



HAL
open science

Genetic differences among isolates from dairy ruminant species: a single-dye DNA micro-array approach

E. Vautor, V. Magnone, G. Rios, K. Le Brigand, D. Bergonier, G. Lina, H. Meugnier, P. Barbry, Régis Thiery, Michel Pépin

► To cite this version:

E. Vautor, V. Magnone, G. Rios, K. Le Brigand, D. Bergonier, et al.. Genetic differences among isolates from dairy ruminant species: a single-dye DNA micro-array approach. *Veterinary Microbiology*, 2008, 133 (1-2), pp.105. 10.1016/j.vetmic.2008.06.006 . hal-00532442

HAL Id: hal-00532442

<https://hal.science/hal-00532442>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Genetic differences among *Staphylococcus aureus* isolates from dairy ruminant species: a single-dye DNA micro-array approach

Authors: E. Vautor, V. Magnone, G. Rios, K. Le Brigand, D. Bergonier, G. Lina, H. Meugnier, P. Barbry, R. Thiéry, M. Pépin

PII: S0378-1135(08)00218-6
DOI: doi:10.1016/j.vetmic.2008.06.006
Reference: VETMIC 4066

To appear in: *VETMIC*

Received date: 3-1-2008
Revised date: 2-6-2008
Accepted date: 4-6-2008

Please cite this article as: Vautor, E., Magnone, V., Rios, G., Le Brigand, K., Bergonier, D., Lina, G., Meugnier, H., Barbry, P., Thiéry, R., Pépin, M., Genetic differences among *Staphylococcus aureus* isolates from dairy ruminant species: a single-dye DNA micro-array approach, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.06.006

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1 **Genetic differences among *Staphylococcus aureus***
2 **isolates from dairy ruminant species: a single-dye**
3 **DNA micro-array approach.**

4
5 E. Vautor ^{a,*}, V. Magnone ^{b,c}, G. Rios ^{b,c}, K. Le Brigand ^{b,c},
6 D. Bergonier ^d, G. Lina ^e, H. Meugnier ^e, P. Barbry ^{b,c} R. Thiéry ^a,
7 M. Pépin ^a

8
9 ^a*Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Unité Pathologie des Petits*
10 *Ruminants, Sophia-Antipolis, France*

11 ^b*CNRS and* ^c*Université de Nice-Sophia Antipolis, Institut de Pharmacologie Moléculaire et*
12 *Cellulaire UMR 6097, Valbonne, France*

13 ^d*Ecole Nationale Vétérinaire (ENVT), UMR 1225 INRA-ENVT Interactions Hôtes Agents-*
14 *Pathogènes, Toulouse, France*

15 ^e*INSERM U851, Centre National de Référence des Staphylocoques, Université Lyon 1,*
16 *Hospices Civils de Lyon, Lyon, France*

17
18 *Corresponding author : AFSSA Sophia-Antipolis, 105 route des Chappes, BP111, F-06902
19 Sophia-Antipolis (France). Phone: 33 4 92943711. Fax: 33 4 92943701 ; E-mail:
20 e.vautor@sophia.afssa.fr

21

22 **Abstract**

23

24 *Staphylococcus aureus* is recognized worldwide as a major pathogen causing clinical or
25 subclinical intramammary infections in lactating sheep, goats and cows. The present study
26 was carried out to compare 65 *S. aureus* isolates mainly obtained from nasal carriage and
27 subclinical mastitis in dairy sheep and 43 *isolates* obtained from subclinical mastitis from 22
28 goats and 21 cows. A DNA microarray, containing probes against 190 true or putative
29 virulence factors, was used to detect the presence of the virulence genes. Their
30 presence/absence was independently assessed by PCR for the genes of interest. Sheep isolates
31 obtained from the nostrils or the udders did not show any significant tissue specific virulence
32 factor. The dominant pulse-field electrophoresis profile (OV/OV'), associated with *spa* clonal
33 complex *spa*-CC1773, matched mainly with the *agr* group III and was only found in ovine
34 and caprine isolates. This clone was more specifically characterized by the prevalence of the
35 following virulence genes: *lpl4*, *ssl6*, *bsaA1*, *bsaB*, *bsaP*, SAV0812. Moreover, seven
36 virulence-associated genes (*lpl1*, *sel*, *sec*, *tst*, *lukF*-PV like component, *lukM*, SAV0876) were
37 associated with isolates from small ruminants, while the *egc* cluster, *fhuD1*, *abiF* and
38 SAV2496 with bovine isolates. This genomic study suggests the existence of lineage- and
39 host-specific genes leading to the development of host-specific pathogenic traits of
40 *Staphylococcus aureus* isolates.

41

42 **Keywords:** *Staphylococcus aureus*; mastitis, host-specific genes; sheep; goats; cows

43 1. Introduction

44

45 *Staphylococcus aureus* is an important cause of mammary gland disease in dairy ruminant
46 species: sheep, goats, cows (Mork et al., 2005a). As in humans and probably in cows and
47 goats, the nasal carriage of *S. aureus* in sheep is frequent. The upper respiratory tract is a
48 possible reservoir for the contamination of udders and milk in dairy farms (Vautor et al.,
49 2005). For dairy cows and sheep, it has been shown that several *S. aureus* strains are shared
50 within and between many herds, even at a long distance. The existence of such dominant
51 strains of *S. aureus* suggests that these clones have special properties, in particular special
52 virulence-associated genes to overcome host defenses and to establish a successful
53 intramammary infection in ewes, goats or in cows (Stephan et al., 2001; Vautor et al., 2003).
54 Although many putative virulence factors have been identified in *S. aureus* by whole-genome
55 sequencing (Kuroda et al., 2001), the potential differences of pathogenicity among isolates
56 from naturally occurring infections remains largely unknown.

