Molecular characteristics of *bap*-positive *Staphylococcus aureus* strains from dairy cow mastitis.

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bap-positive *S. aureus* genetic characteristics

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Summary

The biofilm-associated protein (Bap) of *Staphylococcus aureus* (*S. aureus*) is a high molecular weight cell-wall-anchored protein involved in biofilm formation, firstly described in bovine mastitis strains from Spain. So far, studies regarding Bap were mainly based on the Spanish strain V329 and its mutants, but no information on the genetic variability of *bap*-positive *S. aureus* strains is yet available in the literature. The present study investigated the molecular characteristics of 8 *bap*-positive *S. aureus* strains from subclinical bovine mastitis, isolated in 5 herds; somatic cells counts of milk samples were also registered. Strains were characterized using MLST, SPA typing and microarray and the results were compared with V329. All isolates from this study and V329 were assigned to ST126, t605, but some molecular differences were observed. Only herd A and B strains harboured the genes for β-lactams resistance; the leukocidin D/E gene, a type I site-specific deoxyribonuclease subunit, 3rd locus gene and serin-protease A and B were carried by all strains, but not by V329, while serin-protease E was absent in V329 and in another isolate. Four isolates and V329 harboured the fibronectin-binding protein B gene. Somatic cells counts showed the highest value in the milk sample affected by the only strain carrying all the virulence factors considered. Potential large variability of virulence was evidenced among V329 and all *bap*-positive *S. aureus* strains considered: the carriage of *fnb* could enhance the accumulation of biofilm, but the lack of *lukD/E* and *splA, B* or *E* might decrease the invasiveness of strain.

Key words: *Staphylococcus aureus*, *bap* gene, biofilm, dairy cow mastitis.

Introduction

The biofilm-associated protein (Bap) of *Staphylococcus aureus* (*S. aureus*) is a high molecular weight cell-wall-anchored protein of 2,276 amino acids and was firstly described in bovine mastitis strains from Spanish dairy herds (Cucarella *et al.* 2001). Bap was shown to be involved in intercellular adhesion and accumulation in multilayer cell clusters, and also in primary attachment to abiotic surfaces. These functional characteristics confer a strong biofilm-forming phenotype to strains carrying the *bap* gene (Lasa & Penades 2006).

Bap was the first described member of a family of surface proteins (BAP) now detected in other staphylococcal species and unrelated Gram-positive or Gram-negative species (Tormo *et al.* 2005, Latasa *et al.* 2006). In *S. aureus*, *bap* is carried in a transposon-like element located within the SaPIbov2 pathogenicity island.

In vitro experiments showed that *bap*-positive *S. aureus* was significantly less able to attach to immobilized fibrinogen and fibronectin, probably for an interference of Bap with functional properties of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM). Nevertheless, in the experimental infection, the same *bap*-positive strain was more able to persist inside the mammary gland (Cucarella *et al.* 2002). Also, *bap*-mediated biofilm demonstrated higher resistance to antimicrobials (Cucarella *et al.* 2004).

The location of *bap* in a mobile genetic element could allow horizontal gene transfer among *S. aureus* strains (Tormo *et al.* 2005). However, the low frequency of *bap*-positive *S. aureus* strains seems to indicate that such event is not common (Vautor *et al.* 2008). In fact, since the first description, several attempts to identify *bap* carriage in *S. aureus* were unsuccessfully performed on isolates from different animal species (Vasudevan *et al.* 2003, Vancraeynest *et al.* 2004, Nitzsche *et al.* 2007, Vautor *et al.* 2008, Szweda *et al.* 2012). Recently, Darwish *et al.* (2013)

detected *bap* in one *S. aureus* strain from bovine mastitis in Egypt and Goyal *et al.* (2014) identified 11 *S. aureus bap*-positive isolates from cattle and dog clinical specimens in India.

