC* (Q6GD50)	112	*sek*
38	*kdpC-SCC*	75	*mupA*	113	*sel*

Supplementary table 1: list of 329 gene/sequence probes in the Allere microarray chip

114	*selm*		**Epithelial differentiation inihibitor**	188	*ssl05*/set3_probe 2 (612)
115	*seln* (*consensus*)			189	*ssl05*/set3 (MRSA252)
116	*seln* (other than RF122)	151	*edinA*	190	*ssl06*/set21
117	*selo*	152	*edinB*	191	*ssl06* (NCTC8325+MW2)
118	*egc* (total)	153	*edinC*	192	*ssl07*/set1
119	*seq*		**ACME locus**	193	*ssl07*/set1 (MRSA252)
120	*ser*	154	*ACME* (total)	194	*ssl07*/set1 (AF188836)
121	*selu*	155	*arcA-SCC*	195	*ssl08*/set12_probe 1
122	ORF CM14_ probe1	156	*arcB-SCC*	196	*ssl08*/set12_probe 2
123	ORF CM14_ probe2	157	*arcC-SCC*	197	*ssl09*/set5_probe 1
	hlg and leukocidins	158	*arcD-SCC*	198	*ssl09*/set5_probe 2
124	*lukF*		**Proteases**	199	*ssl09*/set5 (MRSA252)
125	*lukS*	159	*aur* (*consensus*)	200	*ssl10*/set4
126	*lukS* (ST22+ST45)	160	*aur* (other than MRSA252)	201	*ssl10* (RF122)
127	*hlgA*	161	*aur* (MRSA252)	202	*ssl10*/set4 (MRSA252)
128	*lukF-PV*	162	*splA*	203	*ssl11*/set2 (COL)
129	*lukS-PV*	163	*splB*	204	*ssl11*+set2(Mu50+N315)
130	*lukF-PV* (P83)	164	*splE*	205	*ssl11*+set2(MW2+MSSA476)
131	*lukM*	165	*sspA*	206	*ssl11*/set2 (MRSA252)
132	*lukD*		**Superantigen/Enterotoxin-like**	207	*setB3*
133	*lukE*	166	*setC*	208	*setB3* (MRSA252)
134	*lukX*	167	*ssl01*/set6_probe1_11	209	*setB2*
135	*lukY*	168	*ssl01*/set6_probe2_11	210	*setB2* (MRSA252)
136	*lukY* (ST30+ST45)	169	*ssl01*/set6_probe1_12	211	*setB1*
	Haemolysins	170	*ssl01*/set6_probe2_12		**Capsule and biofilm genes**
137	*hl*	171	*ssl01*/set6_probe4_11	212	*cap 1* (total)
138	*hla*	172	*ssl01*/set6_probeRF122	213	*capH1*
139	*hlIII* (*consensus*)	173	*ssl01*/set6 (COL)	214	*capJ1*
140	*hlIII* (other than RF122)	174	*ssl01*/set6 (Mu50+N315)	215	*capK1*
141	*hlb*_probe 1	175	*ssl01*/set6 (MW2+MSSA476)	216	*cap 5* (total)
142	*hlb*_probe 2	176	*ssl01*/set6 (MRSA252)	217	*capH5*
143	*hlb*_probe 3	177	*ssl01*/set6 (RF122)	218	*capJ5*
144	un-disrupted *hlb*	178	*ssl01*/set6 (other alleles)	219	*capK5*
	hlb-conversion phages	179	*ssl02*/set7	220	*cap 8* (total)
145	*sak*	180	*ssl02*/set7 (MRSA252)	221	*capH8*
146	*chp*	181	*ssl03*/set8_probe 1	222	*capI8*
147	*scn*	182	*ssl03*/set8_probe 2	223	*capJ8*
	Exfoliative toxins	183	*ssl03*/set8 (MRSA252, SAR424)	224	*capK8*
148	*etA*	184	*ssl04*/set9	225	*icaA*
149	*etB*	185	*ssl04*/set9 (MRSA252, SAR425)	226	*icaC*
150	*etD*	186	*ssl05*/set3_probe 1	227	*icaD*
		187	*ssl05*/set3 (RF122, probe-611)	228	*bap*

Supplementary table 1: (cont.)