57 The accessory gene regulator (*agr*) locus of *S. aureus* encodes a two-component signal
58 transduction system that leads to down-regulation of surface proteins and up-regulation of
59 secreted proteins during growth *in vitro*. Four allelic groups of *agr* that are related to the
60 genetic background and to the presence of virulence factors have been characterized in human
61 strains (Jarraud et al., 2002). However the relative distribution of the *agr* groups in *S. aureus*
62 isolates obtained from dairy species with diagnosed mastitis, is still unknown.

63 In this study, 108 *S. aureus* isolates were obtained from sheep, goats and cows. A single-
64 dye microarray approach was used: (i) to assess the presence of 190 virulence genes or alleles
65 (e.g. *agr* groups, toxin genes, exoenzyme genes,...) in *S. aureus* isolates that possess distinct
66 PFGE profiles and *spa* types, (ii) to assess whether the nasal carriage isolates from sheep had
67 specific adaptative virulent genes in comparison to udder isolates, (iii) to identify genetic

68 polymorphism of *S. aureus* associated with the relative host specificity in cows, goats and
69 sheep.

70

71

72 **2. Materials and methods**

73

74 *2.1. Bacterial isolates* A total of 108 *S. aureus* isolates were included in this study. The
75 isolates from dairy sheep (n = 65) were mainly collected (i) during two field surveys in the
76 southeast of France in farms without any obvious epidemiological link (Vautor et al., 2003;
77 Vautor, et al., 2005) and (ii) from field surveys in two other regions specialized in sheep dairy
78 production located in the southwest of France (Roquefort and Atlantic Pyrenees areas)
79 between 1995 to 2003. They were obtained from animals presenting subclinical mastitis (n =
80 34), nasal carriage (n = 21) and other clinical features or origins (n = 10).

81 The isolates from goats (n = 22) and dairy cows (n = 21) were collected from
82 subclinical mastitis cases throughout the main French areas of dairy production, in farms
83 without any epidemiological link. The bovine strain V329 from Spain was kindly provided by
84 Dr. José R. Penadés. Seventy-five dairy farms were involved in the study (32 for sheep, 22 for
85 goats and 21 for cows).

86 All the isolates were coagulase-positive and were confirmed as *S. aureus* by PCR
87 performed on the 23S rDNA gene (Straub et al., 1999).

88

89 *2.2. PFGE (pulsed-field gel electrophoresis)*

90

91 Briefly, a contour clamped homogeneous electrophoresis technique (CHEF) on the
92 GenePath™ system (Bio-Rad Laboratories, Hercules, CA, USA) was used according to the
93 manufacturer's instructions. The DNA was digested by *Sma*I. The Isolates of *S. aureus* were

94 classified in groups of identical or related strains by visual comparison of patterns. All the
95 strains with a pulsotype closely or possibly related to OV were named OV' (less than 6-band
96 differences). The Strains with patterns of with more than 6-band differences were classified
97 as unrelated or different and named "not OV/OV" (Vautor et al., 2003; Vautor et al., 2005).

98

99 2.3. *spa* typing

100

101 The polymorphic X region of the protein A gene (*spa*) was amplified using the
102 primers spa-1113f (5' TAA AGA CGA TCC TTC GGT GAG C 3') and spa-1514r (5' CAG
103 CAG TAG TGC CGT TTG CTT 3'), and sequenced. Applying the recently developed
104 algorithm BURP (Based Upon Repeat Patterns) the *spa* types (*spa*-t) were clustered into
105 different groups with a calculated cost between members of a group less or equal to 5. The
106 BURP *spa* clonal complexes (*spa*-CCs) were automatically assigned by Ridom Staph Type
107 software using the code system described on the Ridom SpaServer website
108 (www.spa.ridom.de).

109

110 2.4. Single-dye DNA microarrays

111

112 2.4.1. *DNA extraction*. The isolates were subcultured in trypticase agar soy plates and
113 incubated overnight at 37°C in Luria-Bertani broth. Bacterial cells were harvested (1.5 ml)
114 and lysed in a solution of lysozyme (20 mg.ml⁻¹) and lysostaphine (200 µg.ml⁻¹). DNA was
115 extracted with the DNeasy®Tissue kit (Qiagen, Courtaboeuf, France) according to the
116 manufacturer's recommendations. The nucleic acid concentration was determined by
117 measuring the absorbance with a spectrophotometer.

118 2.4.2. *DNA labelling*. Two µg of DNA were labelled with fluorescent Cy5-dUTP (GE
119 Healthcare, Orsay, France) by random-primer labelling with the Bio Prime DNA labelling

120 system kit (Invitrogen, Cergy Pontoise, France) for 3 hours at 37°C. The Labelled DNA was
121 then purified from the reaction mixture by using the QIAquick PCR purification kit (Qiagen,
122 Courtaboeuf, France), following the manufacturer's protocol. The bound DNA was eluted
123 from the column with 53 µl DNAase-free distilled water and stored at -20°C until used for
124 hybridization.

125 *2.4.3. Array design.* The array was designed to include *S. aureus* genes that are truly or
126 potentially associated to virulence: toxins, leukocidin, coagulase, adhesins, proteases,
127 nucleases, lipoproteins, lipases, capsular polysaccharides, etc. They were identified in the
128 genome of strain Mu50, Genbank accession number BA000017 (Kuroda et al., 2001). The
129 selected genes were completed by 28 additional virulence-associated genes from other *S.*
130 *aureus* strains. Details are given in the supplemental material (Appendix A). Positive and
131 negative control genes were included. Single oligonucleotide probes were designed for each
132 gene (188 genes) using the OligoArray program (Rouillard et al., 2002). Each probe was a 65
133 mer with a calculated T_m of approximately 83°C and with a minimal internal structure. The
134 identification of the genes and the probe's sequences are available in the public database
135 ArrayExpress® under the accession number E-TABM-288 (Brazma et al., 2003).