So far, studies regarding the virulence of *bap*-carrying *S. aureus* isolates were mainly based on wild type strain V329 and its mutants (Di Poto *et al.* 2009, Shukla & Rao 2013). The other Spanish isolate strain V858 was compared with V329 for the variation in number of a conserved *tandem* repeat in the C region of the *bap* gene, showing differences that could be attributed to homologous recombination (Cucarella *et al.* 2004). Nevertheless, information regarding the epidemiology, prevalence and genetic variability of *bap*-positive *S. aureus* strains is, to the best of our knowledge, not yet available in the literature. Therefore, the aim of the present study was to screen dairy cows with subclinical mastitis for bap-positive *S. aureus* and to investigate the molecular characteristics of *bap*-positive *S. aureus* isolates, to assign them to sequence types and to compare them to the Spanish prototypic strain V329.

Materials and Methods

Isolation of *S. aureus* strains and DNA extraction

The 8 *S. aureus* isolates considered in the paper were isolated from quarter milk samples aseptically taken from lactating cows in 5 different Italian dairy herds. Bacteriological analyses were performed according to a previously published protocol (Hogan *et al.* 1999) and milk somatic cells were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska MN, USA). Briefly, an aliquot of 10 µL of each sample was spread onto blood-agar plates (5% bovine blood; Oxoid, UK), and incubated at 37°C. Plates were evaluated after 24 and 48h, and colonies of growth were isolated. The phenotypic identification as *S. aureus* based on standard biochemical tests, was further confirmed by PCR (Pilla *et al*. 2013). Thereafter, the isolates were

frozen at -80° C in MicroBank Bacterial Preservation System (Thermo Fisher Scientific, USA) for further molecular analysis.

After thawing, each isolate was subcultured on 5% bovine blood agar plate (Oxoid, USA) and DNA was extracted using DNeasy kit (QIAgen, Germany) according to manufacturer's instructions. DNA amount and purity were tested with a ND-100 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

PCR analysis for *bap*

PCR to detect *bap* was performed using primers and conditions described by Cucarella *et al.* (2004). The PCR products were analysed by electrophoresis on 0.8% agarose gel with ethidium bromide (0.5 µg/mL) in TAE buffer. The expected *bap* amplicon size was 971 bp. As positive control, the Spanish reference strain V329 was used. The PCR results were further confirmed by sequencing. The *bap* gene was also covered by the microarray analysis (see below).

Multilocus Sequence Typing (MLST) and spa typing

All *bap*-carrying strains were genotyped by MLST, using the procedure described at the *S. aureus* MLST website (http://saureus.mlst.net/misc/info.asp) and by Enright *et al.* (2000). The spa typing analysis was performed following Shopsin et al. (1999) and spa types were assigned using the Ridom SpaServer (http://www.spaserver.ridom.de).

Strain characterization by DNA microarray

The strains were further characterized using a DNA microarray based assay (StaphyType; Alere Technologies, Jena, Germany), which detects a total of 333 different sequences, including accessory gene regulator (*agr)* alleles, genes coding for virulence factors (toxins, enterotoxins, putative toxins, hemolysins, proteases, and biofilm formation molecules) and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsule type-specific

genes, and numerous antimicrobial resistance genes. With regard to *bap*, the binding sites or probes and primers used in the array analysis were designed in a conserved region from 1,807 – 2,770; this region (B) is also homologous in other *Staphylococcus* species, while A, C and D regions show the major differences among species (Thormo *et al.* 2005). Microarray analyses were performed following the recommendations of the manufacturer. The recorded hybridization patterns were analyzed using a designated reader and software (ArrayMate and IconoClust, both by Alere Technologies).

Results

PCR analysis for *bap* and array characterization of *bap*-positive strains gave identical results. The *bap* amplicons, that were localised within the constant part of the gene, showed >98% DNA sequence homology with the reference gene (NCBI accession AY220730.1), using BLAST® analysis (http://blast.ncbi.nlm.nih.gov/).

Bacteriological analysis of quarter milk samples showed minor differences in the shedding of the 8 *bap*-carrying *S. aureus* strains in the milk. Overall, the counts were higher than 2,000 CFU/mL, and only 1 out of the 2 isolates from herd D was present in the milk in low counts (300 CFU/mL). To the contrary, SCC values ranged 1-998 cells/μL, with the lowest value associated with *S. aureus* herd C strain 1, and the highest with the only strain detected in herd B. Large SCC variations were observed not only among herds, but also within them (Table 1).

