Existence of two groups of *Staphylococcus aureus* strains isolated from bovine mastitis based on biofilm formation, intracellular survival, capsular profile and *agr*-typing

Marjorie Bardiau[a,b*], Jonathan Caplin[b], Johann Detilleux[c], Hans Graber[d], Paolo Moroni[e,f], Bernard Taminiau[g] and Jacques G. Mainil[a]

[a] Bacteriology, Department of Infectious Diseases, Faculty of Veterinary Medicine and Institute for Fundamental and Applied Research in Animal Health (FARAH), University of Liège, Sart-Tilman, Bât. 43a, B-4000 Liège, Belgium

[b] Environment & Public Health Research Group, School of Environment & Technology, University of Brighton, Cockcroft Building, Lewes Road, Brighton BN2 4GJ, United Kingdom

[c] Biostatistics, Bioinformatics and Animal Selection, Department of Animal Production, Faculty of Veterinary Medicine, University of Liège, Sart-Tilman, Bât. 43a, B-4000 Liège, Belgium

[d] Agroscope, Institute for Food Sciences IFS, Schwarzenburgstrasse 161, 3003 Berne, Switzerland

[e] Università degli Studi di Milano, Department of Health, Animal Science and Food Safety, via Celoria 10, 20133 Milano, Italy

[f] Cornell University, Animal Health Diagnostic Center, Quality Milk Production Services, 240 Farrier Road, Ithaca, NY, 14853, USA

[g] Quantitative Genetics Group, Department of Animal Production, Faculty of Veterinary Medicine, University of Liège, Sart-Tilman, Bât. 43a, B-4000 Liège, Belgium

[*]Corresponding author. Tel.: +32 4 366 95 22; Fax: +32 4 366 42 61; mbardiau@ulg.ac.be

Abstract

Staphylococcus (S.) aureus is recognised worldwide as an important pathogen causing contagious acute and chronic bovine mastitis. Chronic mastitis account for a significant part of all bovine cases and represent an important economic problem for dairy producers. Several properties (biofilm formation, intracellular survival, capsular expression and group *agr*) are thought to be associated with this chronic status. In a previous study, we found the existence of two groups of strains based on the association of these features. The aim of the present work was to confirm on a large international and non-related collection of strains the existence of these clusters and to associate them with case history records. In addition, the genomes of eight strains were sequenced to study the genomic differences between strains of each cluster. The results confirmed the existence of both groups based on capsular typing, intracellular survival and *agr*-typing: strains *cap8*-positive, belonging to *agr* group II, showing a low invasion rate and strains *cap5*-positive, belonging to *agr* group I, showing a high invasion rate. None of the two clusters were associated with the chronic status of the cow. When comparing the genomes of strains belonging to both clusters, the genes specific to the group "*cap5-agr*I" would suggest that these strains are better adapted to live in hostile environment. The existence of these two groups is highly important as they may represent two clusters that are adapted differently to the host and/or the surrounding environment.

Keywords: *Staphylococcus aureus*; bovine mastitis; persistence; capsule; biofilm; intracellular survival

Introduction

Staphylococcus aureus (*S. aureus*) is an important bacteria causing contagious bovine mastitis (Watts, 1988). *S. aureus* strains can cause acute, usually clinical, and chronic, usually subclinical, mastitis. Subclinical mastitis is characterised by a non-alteration of the milk but high somatic cell count, making the milk inappropriate for the consumers if the cell count is too high. This type of mastitis is often chronic and account for up to 30% of all bovine cases (Halasa *et al.*, 2007), which represents an important economic problem for dairy producers with reduction in milk quantity and quality, prolonged costly antibiotic treatments and premature culling. Several properties are thought to be associated to some extent with these chronic infections: biofilm formation, intracellular survival, capsular expression and group accessory gene regulator (*agr*).

Biofilm formation is one of the most important survival mechanisms of bacteria living in the extracellular niche. It impairs the action of both the host immune system and antimicrobial agents (Costerton *et al.*, 1999; Melchior *et al.*, 2006). Second, *S. aureus* can be an intracellular pathogen of a large variety of eukaryotic cells, including epithelial cells of the mammary glands and immune cells (Almeida *et al.*, 1996; Kerro Dego *et al.*, 2002). Therefore, the bacteria are not only protected from the action of commonly used antibiotics in mastitis treatment (mainly β-lactams), and also able to persist in the host without causing any apparent inflammation (Boulanger *et al.*, 2003; Garzoni and Kelley, 2009). The absence of capsular expression enhance the adherence to and the invasion of eukaryotic cells by *S. aureus* (Pohlmann-Dietze *et al.*, 2000; Buzzola *et al.*, 2007; Tuchscherr *et al.*, 2005). In addition, *S. aureus* bacteria that do not express capsule induce chronic mastitis in mice, suggesting that the absence of capsule synthesis may help the bacteria to persist in the mammary

glands (Tuchscherr *et al.*, 2005). Finally, *agr* group I is associated with a persistence or with features that help the bacteria to persist in the udder: strains belonging to *agr* group I are more likely to be internalised in epithelial cells, to persist in murine mammary glands (Buzzola *et al.*, 2007) and to be associated with penicillin resistance (Melchior *et al.*, 2011) than the strains belonging to the other groups.