136 *2.4.4. Printing and slide Processing.* The oligonucleotides were printed on UltraGAPS™
137 Coated Slides (Corning, Schiphol-Rijk, The Netherlands) by means of a ChipWriter Pro
138 (Bio-Rad Laboratories, Richmond, California). Two replicates were printed for each
139 oligonucleotide and the pattern was printed to each slide in duplicate. The spotting solution
140 alone was printed as a negative control.

141 *2.4.5. Slide prehybridization.* Each slide was prehybridized at 42°C for 45min in a solution
142 (5× SSC, 0.1%SDS and 0.1mg/ml BSA), transferred twice in a solution (0.1× SSC) at room
143 temperature, immersed 30 sec in purified water, and finally dried by centrifugation (1600
144 rpm for 2 min).

145 2.4.6. *Hybridization*. The hybridization experiments were performed with the labelled Cy5
146 DNA (target). The target was denatured by heating at 95°C for 5 min and allowed to cool
147 down to room temperature before the addition of 12 µl of the hybridization solution (35%
148 formamide, 5× SSC, 0.1 % SDS) in a 30 µl final volume. The slides were hybridized under a
149 24x50 mm glass coverslip. They were incubated for 16 h at 42°C in hybridization chambers
150 (Corning, Schiphol-Rijk, The Netherlands) protected from the light. Following hybridization,
151 the slides were immersed in a solution (2× SSC, 0.1 % SDS) at 42°C until the coverslip
152 moved freely away from the slide. The slides were incubated twice in the same solution at
153 42°C for 5 min. Then, they were washed 4 times with 0.1×SSC at room temperature. After a
154 final wash in 0.01× SSC for 10 seconds, the slides were dried by centrifugation at 1600 rpm
155 during 2 min.

156 The arrays were scanned in the red channel of a Gene Pix® autoloader 4200 AL scanner or
157 a Gene Pix® 4000B scanner (Molecular Devices Corporation, Sunnyvale, California). The
158 images were processed using GenePix Pro® version 6.0 (Molecular Devices Corporation,
159 Sunnyvale, California). Briefly, the background signals were subtracted and the medians of
160 the four spots were calculated. A cut-off value was determined for each gene in order to
161 classify each gene as “present”, “absent or divergent” or “not interpretable”. The cut-off
162 values were determined independently for each gene with the data of the positive gene
163 (mainly *S. aureus* Mu50 strain) and the negative data (Plasmid pK 0311). Basically, the
164 positive results were defined for each gene by the results of the median of the four spots
165 fluorescence intensity superior to the data of the positive gene minus the standard deviation.
166 The negative results were defined by a results of the median of the intensity inferior to the
167 negative data plus the standard deviation. Between these results the data were considered “not
168 interpretable”. The results were ascertained by PCR for the genes of interest and the cut off
169 adapted. For each gene, the details of each cut-off are given in the supplementary material
170 (Appendix A).

171 2.4.7. *Validation of the microarray data.* The strain Mu50, 7 strains with PCR positive
172 genes (*cna*, *agrI*, *agrII*, *agrIII*, *agrIV*, *seb*, *seh*, *sea*, *sed*, *see*, *seg*, *sei*, *eta*, *etb*) and strains
173 with known phenotypic characteristic (coagulase) were used as positive controls. The plasmid
174 pKO311 from *S. aureus* strain F5 was kindly provided by Dr. K. Omoe, Japan, and was used
175 as a negative control for all the genes except for *sej*. The reproducibility was estimated by
176 carrying out two independent labeling and hybridization experiments on 15 isolates in
177 duplicate. The reproducibility of the data obtained with the strain Mu50 were evaluated over 7
178 independent experiments. PCR tests were performed for the 108 *S. aureus* isolates on
179 particular loci, genes or alleles to confirm the microarray data by using primers described in
180 the literature: *coa* (Goh et al., 1992), *clfB*, *cna*, *sdrC-E*, *sspA(V8)*, *icaA*, *map/eap* (Peacock et
181 al., 2002), *eta*, *etb*, *tst* (Becker et al., 1998), *sak* (van Wamel et al., 2006), *fnbB* (Kuhn et al.,
182 2006), *lukS-PV-lukF-PV*, *lukE-lukD*, *agr* group I-III (Gilot et al., 2002), *sea*, *seb-c*, *sec*, *sed*,
183 *see*, *seg*, *seh*, *sei*, *sej* (Monday et al., 1999), *sek*, *sel*, *sem*, *sen*, *seo*, *seq* (Smyth et al., 2005).
184 PCR results for a few genes were confirmed by using primers designed with Primer3 (Rozen
185 et al., 2000): *fnbB*, *sdrD*, SAV0879, SAV2496. Moreover, *lukM* gene (Jarraud et al., 2002)
186 and *agr* group IV allele (Gilot et al., 2002) were analyzed only by PCR.

187 A subset of 24 genes giving significantly different hybridization signals between isolates
188 was also verified by PCR using Primer3-designed primers (*lpl1*, *lpl4*, *ssl6*, *bsaA1*, *bsaB*,
189 *bsaP*, SAV0812, *lukF-PV* like component, SAV0876, *fhuD1*, *abiF*, SAV2496, ψ ent1 and ψ
190 ent2) or with primers found in the literature for *agr* group III (Gilot et al., 2002), *lukM*
191 (Jarraud et al., 2002), *sec* (Monday et al., 1999), *tst* (Becker et al., 1998), *seg*, *sei* (Monday et
192 al., 1999), *sel*, *sem*, *sen*, *seo* (Smyth et al, 2005). The primer sequences used in PCRs are
193 listed in the supplementary data (appendix A).

194 2.4.8. *Statistical analysis.* The distribution of putative virulence genes or alleles among *S.*
195 *aureus* isolates obtained from different hosts was compared using Fisher's exact test. The 2-
196 tail p-value was calculated and p values less than 0.05 were considered significant .