The investigation of genetic relatedness using MLST and spa typing showed that all field isolates, as well as strain V329, belonged to Sequence Type (ST)126, t605 (Table 1).

Table 1. Results of bacteriological analysis of quarter milk samples infected by *S. aureus* strains carrying *bap* gene, collected in 5 different dairy herds: somatic cell counts (SCC) and *S. aureus*

134 counts in milk. Genetic relatedness of field strains and Spanish V329 strain is also reported as
135 Sequence Type (ST) and SPA Type (t).

***S. aureus* strains and herds**	**CFU/mL**	**SCC/µL**	**ST**	**t**
A1	>2,000	299	126	605
B1	>2,000	998	126	605
C1	>2,000	1	126	605
C2	>2,000	133	126	605
C3	>2,000	65	126	605
D1	300	391	126	605
D2	>2,000	130	126	605
E1	>2,000	179	126	605
V329			126	605

136

137 Nevertheless, some genetic differences among *bap*-positives could be evidenced by array
138 characterization (Table 2).

139 **Table 2**. Genetic differences among *S. aureus* V329 and *bap*-positive strains from the 5 dairy
140 herds (A, B, C, D and E), revealed by the array characterization.

	S. aureus strains and herds								
Genes	A1	B1	C1	C2	C3	D1	D2	E1	V329
β-lactamase, repressor and regulatory protein (*blaZ, blaI, blaR*)	+	+	-	-	-	-	-	-	-
leukocidin D/E (*lukD/E*)	+	+	+	+	+	+	+	+	-
serin-protease A, B (*splA, splB*)	+	+	+	+	+	+	+	+	-
serin-protease E (*splE*)	-	+	+	+	+	+	+	+	-
fibronectin-binding protein B (*fnbB*)	+	+	+	-	-	+	-	-	+
type I site-specific deoxyribonuclease subunit, 3rd locus (*hsdS3*)	+	+	+	+	+	+	+	+	-

All strains carried the *ica* operon and shared the capsular polysaccharide (CP) serotype 5; among the regulatory genes, both δ-haemolysin and accessory gene regulator allele II (*agrII*) were overall present.

All tested strains were methicillin-susceptible, lacking the *mecA* gene (*mecC* was ruled out for representative isolates); they also did not harbour any of the antibiotic resistance genes comprised in the array. Exceptions were two isolates, from herds A and B, which harboured the β-lactamase operon (including repressor and regulatory genes; *blaZ*, *blaI*, *blaR*). The genes coding for toxic shock syndrome toxin 1 and enterotoxins were absent, those for staphylococcal superantigen-like proteins (*ssl*) were present in all tested isolates; only *ssl11* was not detected being either absent, or present in an unknown, undetectable allele.

All isolates were negative for Panton Valentine leukocidin genes as well as for the cattle-associated leukocidin genes *lukM/lukF-P83*, while all *S. aureus* strains but V329 harboured both components of leukocidin D/E (*lukD/E*). Analogously, serin-protease genes A and B (*splA*, *splB*) were present in all field strains, but not in V329, while serin-protease E (*splE*) was absent from V329, as well as from one herd A isolate.

Among MSCRAMMs comprised in the array, only 3 adhesins were not detected: the collagen-binding adhesin, the *S. aureus* surface protein G and the Ser-Asp rich fibrinogen-/bone sialoprotein-binding protein D. The fibronectin-binding protein B (*fnbB*) was carried by 4 strains and V329.

Other lineage-specific markers such as type I site-specific deoxyribonuclease subunit (*hsdS*) was carried by all strains, indicating their affiliation to one lineage. However, the gene *hsdS3* (as defined by GenBank BA000017.4; 1,935,723 to 1,936,952) was absent from V329 but present in the field isolates.