	Adhesion factors (MSCRAMM)	270	*map* (COL)	303	*lmrP* (other than RF122)_probe2
229	*bbp* (total)	271	*map* (MRSA252)		
230	*bbp* (*consensus*)	272	*map* (Mu50+MW2)	304	*lmrP* (RF122)_probe1
231	*bbp* (COL+MW2)	273	*sasG* (total)	305	*lmrP* (RF122)_probe2
232	*bbp* (MRSA252)	274	*sasG* (COL+Mu50)		**Type I restriction/modification system, single sequence specificity protein**
233	*bbp* (Mu50)	275	*sasG* (MW2)		
234	*bbp* (RF122)	276	*sasG* (other than MRSA252+RF122)		
235	*bbp* (ST45)			306	*hsdS1* (RF122)
236	*clfA* (total)	277	*sdrC* (total)	307	*hsdS2* (Mu50+N315+COL+ USA300+NCTC8325)
237	*clfA* (consensus)	278	*sdrC* (*consensus*)		
238	*clfA* (COL+RF122)	279	*sdrC* (B1)	308	*hsdS2* (MW2+MSSA476)
239	*clfA* (MRSA252)	280	*sdrC* (COL)	309	*hsdS2* (RF122)
240	*clfA* (Mu50+MW2)	281	*sdrC* (Mu50)	310	*hsdS2* (MRSA252)
241	*clfB* (total)	282	*sdrC* (MW2+MRSA252+RF122)	311	*hsdS3* (all other than RF122+ MRSA252)
242	*clfB* (consensus)				
243	*clfB* (COL+Mu50)	283	*sdrC* (other than MRSA252+RF122)	312	*hsdS3* (COL+USA300+NCTC 8325+MW2+MSSA476+RF122)
244	*clfB* (MW2)				
245	*clfB* (RF122)	284	*sdrD* (total)	313	*hsdS3* (Mu50+N315)
246	*cna*	285	*sdrD* (*consensus*)	314	*hsdS3* (CC51+ MRSA252)
247	*ebh* (*consensus*)	286	*sdrD* (COL+MW2)	315	*hsdS3* (MRSA252)
248	*ebpS* (total)	287	*sdrD* (Mu50)	316	*hsdSx* (CC25)
249	*ebpS*_probe 612	288	*sdrD* (other)	317	*hsdSx* (CC15)
250	*ebpS*_probe 614	289	*vwb* (total)	318	*hsdSx* (*etd*)
251	*ebpS* (01-1111)	290	*vwb* (consensus)		**Miscelaneous genes**
252	*ebpS* (COL)	291	*vwb* (COL+MW2)	319	Q2FXC0
253	*eno*	292	*vwb* (MRSA252)	320	Q2YUB3
254	*fib*	293	*vwb* (Mu50)	321	Q7A4X2
255	*fib* (MRSA252)	294	*vwb* (RF122)		**Hyaluronate lyase**
256	*fnbA* (total)		**Immunodominant Ag B**	322	*hysA1* (MRSA252)
257	*fnbA* (*consensus*)	295	*isaB*	323	*hysA1* (MRSA252+RF122) and/or hysA2 (*consensus*)
258	*fnbA* (COL)	296	*isaB* (MRSA252)		
259	*fnbA* (MRSA252)		**Defensin resistance**	324	*hysA1* (MRSA252+RF122) and/or hysA2 (COL+USA300)
260	*fnbA* (Mu50+MW2)	297	*mprF* (COL+MW2)		
261	*fnbA* (RF122)	298	*mprF* (Mu50+MRSA252)	325	*hysA2* (all other than MRSA252)
262	*fnbB* (total)		**Transferrin binding protein**		
263	*fnbB* (COL)	299	*isdA* (*consensus*)	326	*hysA2* (COL+USA300+ NCTC8325)
264	*fnbB* (COL+Mu50+MW2)	300	*isdA* (MRSA252)		
265	*fnbB* (Mu50)	301	*isdA* (other than MRSA252)	327	*hysA2* (all other than COL+USA300+NCTC8325)
266	*fnbB* (MW2)		**Putative transporter**		
267	*fnbB* (ST15)	302	*lmrP* (other than RF122)_probe1	328	*hysA2* (all other than COL+USA300+NCTC8325)
268	*fnbB* (ST45-2)				
269	*map* (total)			329	*hysA2* (MRSA252)

Supplementary table 1: (cont.)