197 The microarray data are available in the public database ArrayExpress® under the
198 accession number E-TABM-288 (Brazma et al., 2003).

199

200 3. Results

201

202 All raw data from this study are given in the supplementary material (Appendix A).

203

204 3.1 PFGE patterns, *spa*-clonal complexes and *agr* groups.

205

206 All the *S. aureus* strains included in this study were classified using PFGE, *spa* typing, *agr*
207 groups and compared with the dominant PFGE pulsotype found in the ewe suclinical mastitis
208 isolates of the southeast of France. The PFGE patterns, classified as OV/OV' or not OV/OV'
209 (Vautor et al, 2003), the *spa*-clonal complexes (*spa*-CC), and the *agr* groups are shown in
210 Table 1. The genetically related OV and OV' PFGE profiles were only found in small
211 ruminant isolates. These PFGE profiles (n = 45), clustered as a PFGE group, belonging
212 mainly to the *spa*-clonal complex 1773 except for two strains that belong to *spa*-CC 2678 and
213 a singleton (*spa*-type 3572).

214 In this study we had incorporated the *spa* types with two repeats in the *spa* clustering
215 because 20 isolates out of 108 had only two *spa* repeats. Thus reliable deduction about the
216 ancestries could be made from these types. The *spa*-CC 1773 clustered different *spa*-types
217 (1736, 1773, 3568, 3569, 3570, 3571, 3575, 3576, 524, 529, 605) ranging from 2 to 9 repeats.
218 The dominant repeat were for *spa*-type 1773: 04-82-17-25-17, *spa*-type 3568: 04-39-17 and
219 *spa*-type 524: 04-17. The occurrence of the *spa*-types detected in this study was very low
220 (0,02 %), when compared with the 3605 *spa*-types present in the Ridom SpaServer database
221 (spaserver2.ridom.de). This database contains 53936 strains from 48 countries mostly from
222 human.

223 The PFGE technique was more discriminant than *spa* typing. For example, many of the
224 not OV/OV' PFGE profiles were incorporated in the same *spa* clonal complex *spa*-CC 2678
225 (data not shown). The strains depicting OV/OV' PFGE profiles (and *spa*-CC 1773) were
226 predominant in the southeast of France. This subset of strains was further used for the
227 comparative genetic study between subclinical and nasal carriage isolates.

228 In the southwest areas where dairy ewe husbandry is practiced, the OV and OV' PFGE
229 profiles were minor ones. In this area we found 2 isolates with OV/OV' PFGE profiles
230 associated with *spa*-CC 1773. A third isolate did not show a OV/OV' profile but belonged to
231 *spa*-CC 1773.

232 Among the bovines isolates, we found 7 strains associated with the *spa*-clonal complex
233 1773 [*spa*-type 524 (n = 4); *spa*-type 529 (n = 1); *spa*-type 1736 (n = 1); *spa*-type 605 (n =
234 1)]. The *spa* types 1773, 3568, 3569, 3570, 3571, 3574, 3575, 3576 clustered in *spa*-CC 1773
235 found in the small ruminant isolates were never found in the bovine isolates.

236 Isolates displaying the OV/OV' PFGE profiles and *spa*-CC 1773 were mostly classified
237 into the *agr* group III (43 out of 45), whereas those with the "not OV/OV'" were more
238 frequently classified into the *agr* groups I or II (56 out of 63).

239

240 3.2. Hybridization and PCR results.

241

242 In first place, the DNA microarray data were used to screen and classify the genes as
243 "present", "absent/divergent" or "not interpretable". The results obtained by DNA microarray
244 and PCR analysis were compared for 38 distinct genes. Seventy-eight percent of the tested
245 genes gave the same results by both techniques, whereas 14% was discordant. In case of
246 doubt, some PCR results were confirmed two or more times. When the results between DNA
247 microarray and PCR were discordant, only the positive results, except for SAV0812 (Table2),

248 were taken into account into the final statistical analysis, after elimination of all possible
249 artifacts.

250 One hundred and fifteen virulence-associated genes or alleles (61%) defined in this study
251 were present in at least 90% of the *S. aureus* isolates. In contrast, 15 genes and alleles (8%)
252 were absent in more than 90% of the isolates (supplementary data). Thirty-seven genes or
253 alleles (*bsaD*, *capH-K*, *fnbB*, *hsdS*, *vwb*, *lpl2*, *lpl3*, *lpl5*, *lpl6*, *lpl7*, SAV0288, SAV0368,
254 SAV0370, SAV1130, SAV1166, SAV1602, SAV1945, *sdrC*, *sdrD*, *splA-D*, *splF*, *cna*, *map*-
255 ND2C, *seb*, *sed*, *set15*, *set16*, *set19*, *set21*, *agr* group II) were not informative since they were
256 not statistically associated to any subset of *S. aureus* isolates. These genes were present or
257 were “not interpretable“ by DNA microarray analysis for more than 20% of isolates. Twenty-
258 four genes or alleles (12.8%) were found significantly associated with the PFGE group
259 OV/OV⁺/spa-CC 1773 or with isolates obtained from small ruminant species (sheep and
260 goats *versus* cows). Results are summarized in Table 2 and Table 3.

261 On the other hand, no genes were found statistically associated with nasal carriage isolates
262 *versus* mastitis isolates from dairy sheep in the same geographical location (southeast of
263 France).

264 4. Discussion

265

266 A combined PCR and single-dye microarray approach allowed to screen for the presence
267 of 190 genes among 108 *S. aureus* isolates, including some from the previously characterized
268 OV/OV' lineage (Vautor et al., 2003; Vautor et al., 2005). To gain insight into the host
269 specificity of *S. aureus* isolates, the gene patterns observed in isolates from cows and small
270 ruminants were compared. The results were also examined in terms of geographical and tissue
271 specificity.