In a previous study (Bardiau *et al.*, 2014), we correlated *agr*-typing, capsular expression, biofilm formation, and intracellular survival in a collection of Belgian *S. aureus* strains from bovine mastitis (with no case history records). We found the existence of two groups based on the association of these features: *cap5*-positive strains belonging to *agr* group I, which in vitro test negative for CP5 ELISA and show a high invasion rate in MAC-T cells, and *cap8*-positive strains belonging to *agr* group II, which express CP8 in vitro and show a low invasion rate in MAC-T cells. We hypothesised that the first group may correspond to strains adapted to the intracellular niche leading to chronic infection and that the second group may correspond to strains better adapted to the extracellular niche leading to acute infection.

The aim of this work was therefore to confirm on a large European and non-related collection of strains the existence of these two groups based on features described to be associated with long-lasting infections and to find out if these two clusters are associated with a persistence of the disease. We therefore investigated the association between *agr*-typing, capsular antigen identity and expression, biofilm formation, intracellular survival and the case history data of a collection of *S. aureus* strains isolated from cases of chronic and acute bovine mastitis in four countries (Belgium, Italy, Canada and Switzerland). Moreover, the genomes of eight strains were

sequenced and compared to assess of the genomic differences between the formed clusters.

Materials and methods

Bacterial isolates

A total of 168 bovine *S. aureus* isolates, were collected from chronic and acute bovine intramammary infection in four countries. One hundred and two isolates were collected from chronic cases in Belgium (n=7), Canada (n=45), Italy (n=25) and Switzerland (n=25). Sixty-six isolates were collected from acute cases in Canada (n=46) and Italy (n=20). Strains have been isolated and identified as *Staphylococcus aureus* according to the protocol described in Ote *et al.* (2011). Chronic mastitis was defined as recurring isolates in the same quarter of the same animal. Four *S. aureus* reference strains were included in this collection: ATCC 29740 (N305), ATCC 31885 (NL6), ATCC 49521 (Lowenstein) and ATCC 49525 (Wright).

Capsular genotyping and serotyping

Capsular genotyping was performed using PCR detection of the capsule-encoding genes *cap5* and *cap8* genes as previously described (Ote *et al.*, 2011). Capsular serotyping was performed in in triplicates and in two independent experiments by enzyme-linked immunosorbent assay (ELISA) using specific monoclonal and polyclonal antibodies (kindly provided by GSK Biologicals, Belgium) against CAP5 and CAP8 as previously described (Bardiau *et al.*, 2014). For the serotyping, OD values were compared to those obtained with *S. aureus* CP reference strains, namely the CP5-positive strain ATCC 49521 and CP8-positive strain ATCC 49525, and isolates that tested negative for CP5- and CP8- ELISA were defined as non-typeable (NT).

Invasion assay

Bovine mammary epithelial cells (MAC-T) were used for in vitro bacterial internalisation assays as previously described (Bardiau *et al.*, 2014; Boulanger *et al.*, 2007; Brouillette *et al.*, 2003). Briefly, cell monolayers (~2.5 x 10^5 cells/well) were inoculated with 10^7 CFU of *S. aureus* (MOI~40) and incubated at 37°C in 5% CO_2 for three hours. After the removal of extracellular bacteria by phosphate buffered saline (PBS) washing and lysostaphin treatment, the MAC-T cells were detached and lysed by addition of 900 µl/well of sterile distilled water containing 0.025% Triton X-100. The cell lysates were carefully suspended, serially diluted, and plated on Columbia sheep blood agar plates to quantify intracellular staphylococci. Results are expressed as a percentage of the initial inoculum and classified using the same criteria as in our previous work (Bardiau *et al.*, 2014).

