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

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The biofilm-associated protein (Bap) of *Staphylococcus aureus* is a high molecular weight cell-wall-anchored protein involved in biofilm formation, first 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 *Staph. aureus* strains is yet available in the literature. The present study investigated the molecular characteristics of 8 *bap*-positive *Staph. aureus* strains from subclinical bovine mastitis, isolated in 5 herds; somatic cell counts (SCC) of milk samples were also registered. Strains were characterised 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. SCC 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 *Staph. 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.

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

The biofilm-associated protein (Bap) of *Staphylococcus aureus* is a high-molecular weight cell-wall-anchored protein of 2276 amino acids and was first 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 & Penadés, 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 *Staph. aureus, bap* is carried in a transposon-like element located within the SaPIbov2 pathogenicity island.

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Experiments in vitro showed that *bap*-positive *Staph. aureus* was significantly less able to attach to immobilised fibrinogen and fibronectin, probably because of an interference of Bap with functional properties of the microbial surface components recognising 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 *Staph. aureus* strains (Tormo et al. 2005). However, the low frequency of *bap*-positive *Staph. aureus* strains seems to indicate that such an event is not common (Vautor et al. 2008). In fact, since the first description, several attempts to identify *bap* carriage in *Staph. 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 & Asfour (2013) detected *bap* in one *Staph. aureus* strain from bovine mastitis in Egypt and Goyal et al. (2014) identified 11 *Staph. aureus bap*-positive isolates from cattle and dog clinical specimens in India.

So far, studies regarding the virulence of *bap*-carrying *Staph. aureus* isolates have been 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 and genetic variability of *bap*-positive *Staph. 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 *Staph. aureus* and to investigate the molecular characteristics of *bap*-positive *Staph. aureus* isolates, to assign them to sequence types and to compare them to the Spanish prototypic strain V329.

Materials and methods

Isolation of Staph. aureus *strains and DNA extraction*

The 8 *Staph. 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, 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 48 h, and colonies of growth were isolated. Phenotypic identification as *Staph. 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 the 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). 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. 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 *Staph. 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 characterisation by DNA microarray

The strains were further characterised 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, haemolysins, proteases, and biofilm formation molecules) and microbial surface components recognising 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 1807–2770; this region (B) is also homologous in other *Staphylococcus* species, while A, C and D regions show the major differences among species (Tormo et al. 2005). Microarray analyses were performed following the recommendations of the manufacturer. The recorded hybridisation patterns were analysed using a designated reader and software (ArrayMate and IconoClust, both by Alere Technologies).

Results

PCR analysis for *bap* and array characterisation 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 *Staph. aureus* strains in the milk. Overall, the counts were higher than 2000 CFU/µl, and only 1 out of the 2 isolates from herd D was present in the milk in low counts (300 CFU/µl). To the contrary, SCC values ranged 1–998 cells/µl, with the lowest value associated with *Staph. aureus* herd C strain 1, and the highest with the only strain detected in herd B. Large variations in SCC were observed not only among herds, but also within them (Table 1).

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Table 1. Results of bacteriological analysis of quarter milk samples infected by *Staph. aureus* strains carrying bap gene, collected in 5 different dairy herds: somatic cell counts (SCC) and *Staph. aureus* counts in milk. Genetic relatedness of field strains and Spanish V329 strain is also reported as Sequence Type (ST) and SPA Type (t)

Staph. aureus strains and herds	CFU/ml	SCC/μl	ST	*t*
A1	>2000	299	126	605
B1	>2000	998	126	605
C1	>2000	1	126	605
C2	>2000	133	126	605
C3	>2000	65	126	605
D1	300	391	126	605
D2	>2000	130	126	605
E1	>2000	179	126	605
V329			126	605

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

Genes	*Staph. aureus* strains and herds								
	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*)	+	+	+	+	+	+	+	+	−

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

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 *Staph. 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 *Staph. 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 small number of strains examined in the study poses some limitation in data analysis, but it should be emphasised that bap-positive *Staph. aureus* has been rarely isolated from dairy cow mastitis. Moreover, the 8 strains tested had been collected in different and unrelated herds, situated in different regions, in Northern or in Southern Italy. The results of the study showed some molecular differences among *bap*-positive *Staph. aureus* strains considered, even though all of them belonged to ST126, t605. 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 *Staph. aureus* replication in vivo by directly killing mice

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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 hospitalised 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 with the other *bap*-positive strains.

Fibronectin-binding proteins are important adhesins for *Staph. aureus* infection. Even though *fnbA* plays a major role in infections both in vitro and in vivo, 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 al. 2014). Such expression was shown throughout the growth cycle of an MRSA field strain, not only during the exponential phase of growth as previously thought (Geoghegan et al. 2013). Therefore, we may hypothesise a more active production of biofilm in V329 and in those field strains harbouring not only *ica* operon and *bap*, but also both *fnb* genes. An interesting finding was the demonstration of the genes for bone sialoprotein-binding and cell wall associated fibronectin-binding protein in all bap-positive ST126 strains, and the absence in bap-negatives belonging to the same ST, detected in other Italian herds (data not shown). Notably, bone sialoprotein-binding protein is regarded as an important virulence factor, closely related to biofilm formation (Vancraeynest et al. 2004).

The β-lactamase operon was carried only by 2 isolates, from 2 herds in Northern Italy. This finding diverges from that observed in bap-negative ST126 strains.

Type I site-specific deoxyribonuclease is a mechanism blocking transfer of resistance genes and other mobile genetic elements into *Staph. aureus* isolates between isolates of different lineages and from other species (Waldron & Lindsay, 2006). Therefore, a key role of *hsdS* was suggested in controlling genetic exchange and evolution of *Staph. aureus*. The partial lack of such mechanism might be the result of a random deletion especially in a laboratory strain kept in vitro, i.e., without outside selective pressures, but it could also be speculated that that such genetic configuration might confer lower stability to V329 strain in comparison with the others.

The association between gene carriage and virulence of *Staph. aureus* field strains could not be analysed owing to the reduced number of strains. Nevertheless, it should be noted that SCC showed the highest value reaching 1000 cells/µl, in herd B quarter milk sample, the only one affected by a *Staph. aureus* carrying all virulence factors considered.

Conclusion

The results of the study showed important genetic differences among the 8 *Staph. 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 *Staph. 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. The demonstration of such genetic differences among bap-positive *Staph. aureus* requires further research, in order to expand the results of the present study, investigating the association between gene carriage and virulence of the strains.

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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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