272 From a technical point of view, most probably a one-colour-dye microarray is inferior to a
273 two-colour one but, at a reasonable cost it made possible the screening of more than one
274 hundred isolates for 190 genes. In some circumstances, the microarray data were not
275 exploitable due to high background, artifacts or because differences between the positive and
276 the negative results were considered as: "not interpretable". Another limitation of the study
277 was that primers and probes used for PCR and microarrays could not be systematically chosen
278 to match the same gene locations, which could explain some of the discordant results.
279 Besides, allelic variations between veterinary *S. aureus* isolates and the Mu50 strain that was
280 used to design most microarray probes could have occur.

281 4.1. Regional diversity in *S. aureus* sheep isolates.

282 A major lineage of *S. aureus* (PFGE group OV/OV' and *spa*-CC 1773) has been identified
283 to be linked with a geographical region (isolates from sheep in southeast of France). Mork et
284 al., (2005a) and Aires-de-Sousa et al., (2007) carried out studies using PFGE on 905 and 14
285 isolates, respectively. They did not find any peculiar genotype related to the three dairy
286 species. In another report on 384 *S. aureus* isolates from sheep udders in four regions of
287 Norway (Mork et al., 2005b), 59% of the isolates belonged to five closely related pulsotypes,
288 that differed from the OV/OV' type previously characterized in our laboratory (Vautor et al.,
289 2003).

290 4.2. *Agr* group, PFGE group, *spa* clonal complex and lineage specific genes

291 The *agr* group I and III represented 44 % (47/108) and 46% (50/108) of the isolates,
292 respectively. The predominance of the *agr* group III was the consequence of the over-
293 representation of the isolates from the dairy sheep in the southeast of France (46/108). All but
294 2 isolates out of 45, with the OV / OV' PFGE profiles and *spa*-CC 1773, belonged to the *agr*
295 group III. This observation is in agreement with Jarraud et al. (2002), who demonstrated a
296 relationship between the genetic background of the strains and the *agr* allele group. In a study
297 carried out by De Santis et al (2005) on strains isolated from sheep with subclinical mastitis in
298 Sardinia, 26 strains (81%) out of 32 belonged to the *agr* group III.

299 Two genes were more frequently found in isolates from the OV/OV' PFGE group/*spa*-CC
300 1773: *lpl4* (coding for a probable lipoprotein) (Kuroda et al., 2001) and *ss16* (coding for a
301 staphylococcal superantigen-like protein previously named *set6*). Interestingly, these two
302 genes are localized in the same staphylococcal genomic island type I vSa α in strain Mu50.
303 The SAV0812 gene, coding for a protein similar to a secreted von Willebrand factor-binding
304 protein, was also specific for the OV/OV' PFGE group/*spa*-CC 1773. This gene is truncated
305 in some strains (e.g. RF122, MW2, COL, NCTC8325, MRSA252, MSSA476). This may
306 explain the discrepancies found between the microarray data and the PCR results (Table 2),
307 since the microarray probe was located in the truncated part of the gene whereas the PCR
308 primers were not. We found that the OV/OV' lineage was also characterized by the absence
309 of three bacteriocin genes (*bsaA1*, *bsaB*, *bsaP*). These genes are located in the same genomic
310 island (type II vSa β) in strain MW2 and NCTC8325 (Baba et al., 2002).

311 4.3. Tissue specificity of *S. aureus* virulence genes.

312 No genes were found specific to the isolates collected from the nostrils of sheep in
313 comparison to udder isolates. This is in agreement with Lindsay et al. (2006) who failed to
314 find, in human isolates, any genes significantly associated with invasive isolates by
315 comparing 61 community-acquired invasive isolates of *S. aureus* to 100 nasal carriage

316 isolates from healthy donors. However, since our study was performed in field conditions, it
317 is possible that *S. aureus* isolates belonging to the resident flora of the nostrils could not be
318 detected due the overwhelming presence of *S. aureus* isolates that are present in the milking
319 room atmosphere (Vautor et al., 2005).

320 4.4. Host specificity of *S. aureus* virulence genes

321 Small ruminant isolates had a set of genes that was not statistically found in bovine
322 isolates. Among them there are the *lpl1* gene (located in the genomic island of Mu50 type
323 *IvSa α* and coding for a probable lipoprotein), and the SAV0876 gene (coding for a
324 hypothetical integrated prophage-related protein). The leukotoxin *lukF*-PV-like component
325 (*LukF*^{PV}-PV) is linked with *lukM* (Kaneko et al., 1997) and was found with a higher frequency
326 in small ruminants isolates. *LukF*^{PV}-PV/*LukM* is the most active *S. aureus* leukotoxin on
327 bovine neutrophils (Barrio et al., 2006). This leukotoxin is of importance because
328 phagocytosis by polymorphonuclear cells (PMNs) and macrophages is regarded as one of the
329 most important defense mechanisms of the mammary gland. It has been shown that the small
330 ruminant isolates were more leukotoxic for bovine PMNs than the bovine isolates, but PMNs
331 of small ruminants were more resistant to *lukM* than bovine PMNs. The higher amount of
332 leukotoxins secreted by *S. aureus* isolates from small ruminants may be seen as an adaptation
333 to the higher resistance of small ruminants PMNs to *lukM* in comparison to bovine PMNs
334 (Rainard et al., 2003). In addition, the cluster *sel*, *sec3* and *tst* (belonging to the toxic shock-
335 syndrome toxin 1 island, SaPI_{m1}) was more prevalent in small ruminants isolates. In another
336 study, the gene encoding the toxic shock-syndrome protein was more frequently associated to
337 veterinary strains (especially those causing mastitis), than to human isolates (van Leeuwen et
338 al., 2005). It was also reported that these genes were significantly associated with small
339 ruminant isolates in comparison to bovine ones, by studying isolates from various countries
340 (Smyth et al., 2005; Vimercati et al., 2006).