Biofilm production

Biofilm formation was evaluated by spectrophotometry in microplates using safranin staining as previously described (Bardiau *et al.*, 2014). Briefly, overnight cultures were diluted 1:100 in tryptic soy broth (TSB) containing 0.25% glucose (TSB_{glc}), transferred into wells of sterile 96-well polystyrene tissue culture (TC) plates and incubated at 37°C. TSB_{glc} without bacteria served as negative control. After 24h, the plates were stained with safranin 0.1% (w/v) for 10 min. A mixture of 50% ethanol and 50% acetic acid was added to each well and plates were incubated at room temperature for 15 min. Finally, the OD of each well was measured at 490 nm using a microplate reader. The results were collected from at least two independent experiments in which the biofilm formation of each culture tested was evaluated in triplicate. The quantitative classification of biofilm production based on cut-off value (ODc) and average OD values was carried out leading to four categories of strains: no

biofilm producer (OD ≤ ODc); weak biofilm producer (ODc < OD ≤ 2 × ODc); moderate biofilm producer (2 × ODc < OD ≤ 4 × ODc); strong biofilm producer (4 × ODc < OD) (Stepanovic *et al.*, 2007).

agr-typing

agr-groups were determined by multiplex PCR as previously described (Gilot *et al.*, 2002). In brief, multiplex PCRs were performed with the following primers: Pan (5'-ATG CAC ATG GTG CAC ATG C-3'), agr1 (5'-GTC ACA AGT ACT ATA AGC TGC GAT), agr2 (5'-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3'), agr3 (5'-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3'), and agr4 (5'-CGA TAA TGC CGT AAT ACC CG-3'). Amplifications were performed with the following PCR program: 1 cycle at 94°C for 1 min; 26 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and finally 1 cycle at 72°C for 10 min. All PCR products were separated by electrophoresis in 1.5% (w/v) agarose gel.

Statistical analysis

For calculation of the statistical significance of the observed frequency distributions, contingency tables of the expected values were determined and Chi-square tests were performed. *P* values lower than 0.05 were considered significant. K-means clustering method, adapted for the clustering of categorical data, was used to group similar strains into homogeneous groups (procedure fastclus, sas 9.1).

Genomes sequencing

Eight strains (four from group 1, named "*cap5-agr*I", isolated in Belgium and in Italy and four from group 2, named "*cap8-agr*II", isolated in Belgium and in Italy) were sequenced to assess of the genomic differences between strains of both groups. MiSeq next-generation sequencing NGS was performed according to the manufacturer's instructions using Nextera Mate Pair Library Preparation kit with

2x250bp paired-end (Illumina). Assembly was performed *de novo* using Platanus genome assembler. Annotations were performed using RAST server (Aziz *et al*., 2008). The eight *S. aureus* genomic sequences have been deposited at GenBank under the accession numbers xxx. Genomic analyses (Multi-Locus Sequences Typing-MLST and virulence factors) were performed using the Center for Genomic Epidemiology website platform (Larsen *et al*., 2012; Zankari *et al*., 2012; Joensen *et al*., 2014; Kaas *et al*., 2014).

Results

Capsular serotyping, invasion assay, biofilm formation and *agr*-typing (Table 1)

Ninety-seven strains (58%) and 71 strains (42%) harboured the *cap5* and *cap8* genes respectively. We then assessed the expression of the CP5 and CP8 polysaccharides by ELISA analysis. Eighty-two (49%) *S. aureus* isolates expressed CP5, 69 (41%) expressed CP8, and 17 (10%) were defined as non-typeable.

We chose to divide the distribution of the strains in the collection into 2 populations as we did in our previous study (Bardiau *et al*, 2014). Thus, we determined that 74 strains (44%) showed a lower invasion rate, while 94 strains (56%) showed a higher invasion rate.

Eighteen strains (11%) did not produce any biofilm, 85 (51%) produced weak biofilms, 49 (29%) produced moderate biofilms, while 16 (10%) produced strong biofilms.

In the collection of strains, three of the four *agr*-groups were detected by PCR, with two (*agr* group I and II) representing 99% of the strains: 97 (58%) strains belonged to *agr* group I, 69 (41%) belonged to *agr* group II, and two strains (1%) belonged to *agr* group IV.

Statistical associations and clustering method

We could find the following statistical associations (Table 1): (i) we first highlighted that more *cap8*-positive strains expressed CP8, whereas more *cap5*-positive strains were defined as non-typeable; (ii) we then observed that more *cap8*/CP8-strains showed a low invasion rate, whereas more *cap5*/NT or *cap5*/CP5 strains showed a higher invasion rate; (iii) furthermore, the intracellular survival capacity and the capsular serotype of the strains were associated with the *agr* groups: strains belonging to *agr* group I showed a higher internalisation rate and were mainly *cap5* positive, whilst strains belonging to *agr* group II showed a lower internalisation rate and were mainly *cap8* positive; (iv) no association could be observed between the biofilm formation and any other results (intracellular survival, capsular serotype and *agr*-typing).