Discussion

The study showed some molecular differences among *bap*-positive *S. aureus* strains considered, even though all of them grouped in ST126. It should be noted, that the 8 strains isolated from subclinical dairy cow mastitis, had been collected in different and unrelated herds, situated in different regions, in Northern or in Southern Italy. The major divergences regarded two virulence factors, namely *lukD/E* and serin-proteases, which were absent exclusively in the prototypic strain V329. Both factors affect the host's immune response, targeting the neutrophils. It was previously demonstrated, that the leukocidin promotes *S. aureus* replication *in vivo* by directly killing mice phagocytes recruited to sites of infection (Alonzo III *et al.* 2012). The proteases affect human neutrophil functions, increasing bacterial resistance to phagocytosis (Kolar *et al.* 2012). In a study including hospitalized patients with or without invasive endocarditis, the genes encoding *splA* and *splB* were significantly associated with invasive isolates. The same was shown for *lukD/E* (Rasmussen *et al.* 2013). Therefore, the lack of these genes could result in a decreased / attenuated virulence of V329 strain compared to the other *bap*-positive strains.

Fibronectin-binding proteins are important adhesins for *Staphylococcus aureus* infection. Even though *fnbA* is plays a major role in both *in vitro* and *in vivo* infections, nevertheless synergism between *fnbA* and *fnbB* was demonstrated to be crucial in human medicine, for the induction of severe infections ending in septic death in (Shinji *et al.* 2011). Loss of these MSCRAMMs reduced the initial adherence of bacteria, indicating that these genes are involved in primary attachment. Also, expression of both fibronectin-binding proteins increased bacterial aggregation, suggesting that they can promote the accumulation phase of biofilm (McCourt *et*

187 *al.* 2014). Such expression was shown throughout the growth cycle of an MRSA field strain, not
188 only during the exponential phase of growth as previously thought (Geoghegan *et al.* 2013).
189 Therefore, we may hypothesize a more active production of biofilm in V329 and in those field
190 strains harbouring not only *ica* operon and *bap*, but also both *fnb* genes. An interesting finding
191 was the demonstration of the genes for bone sialoprotein-binding and cell wall associated
192 fibronectin-binding protein in all bap-positive ST126 strains, and the absence in bap-negatives
193 belonging to the same ST, detected in other Italian herds (data not shown). Notably, bone
194 sialoprotein-binding protein is regarded as an important virulence factor, closely related to
195 biofilm formation (Vancraeynest *et al*. 2004).
196 The β-lactamase operon was carried only by 2 isolates, from 2 herds in Northern Italy. This
197 finding diverges from that observed in bap-negative ST126 strains.
198 Type I site-specific deoxyribonuclease is a mechanism blocking transfer of resistance genes and
199 other mobile genetic elements into *S. aureus* isolates between isolates of different lineages and
200 from other species (Waldron *et al.* 2006). Therefore, a key role of *hsdS* was suggested in
201 controlling genetic exchange and evolution of *S. aureus*. The partial lack of such mechanism
202 might be the result of a random deletion especially in a laboratory strain kept *in vitro*, i.e.,
203 without outside selective pressures, but it also could speculated that that such genetic
204 configuration might confer higher stability to V329 strain, or also lead to a low probability to
205 acquire those virulence factors that were detected in the other isolates.
206 The association between gene carriage and virulence of *S. aureus* field strains could not be
207 analyzed, due to the reduced number of strains. Nevertheless, it should be evidenced that SCC
208 showed the highest value reaching 1,000 cells/μL, in herd B quarter milk sample, the only one
209 affected by a *S. aureus* carrying all virulence factors considered.

Conclusion

The results of the study showed important genetic differences among the 8 *S. aureus* field strains considered and with the Spanish prototypic strain V329. Such results indirectly confirm previous findings (Cucarella *et al.* 2004), which demonstrated variation in the C region of the *bap* gene, hypothetically attributed to homologous recombination. The information is now expanded to a higher level, evidencing potential large variability of virulence among V329 and all *bap*-positive *S. aureus* strains considered. The best example is the carriage by V329, but not by all the other strains, of an important virulence factor such as fibronectin-binding protein B gene, and the absence of leukocidin D/E and serin-protease exclusively in V329. While the carriage of *fnb* could enhance the accumulation of biofilm, on the other hand the lack of *lukD/E* and *splA, B* or *E* decrease the invasiveness ability of strain.

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Conflicts of interest

R.E. and S.M. are employees of Alere technologies, the company that manufactured the arrays used for this study. This had no influence on study design and implementation. The other authors declare no competing interests.

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