341 In contrast, small ruminant isolates statistically lacked different genes. The *abiF* gene
342 (SAV0368 in Mu50) codes for an abortive infection phage resistance determinant (Garvey et
343 al., 1995). The Abortive infection (Abi) systems, also called phage exclusion, is widespread in
344 bacteria and a recent analysis indicates that Abis might have an additional role apart from
345 conferring phage resistance (Chopin et al., 2005). In the dairy environment, there is a large
346 biodiversity of the lactococcal phage population, so the acquisition of this *abiF* gene through
347 lateral transfer is probable. However, there is no explanation about its lesser frequency in
348 small ruminants isolates. The SAV2496 gene codes for a hypothetical protein belonging to
349 surface proteins from Gram-positive cocci such as a cell wall surface anchor family protein,
350 which also less frequent among isolates from small ruminants. The operon *egc* (enterotoxin
351 gene cluster) comprises the five enterotoxin genes *seg*, *sei*, *sem*, *sen*, *seo* and two pseudogenes
352 Ψ *ent1* and Ψ *ent2*. This cluster forms a putative nursery of superantigens in *S. aureus*
353 (Jarraud et al., 2001). Smyth et al. (2005) found that the *egc* cluster (complete and
354 incomplete) was present with a lower frequency amongst the small ruminants isolates in
355 comparison to bovine ones, which was also observed in the present study. Some surveys on *S.*
356 *aureus* human clinical isolates showed that *egc* cluster was coupled with clonal lineages (van
357 Belkum et al., 2006). The *fhuD1* gene (SAV0803 in Mu50) was less prevalent in the small
358 ruminants isolates than in the bovine ones. This gene, coding for a receptor for the transport
359 of ferric hydroxamate (source of iron), is redundant to *fhuD2* (SAV2284 in Mu50) (Sebulsky
360 et al., 2001) and may play a prominent role during the growth of *S. aureus* in the host udder.

361 In general terms, the mechanisms explaining the relative host-specificity of *S. aureus* are
362 still unknown, and more studies on the protein production of the specific genes have to be
363 realized.

364

365 **Acknowledgements**

366

367 The support of the Réseau National Génopole™ and of the CNRS (Centre National de la
368 Recherche Scientifique) are greatly appreciated.

369 We thank Dr J.P. Rossec, Dr K. Omoe, Dr J.L. Martel, Dr P. Mercier, and Dr J. Penades
370 for kindly providing some of the isolates used in this work. We acknowledge G. Rossi for his
371 excellent technical assistance and C. Thiéry for checking the manuscript for English language.

372

373 **Appendix A. Supplementary data.**

374 Supplementary data associated with this article can be found, in the online version, at
375 [doi:XXXXXXXj.vetmic.2008yyyy](https://doi.org/10.1016/j.vetmic.2008yyyy) .

376

377

378 **References**

379

380 Aires-de-Sousa, M., Parente, C.E., Vieira-da-Motta, O., Bonna, I.C., Silva, D.A., de
381 Lencastre, H., 2007. Characterization of *Staphylococcus aureus* isolates from buffalo,
382 bovine, ovine, and caprine milk samples collected in Rio de Janeiro State, Brazil. Appl.
383 Environ. Microbiol. 73, 3845-3849.

384 Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N.,
385 Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., Hiramatsu, K., 2002.
386 Genome and virulence determinants of high virulence community-acquired MRSA. The
387 Lancet 359, 1819-27.

388 Barrio, M.B., Rainard, P., Prevost, G., 2006. LukM/LukF'-PV is the most active
389 *Staphylococcus aureus* leukotoxin on bovine neutrophils. Microbes. Infect. 8, 2068-
390 2074.

391 Becker, K., Roth, R., Peters, G., 1998. Rapid and specific detection of toxigenic
392 *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for
393 amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin
394 genes, and toxic shock syndrome toxin 1 gene. J. Clin. Microbiol. 36, 2548-2553.

395 Brazma, A., Parkinson, H., Sarkans, U., Shojatalab, M., Vilo, J., Abeygunawardena, N.,
396 Holloway, E., Kapushesky, M., Kemmeren, P., Lara, G.G., Oezcimen, A., Rocca-Serra,
397 P., Sansone, S.A., 2003. ArrayExpress—a public repository for microarray gene
398 expression data at the EBI. Nucleic Acids Res. 31, 68-71.

399 Chopin, M.C., Chopin, A., Bidnenko, E., 2005. Phage abortive infection in lactococci:
400 variations on a theme. Curr. Opin. Microbiol. 8, 473-479.