All the strains grouped into two clusters by the K-Means statistical method (Table 2). The cluster 1 grouped strains that were *cap8*-positive, belonged to *agr* group II, showed a low invasion rate in MAC-T cells and formed weak or no biofilm in TSB_{glc}. The cluster 2 grouped strains that were *cap5*-positive, belonged to *agr* group I, showed a high invasion rate in MAC-T cells and formed weak, moderate or strong biofilm in TSB_{glc}.

These two groups were associated with the case history record (chronic or acute mastitis). However, no association was seen between the clusters and the chronic status of the infection (Table 2).

Cluster-associated variable gene content

In order to further evaluate the genomic differences between the strains belonging to both clusters, we sequenced eight genomes: four strains belonging to the group "*cap5-agr*I" (two isolated in Belgium and two isolated in Italy) and four strains

belonging to the group "*cap8-agr*II" (two isolated in Belgium and two isolated in Italy). The size of the sequenced genomes varied from 2.55 Mb to 2.77 Mb and the number of coding sequences from 1944 to 2012. MLST and virulence genes profile were determined for all sequenced strains (Table 3). Six strains belong to five different ST (ST115, ST504, ST8, ST97, ST479) and the ST of two strains could not be identified. Some virulence genes could not be found in any strain: *aap* coding for the accumulation-associated protein, *sea, sec, seg, sei, sel, sen, seo* respectively coding for the enterotoxins A, C, G, I, L, N and O, *etb* coding for the exfoliative toxins B, *cna* coding for the collagen-binding protein. Some virulence genes were detected in all strains: the *cap* operon coding for the capsule, *lukF'-PV* coding for the Panton-Valentine leukocidin chain F, *hlb* coding for the beta-haemolysin precursor, *hlg* coding for the gamma-haemolysin precursor, *atl* coding for the bifunctional autolysin Atl, *etA* coding for the exfoliative toxin A, *splA* coding for the serine protease SplA, *sspA* coding for the serine V8 protease, *vwb* coding for the von Willebrand factor-binding protein, *eap* coding for the extracellular adherence protein, *spa* coding for the protein A. When comparing the presence of the virulence genes in the strains regarding their groups, *lukM*, the gene coding for the leukocidin LukM, was present in most of the "*cap8-agr*II" strains and none of the "*cap5-agr*I" strains and three genes, *hld*, *clfA* and *sdrC* respectively coding for the delta-haemolysin precursor, the clumping factorA and the Bone sialoprotein-binding protein, were present in most if not all "*cap5-agr*I" strains and in only one if not none "*cap8-agr*II" strains.

When comparing all genomes, nine genes appeared to be exclusively specific to the "*cap5-agr*I" strains and one to the "*cap8-agr*II" strains. When relaxing the stringency of the comparison (presence in minimum three out of four strains in one

group and absence in minimum three out of four strains in the other one), we could identify 51 (and therefore a total of 60 genes when including the nine exclusively specific) and 13 (and therefore a total of 14 genes when including the one exclusively specific) genes more specific to cluster 1 "*cap5-agr*I" and cluster 2 "*cap8-agr*II" isolates, respectively (Figure 1). The 60 genes over-represented in the cluster 1 "*cap5-agr*I" were assigned to the following functional categories: antibiotic and heavy metal resistance (β-lactamase, cadmium and arsenic resistance proteins), capsular proteins (*cap5*), DNA-related proteins (DNA-invertase, transposase, putative primase, transcriptional regulator, mutator mutT, cl-like repressor), putative membrane protein, proline/glycine betaine transporter, pathogenicity island (Staphylococcal Pathogenicity Island 2), fibrinogen-binding protein, δ-haemolysin and hypothetical proteins. The 14 genes over-represented in the cluster 1 "*cap8-agr*II" were assigned to the following functional categories: exotoxin homologue (genomic island nu Sa alpha2), DNA-related proteins (DBA-cytosine methyltransferase, transcriptional regulator), intracellular protease/amidase (Thij/Pfpl family), pathogenicity island (Staphylococcal Pathogenicity Island 1 Orf21), phage minor head and hypothetical proteins.

Discussion

In a previous study (Bardiau *et al.*, 2014), it was found that *S. aureus* strains isolated from Belgian bovine mastitis could be divided into several clusters based on features potentially associated with long-lasting infections (biofilm formation, capsular profile, *agr*-typing and intracellular survival). In this present work, we aimed to confirm using a large international and non-related collection of strains the existence of these clusters and to find out whether they could be associated with a persistence of the infection of the udder. Therefore, we studied the association between these features and the case history of strains isolated from chronic and acute bovine mastitis from four countries (Belgium, Canada, Italy, Switzerland). We also investigated the genomic characteristics of eight strains belonging to the different groups.

First, we find the same kind of associations than in our first study when comparing results two by two, except for the biofilm formation that was not associated with any other features. When correlating the results with the country of origin, the repartition is evenly distributed (data not shown); therefore the country of origin does not introduce any bias in the statistical analysis of the results.

When strains were clustered (Bardiau *et al.*, 2014), similar correspondences were observed: one cluster of strains that are *cap8*-positive, belong to *agr* group II, show a low invasion rate in MAC-T cells and form weak or no biofilm in TSB_{glc}, and one cluster of strains that are *cap5*-positive, belong to *agr* group I, show a high invasion rate in MAC-T cells and form weak, moderate or strong biofilm in TSB_{glc}. Our previous hypothesis (Bardiau *et al.*, 2014) was that the first group of "*cap8-agr*II" strains are better adapted to an extracellular niche and therefore could be associated with acute mastitis and that the second group of "*cap5-agr*I" strains are better adapted

to an intracellular niche and therefore could be associated with chronic mastitis. However, no correlation is found in this study between any feature of the strain and the case history data. The fact that those case history data were determined on an individual basis in each country of origin and not as part of the same study using the same criteria may of course have introduced a bias in this study and therefore explain in part this absence of correlation.

We analysed the presence of virulence genes in the eight sequenced strains. The relative presence of the genes vary from 0% to 100% and no clear genotype subtype regrouping several strains appears as previously shown by our group (Ote *et al*., 2011). Nevertheless, four genes are specific to one group or the other ("*cap5-agr*I" or "*cap8-agr*II"): *lukM* to "*cap8-agr*II" and *hld*, *clfA* and *sdrC* to "*cap5-agr*I". These results are in accordance with the study published by Peton *et al.* in 2014 in which they have compared two *S. aureus* bovine strains, N305 that produces mild and chronic mastitis and RF122 that produces severe mastitis. In their study, *lukM* is present in RF122 and absent in N305. *lukM* in association with *lukF-PV* form LukM/F', a protein involved in cytotoxicity against polynuclear neutrophils, mainly described during a strong inflammatory reaction in the mammary gland and therefore in severe clinical mastitis. In contrast, in their study, the gamma-haemolysin is more produced in N305 than in RF122. In our study, we observe that the gamma-haemolysin gene is more present in the "*cap5-agr*I" than in the "*cap8-agr*II". The protein encoded by the *sdrC* gene promotes both bacterial adherence to surfaces and biofilm formation (Barbu *et al*., 2014). The clumping factor A promotes bacterial attachment to eukaryotic cells, induces formation of bacterial clumps and decreases the phagocytosis. Both proteins could be implicated in the persistence of the infection by helping the bacteria to survive in hostile environment.

Finally, when analysing the genes over-represented in either group of the eight strains (four from either group) that were sequenced, we can hypothesise that the strains "*cap5-agr*I" are better adapted to live and persist in the environment due to the presence of heavy metal resistance genes and proline/glycine betaine transporter. It has indeed been shown that the proline/glycine betaine confers osmotic protection to various bacterial species including *Staphylococcus sp.* (Amin, 1995), thus it may help the bacteria to survive in high osmolality environments. It is interesting to note that high level of glycine betaine has recently been associated with planktonic versus biofilm producer staphylococci (Junka *et al.*, 2013). The presence of heavy metal resistance genes could also help the bacteria to survive in hostile environments and can facilitate the persistence and dissemination of the bacteria (Srivastava, 2012; Nair, 2014). Antibiotic resistance genes (especially production of β-lactamase), here associated with the strains "*cap5-agr*I", have previously been linked with *agr*I-positive strains (Melchior *et al*, 2011). However several of the genes over-represented in both groups are hypothetical proteins. It would be interesting to investigate their presence in a larger collection of strains to target the ones that seem to be specific to either group and to study their functions.