- 401 De Santis, E., Mureddu, A., Mazzette, R., Scarano, C., Bes, M., 2005. Detection of
402 enterotoxins and virulence genes in *Staphylococcus aureus* strains isolated from sheep
403 with subclinical mastitis, In H. Hogeveen (ed.), Mastitis in dairy production, current
404 knowledge and future solutions, pp. 504-510.
- 405 Garvey, P., Fitzgerald, G.F., Hill, C., 1995. Cloning and DNA sequence analysis of two
406 abortive infection phage resistance determinants from the lactococcal plasmid pNP40.
407 Appl. Environ. Microbiol. 61, 4321-4328.
- 408 Gilot, P., Lina, G., Cochard, T., Poutrel, B., 2002. Analysis of the genetic variability of genes
409 encoding the RNA III-activating components Agr and TRAP in a population of
410 *Staphylococcus aureus* strains isolated from cows with mastitis. J. Clin. Microbiol. 40,
411 4060-4067.
- 412 Goh, S.H., Byrne, S.K., Zhang, J.L., Chow, A.W., 1992. Molecular typing of *Staphylococcus*
413 *aureus* on the basis of coagulase gene polymorphisms. J. Clin. Microbiol. 30, 1642-
414 1645.
- 415 Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch,
416 F., Bonneville, M., Lina, G., 2001. *egc*, a highly prevalent operon of enterotoxin gene,
417 forms a putative nursery of superantigens in *Staphylococcus aureus*. J. Immunol. 166,
418 669-677.
- 419 Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne,
420 J., Vandenesch, F., 2002. Relationships between *Staphylococcus aureus* genetic
421 background, virulence factors, *agr* groups (alleles), and human disease. Infect. Immun.
422 70, 631-641.
- 423 Kaneko, J., Muramoto, K., Y. Kamio, Y., 1997. Gene of LukF-PV-like component of Panton-
424 Valentine leukocidin in *Staphylococcus aureus* P83 is linked with lukM. Biosci.
425 Biotechnol. Biochem. 61, 541-544.
- 426 Kuhn, G., Francioli, P., Blanc, D.S., 2006. Evidence for clonal evolution among highly
427 polymorphic genes in methicillin-resistant *Staphylococcus aureus*. J. Bacteriol. 188,
428 169-178.
- 429 Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A.,
430 Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A.,
431 Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue,
432 R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa,
433 M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M.,
434 Ogasawara, N., Hayashi, H., Hiramatsu, K., 2001. Whole genome sequencing of
435 methicillin-resistant *Staphylococcus aureus*. Lancet 357, 1225-1240.
- 436 Lindsay, J.A., Moore, C.E., Day, N.P., Peacock, S.J., Witney, A.A., Stabler, R.A., Husain,
437 S.E., Butcher, P.D., Hinds, J., 2006. Microarrays reveal that each of the ten dominant
438 lineages of *Staphylococcus aureus* has a unique combination of surface-associated and
439 regulatory genes. J. Bacteriol. 188, 669-676.
- 440 Monday, S.R., Bohach, G.A., 1999. Use of multiplex PCR to detect classical and newly
441 described pyrogenic toxin genes in staphylococcal isolates. J. Clin. Microbiol. 37, 3411-
442 3414.

- 443 Mork, T., Tollersrud, T., Kvitle, B., Jorgensen, H.J., Waage, S., 2005. Comparison of
444 *Staphylococcus aureus* genotypes recovered from cases of bovine, ovine, and caprine
445 mastitis. *J. Clin. Microbiol.* 43, 3979-3984.
- 446 Mork, T., Tollersrud, T., Kvitle, B., Jorgensen, H.J., Waage, S., 2005. Genetic diversity of
447 *Staphylococcus aureus* isolated from ovine intramammary infections in Norway. *Vet.*
448 *Microbiol.* 106, 265-273.
- 449 Peacock, S.J., Moore, C.E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G.,
450 Day, N.P., 2002. Virulent combinations of adhesin and toxin genes in natural
451 populations of *Staphylococcus aureus*. *Infect. Immun.* 70, 4987-4996.
- 452 Rainard, P., Corrales, J.C., Barrio, M.B., Cochard, T., Poutrel, B., 2003. Leucotoxic Activities
453 of *Staphylococcus aureus* Strains Isolated from Cows, Ewes, and Goats with Mastitis:
454 Importance of LukM/LukF'-PV Leukotoxin. *Clin. Diagn. Lab. Immunol.* 10, 272-277.
- 455 Rouillard, J.M., Herbert, C.J., Zuker, M., 2002. OligoArray: genome-scale oligonucleotide
456 design for microarrays. *Bioinformatics.* 18, 486-487.
- 457 Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist
458 programmers. *Methods Mol. Biol.* 132, 365-386.
- 459 Sebulsy, M.T., Heinrichs, D.E., 2001. Identification and characterization of *fhuD1* and
460 *fhuD2*, two genes involved in iron-hydroxamate uptake in *Staphylococcus aureus*. *J.*
461 *Bacteriol.* 183, 4994-5000.
- 462 Smyth, D.S., Hartigan, P.J., Meaney, W.J., Fitzgerald, J.R., Deobald, C.F., Bohach, G.A.,
463 Smyth, C.J., 2005. Superantigen genes encoded by the *egc* cluster and SaPIbov are
464 predominant among *Staphylococcus aureus* isolates from cows, goats, sheep, rabbits and
465 poultry. *J. Med. Microbiol.* 54, 401-411.
- 466 Stephan, R., Annemuller, C., Hassan, A.A., Lammler, C., 2001. Characterization of
467 enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-
468 east Switzerland. *Vet. Microbiol.* 78, 373-382.
- 469 Straub, J.A., Hertel, C., Hammes, W.P., 1999. A 23S rDNA-targeted polymerase chain
470 reaction-based system for detection of *Staphylococcus aureus* in meat starter cultures
471 and dairy products. *J. Food Prot.* 62, 1150-1156.
- 472 Van Belkum, A., Melles, D.C., Snijders, S.V., van Leeuwen, W.B., Wertheim, H.F., Nouwen,
473 J.L., Verbrugh, H.A., Etienne, J., 2006. Clonal distribution and differential occurrence
474 of the enterotoxin gene cluster, *egc*, in carriage- versus bacteremia-associated isolates of
475 *Staphylococcus aureus*. *J. Clin. Microbiol.* 44, 1555-1557.
- 476 Van Leeuwen, W.B., Melles, D.C., Alaidan, A., al Ahdal, M., Boelens, H.A., Snijders, S.V.,
477 Wertheim, H., van Duijkeren, E., Peeters, J.K., van der Spek, P.J., Gorkink, R., Simons,
478 G., Verbrugh, H.A., van Belkum, A., 2005. Host- and tissue-specific pathogenic traits of
479 *Staphylococcus aureus*. *J. Bacteriol.* 187, 4584-4591.
- 480 Van Wamel, W. J., Rooijackers, S.H., Ruyken, M., van Kessel, K.P., van Strijp, J.A., 2006.
481 The innate immune modulators staphylococcal complement inhibitor and chemotaxis
482 inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting
483 bacteriophages. *J. Bacteriol.* 188, 1310-1315.

- 484 Vautor, E., Abadie, G., Guibert, J.M., Chevalier, N., Pepin, M., 2005. Nasal carriage of
485 *Staphylococcus aureus* in dairy sheep. Vet. Microbiol. 106, 235-239.
- 486 Vautor, E., Abadie, G., Guibert, J.M., Huard, C., Pepin, M. 2003. Genotyping of
487 *Staphylococcus aureus* isolated from various sites on farms with dairy sheep using
488 pulsed-field gel electrophoresis. Vet. Microbiol. 96, 69-79.
- 489 Vimercati, C., Cremonesi, P., Castiglioni, B., Pisoni, G., Boettcher, P.J., Stella, A., Vicenzoni,
490 G., Moroni, P., 2006. Molecular typing of *Staphylococcus aureus* isolated from cows,
491 goats and sheep with intramammary infections on the basis of gene polymorphisms and
492 toxins genes. J. Vet. Med .B Infect. Dis. Vet. Public Health 53, 423-428.
- 493
494
495
- 496
497
498
- 499
- 500
501
- 502
503

Table 1

PFGE Patterns, *spa*-CC and *agr* groups of 108 *Staphylococcus aureus* isolates included in this study

Animal species (Region)	Clinical features or origins	Isolates (n)	PFGE Patterns	<i>agr</i> group			
				I	II	III	IV
Dairy sheep (Southeast)	Subclinical mastitis	16	OV/OV ^a	0	0	14	0
			Not OV/OV ^b	1	1	0	0
	Nasal carriage	21	OV/OV'	0	0	13	0
			Not OV/OV'	7	1	0	0
	Others (abscesses, air, clinical mastitis)	9	OV/OV'	0	0	5	0
			Not OV/OV'	2	0	2	0
Dairy sheep (Southwest)	Subclinical mastitis	18	OV/OV'	1	0	3	0
			Not OV/OV'	12	2	0	0
	Clinical mastitis	1	Not OV/OV'	1	0	0	0
Goats	Subclinical mastitis	22	OV/OV'	0	1	8	0
			Not OV/OV'	8	3	2	0
Cows	Subclinical mastitis	21	Not OV/OV'	15	3	3	0
<i>Total</i>	/	<i>108</i>	<i>OV/OV'</i>	<i>1</i>	<i>1</i>	<i>43</i>	<i>0</i>
			<i>not OV/OV'</i>	<i>46</i>	<i>10</i>	<i>7</i>	<i>0</i>

^a OV and OV' were PFGE profiles belonging to the same group (n = 45). The OV/OV' PFGE profiles were determined according to Vautor et al. (2003). There were 12 *spa* types among the 45 isolates. These OV/OV' patterns belonged to the *spa*-clonal complex *spa*-CC 1173 (n = 43), and two other *spa*-CC 2678 and *spa*-type 3572.

^b Not OV/OV' were other PFGE profiles which were not predominant among the 61 patterns (n = 63) (data not shown). There were 36 *spa* types among the 63 isolates. These PFGE profiles did not have a predominant *spa*-clonal complex: *spa*-CC 2678 (n = 25), *spa*-CC 1773 (n = 12), singleton (n = 6), *spa*-CC 164 (n = 4), *spa*-CC 359 (n = 3), *spa*-CC 127 (n = 3), *spa* type 3585/excluded (n = 1), no founder (n = 9).

Table 2

Genes or alleles statistically associated with *S. aureus* strains clustered in PFGE pattern OV/OV'/*spa*-CC 1773 or "not OV/OV'" (DNA microarray data and PCR results)

Gene or allele ^a	PFGE profile		<i>p</i>
	Percentage of OV/OV' strains carrying the gene (out of 45)	Percentage of "not OV/OV' " strains carrying the gene (out of 63)	
	SAV0812 (PCR only)	97,8 %	
SAV0812 (microarray only)	8,9 %	58,7 %	< 0.0001
<i>ssl6</i>	97,8 %	34,9 %	< 0.0001
<i>lpl4</i>	98,8 %	15,9 %	< 0.0001
<i>agr</i> group III	95,5 %	14,3 %	< 0.0001
<i>bsaA1</i>	2,2 %	63,5 %	< 0.0001
<i>bsaB</i>	0,0 %	60,3 %	< 0.0001
<i>bsaP</i>	0,0 %	55,6 %	< 0.0001

^a In bold characters: genes specific to PFGE pattern OV/OV'/*spa*-CC 1773.

Table 3

Genes or alleles statistically associated with *S. aureus* isolates from small ruminants *versus* those coming from cows (DNA microarray data and PCR results)

Gene or allele ^a	Percentage of isolates from small ruminants carrying the gene (out of 87 isolates)	Percentage of isolates from cows carrying the gene (out of 21 isolates)	<i>p</i>
<i>lpl1</i>	96,6 %	47,6 %	< 0.0001
SAV0876	89,7 %	28,6 %	< 0.0001
lukM	85,1 %	19,0 %	< 0.0001
lukF-PV like component	81,6 %	19,0 %	< 0.0001
<i>sec</i>	58,6 %	23,8 %	0.006
<i>tst</i>	47,1 %	14,3 %	0.006
<i>sel</i>	44,8 %	9,5 %	0.002
<i>abiF</i>	20,7 %	85,7 %	< 0.0001
<i>sem</i>	16,1 %	47,6 %	0.006
<i>seo</i>	13,8 %	52,4 %	0.001
<i>sen</i>	12,6 %	47,6 %	0.0009
<i>sei</i>	11,5 %	47,6 %	0.0005
ψ <i>ent1</i>	11,5 %	42,9 %	0.002
<i>seg</i>	10,3 %	39,0 %	0.004
ψ <i>ent2</i>	10,3 %	47,6 %	0.0003
SAV2496	10,3 %	66,7 %	< 0.0001
<i>fhuD1</i>	6,9 %	81,0 %	< 0.0001

^a In bold characters: genes specific to small ruminants (sheep and goats).