Conclusion

In conclusion, we confirm the existence of two groups of *S. aureus* strains isolated from bovine mastitis based on capsular typing, intracellular survival and *agr*-typing. Strains *cap8*-positive, belonging to *agr* group II, show a low invasion rate and strains *cap5*-positive, belonging to *agr* group I, show a high invasion rate. Despite the fact that we could not correlate these groups with the case history data, the existence of these two groups is highly important as they may represent two clusters that are adapted differently to the host and/or the surrounding environment. When comparing

the genomes of strains belonging to both clusters, only 14 genes are over-represented in the group "*cap8-agr*II", but 60 genes are over-represented in the group "*cap5-agr*I". The genes specific to the group "*cap5-agr*I" would suggest that these strains are better adapted to live in the environment than the strains belonging to "*cap8-agr*II". The first perspective of this study would be to check for the presence of these genes in a larger collection of strains to assess of their specificity for each cluster. In addition, it would be beneficial to study these features (intracellular survival, biofilm formation, capsular typing and *agr*-typing) in *S. aureus* strains isolated from other hosts and other pathologies to specify if these clusters are specific to bovine mastitis *S. aureus* strains or are present in all *S. aureus* strains. Finally, a broader study collecting worldwide strains from mastitis with anamnesis done with the same criteria would be ideal to assess of the existence of groups regarding the chronic status of the cow.

Acknowledgements

This work was supported by the Walloon Regional government (SPW-DGARNE, *convention D31-1231*) and by the European Network of Excellence EADGENE (European Animal Disease Genomics Network of Excellence for Animal Health and Food Safety). We thank GSK Biologicals (Belgium) for the kind gift of the capsule antibodies. The Canadian strains have been provided by the Canadian Bovine Mastitis Network with the help of the Natural Sciences and Engineering Research Council of Canada, Alberta Milk, Dairy Farmers of New Brunswick, Nova Scotia, Ontario and Prince Edward Island, Novalait Inc., Milk producers of Canada, Canadian milk network, AAC, ASPC, Technology PEI Inc., the University of Montréal and the University of Prince Edward Island. M.B. is currently a Marie Skłodowska-Curie post-doctoral fellow (European Union FP7 program) at the Environment & Public Health Research Group, School of Environment & Technology, University of Brighton, England, UK.

373 **References**

374

375 Almeida, R.A., Matthews, K.R., Cifrian, E., Guidry, A.J., Oliver, S.P., 1996.
376 *Staphylococcus aureus* invasion of bovine mammary epithelial cells. J. Dairy
377 Sci. 79, 1021-1026.

378 Amin, U.S., Lash, T.D., Wilkinson, B.J., 1995. Proline betaine is a highly effective
379 osmoprotectant for *Staphylococcus aureus*. Arch. Microbiol. 163, 138-142.

380 Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., …
381 Zagnitko, O. 2008. The RAST Server: Rapid Annotations using Subsystems
382 Technology. BMC Genomics. 9, 75.

383 Barbu, EM, Mackenzie, C., Foster, TJ, Höök, M., 2014. SdrC induces
384 staphylococcal biofilm formation through a homophilic interaction. Moel.
385 Microbiol., 94 (1), 172-85.

386 Bardiau, M., Detilleux, J., Farnir, F., Mainil, J. G., Ote, I. 2014. Associations
387 between properties linked with persistence in a collection of *Staphylococcus*
388 *aureus* isolates from bovine mastitis. Vet. Microbiol. 169, 74-79.

389 Boulanger, D., Brouillette, E., Jaspar, F., Malouin, F., Mainil, J., Bureau, F.,
390 Lekeux, P., 2007. Helenalin reduces *Staphylococcus aureus* infection in vitro
391 and in vivo. Vet. Microbiol. 119, 330-338.

392 Boulanger, D., Bureau, F., Melotte, D., Mainil, J., Lekeux, P., 2003. Increased
393 nuclear factor kappaB activity in milk cells of mastitis-affected cows. J. Dairy
394 Sci. 86, 1259-1267.

395 Brouillette, E., Grondin, G., Shkreta, L., Lacasse, P., Talbot, B.G., 2003. *In vivo*
396 and *in vitro* demonstration that *Staphylococcus aureus* is an intracellular

pathogen in the presence or absence of fibronectin-binding proteins. Microb. Pathog. 35, 159-168.

Buzzola, F.R., Alvarez, L.P., Tuchscherr, L.P., Barbagelata, M.S., Lattar, S.M., Calvinho, L., Sordelli, D.O., 2007. Differential abilities of capsulated and noncapsulated *Staphylococcus aureus* isolates from diverse *agr* groups to invade mammary epithelial cells. Infect. Immun. 75, 886-891.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318-1322.

Garzoni, C., Kelley, W.L., 2009. *Staphylococcus aureus*: new evidence for intracellular persistence. Trends Microbiol. 17, 59-65.

Gilot, P., Lina, G., Cochard, T., Poutrel, B., 2002. Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. J. Clin. Microbiol. 40, 4060-4067.

Halasa, T., Huijps, K., Osteras, O., Hogeveen, H., 2007. Economic effects of bovine mastitis and mastitis management: a review. Vet. Q. 29, 18-31.

Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM, 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. J. Clin. Micobiol. 52(5): 1501-1510.

Junka, A.F., Deja, S., Smutnicka, D., Szymczyk, P., Ziółkowski, G., Bartoszewicz, M., Młynarz, P., 2013. Differences in metabolic profiles of planktonic and biofilm cells in *Staphylococcus aureus* - (1)H Nuclear Magnetic Resonance search for candidate biomarkers. Acta Biochim. Pol. 60, 701-706.

Kaas, R.S., Leekitcharoenphon, P., Aarestrup, F.M., Lund, O., 2014. Solving the Problem of Comparing Whole Bacterial Genomes across Different Sequencing Platforms. PLoS ONE; 9(8): e104984.

Kerro Dego, O., van Dijk, J.E., Nederbragt, H., 2002. Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. A review. Vet. Q. 24, 181-198.

Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-Pontén T, Ussery DW, Aarestrup FM and Lund O, 2012. Multilocus Sequence Typing of Total Genome Sequenced Bacteria. J. Clin. Micobiol. 50(4): 1355-1361.

Melchior, M.B., Vaarkamp, H., Fink-Gremmels, J., 2006. Biofilms: a role in recurrent mastitis infections? Vet. J. 171, 398-407.

Melchior, M.B., van Osch, M.H., Graat, R.M., van Duijkeren, E., Mevius, D.J., Nielen, M., Gaastra, W., Fink-Gremmels, J., 2009. Biofilm formation and genotyping of *Staphylococcus aureus* bovine mastitis isolates: evidence for lack of penicillin-resistance in Agr-type II strains. Vet. Microbiol. 137, 83-89.

Melchior, M. B., van Osch, M. H. J., Lam, T. J. G. M, Vernooij, J. C. M., Gaastra, W, Fink-Gremmels, J., 2011. Extended biofilm susceptibility assay for *Staphylococcus aureus* bovine mastitis isolates: Evidence for association between genetic makeup and biofilm susceptibility. J. Dairy Sci. 94, 5926–5937.

Ote, I., Taminiau, B., Duprez, J.N., Dizier, I., Mainil, J.G., 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet. Microbiol. 153, 285-292.

Peton, V., Bouchard, D., Almeida, S., Rault, L., et al. 2014. Fine-tuned characterization of Staphylococcus aureus Newbould 305, a strain associated with mild and chronic mastitis in bovines. Vet. Res. 45:106. 1–15.

Pohlmann-Dietze, P., Ulrich, M., Kiser, K.B., Doring, G., Lee, J.C., Fournier, J.M., Botzenhart, K., Wolz, C., 2000. Adherence of *Staphylococcus aureus* to endothelial cells: influence of capsular polysaccharide, global regulator agr, and bacterial growth phase. Infect. Immun. 68, 4865-4871.

Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukic, S., Cirkovic, I., Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115, 891-899.

Tuchscherr, L.P., Buzzola, F.R., Alvarez, L.P., Caccuri, R.L., Lee, J.C., Sordelli, D.O., 2005. Capsule-negative *Staphylococcus aureus* induces chronic experimental mastitis in mice. Infect. Immun. 73, 7932-7937.

Watts, J.L., 1988. Etiological agents of bovine mastitis. Vet. Microbiol. 16, 41-66.

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV, 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. Jul 10.

Table 1. Capsular profile, intracellular survival, biofilm formation and *agr*-groups frequency distribution.

		Intracellular survival		Biofilm formation				*agr*-groups			
		Low	**High**	**No**	**Weak**	**Moderate**	**Strong**	**I**	**II**	**III**	**IV**
Capsular profile	*cap5*/NT	4	**11**	0	8	6	1	**15**	0	0	0
	cap5/CP5	20	**62**	2	41	26	13	**74**	8	0	0
	cap8/NT	1	1	0	1	1	0	0	2	0	0
	cap8/CP8	**49**	20	16	35	16	2	8	**59**	0	2
Intracellular survival			**Low**	**14**	41	14	5	22	**50**	0	2
			High	4	44	**35**	11	**75**	19	0	0
Biofilm formation							**No**	3	15	0	0
							Weak	47	38	0	0
							Moderate	32	15	0	2
							Strong	15	1	0	0

Significant correlations are highlighted in bold. *cap*, capsule-encoding gene; CP, capsular polysaccharide; NT: non-typeable.

Table 2. Clustering of the strains according to their capsular profile, intracellular survival, biofilm formation and *agr*-groups (K-Means method). The anamnesis data are mentioned for each cluster.

		Clusters	
		1	**2**
Capsular profile	*cap5*/NT	0	15
	cap5/CP5	7	**75**
	cap8/NT	2	0
	cap8/CP8	**61**	8
Intracellular survival	Low	**52**	22
	High	18	**76**
Biofilm formation	No	**15**	3
	Weak	**38**	**47**
	Moderate	16	33
	Strong	1	15
***agr*-groups**	I	0	**97**
	II	**68**	1
	III	0	0
	IV	2	0
Anamnesis	Chronic	41	61
	Acute	29	37
	TOTAL	**70**	**98**

Significant correlations (P<0.001) are highlighted in bold. Capsular profile: *cap*, capsule-encoding gene; CP, capsular polysaccharide; NT, non-typeable. Intracellular survival: presented as the % of the initial inoculums. agr-typing: I, II, III, IV, agr-groups.

Table 3. MLST and virulence factors genes contents of all sequenced strains.

	*cap8-agr*II strains				*cap5-agr*I strains			
	120	**299**	**682**	**685**	**19**	**141**	**663**	**674**
MLST	ST504	ST504	ni	ST479	ST115	ni	ST8	ST97
Biofilm formation								
ica operon	+	+	-	+	+	+	+	+
aap	-	-	-	-	-	-	-	-
cap operon	+	+	+	+	+	+	+	+
Secreted toxins								
lukE	+	+	+	+	-	+	+	+
lukD	-	+	+	-	+	+	+	-
lukM	+	+	-	+	-	-	-	-
lukF'-PV	+	+	+	+	+	+	+	+
lukS	+	+	+	-	+	+	+	+
hla	-	-	-	+	-	+	+	-
hlb	+	+	+	+	+	+	+	+
hlg	+	+	+	+	+	+	+	+
hld	-	-	+	-	+	+	+	+
atl	+	+	+	+	+	+	+	+
tst	+	+	-	-	+	-	-	-
etA	+	+	+	+	+	+	+	+
etB	-	-	-	-	-	-	-	-
sea	-	-	-	-	-	-	-	-
sec	-	-	-	-	-	-	-	-
seg	-	-	-	-	-	-	-	-
sei	-	-	-	-	-	-	-	-
sel	-	-	-	-	-	-	-	-
sen	-	-	-	-	-	-	-	-
seo	-	-	-	-	-	-	-	-
splA	+	+	+	+	+	+	+	+
splE	+	+	-	-	+	+	+	+
sspA	+	+	+	+	+	+	+	+
Colonisation factors								
vwb	+	+	+	+	+	+	+	+
clfA	-	-	-	-	+	+	+	+
clfB	-	+	-	+	-	+	+	+
fnbA	+	+	-	-	-	-	-	-
fnbB	-	-	-	-	-	-	+	-
eap	+	+	+	+	+	+	+	+
sak	-	-	-	-	-	-	+	-
spa	+	+	+	+	+	+	+	+
cna	-	-	-	-	-	-	-	-
ebpS	+	+	+	-	+	+	+	+
sdrC	-	-	+	-	-	+	+	+

Ni: not identified; Genes coding for: *ica*, Intercellular adhesion protein A; *aap*, accumulation-associated protein; *cap*, capsular polysaccharide; *lukE*, leukocidin LukE; *lukD*, leukocidin LukD; *lukM*, leukocidin LukM; *lukF'-PV*, Panton-Valentine leukocidin chain F; *lukS*, leukocidin chain S; *hla*, Alpha-haemolysin precursor; *hlb*, Beta-haemolysin precursor; *hlg*, Gamma-haemolysin precursor; *hld*, Delta-haemolysin precursor; *atl*, Bifunctional autolysin precursor; *tst*, Toxic shock syndrome toxin-1; *etA*, exfoliative toxin A; *etB*, exfoliative toxin B; *sea*, enterotoxin A; *sec*, enterotoxin C; *seg*,

enterotoxin G; *sei*, enterotoxin I; *sel*, enterotoxin L; *sen*, enterotoxin N; *seo*, enterotoxin O; *splA*, Serine protease SplA; *splE*, Serine protease SplE; *sspA*, Serine V8 protease; *vwb*, Secreted von Willebrand factor-binding protein precursor; *clfA*, Clumping factor ClfA; *clfB*, Clumping factor ClfB; *fnbA*, Fibronectin-binding protein A; *fnbB*, Fibronectin-binding protein B; *eap*, extracellular protein; *sak*, staphylokinase; *spa*, Protein A (IgG-binding protein); *cna*, Collagen-binding protein; *ebpS*, Cell surface elastin-binding protein; *sdrC*, serine-aspartate repeat protein.