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Analysis of prophages harbored by the human-adapted subpopulation of *Staphylococcus aureus* CC398

Nathalie van der Mee-Marquet[1,2*], Anna-Rita Corvaglia[3], Anne-Sophie Valentin[1], David Hernandez[3], Xavier Bertrand[4,5], Myriam Girard[3], Jan Kluytmans[6], Pierre-Yves Donnio[7,8], Roland Quentin[1], Patrice François[3]

[1]Service de Bactériologie et Hygiène, Centre Hospitalier Régional Universitaire, and [2]Réseau des Hygiénistes du Centre, Centre Hospitalier Universitaire, F37044 Tours cedex, France. [3]Genomic Research Laboratory, University of Geneva Hospitals, CH-1211 Geneva 14, Switzerland. [4]Service d'Hygiène, Centre Hospitalier Universitaire, and [5]UMR 6249 Chrono-environnement, Université de Franche-Comté, F25030 Besançon cedex, France. [6]Laboratory for Microbiology and infection Control, Amphia Hospital, Breda and VU University Medical Center, Amsterdam, The Netherlands. [7]Service de Bactériologie et Hygiène Hospitalière, Centre Hospitalier Universitaire, and [8]EA1254 Microbiologie-Risques Infectieux, Université de Rennes 1, F35033 Rennes cedex, France.

Short title: prophages in *Staphylococcus aureus* CC398

Corresponding author :
Nathalie van der Mee-Marquet
Laboratoire de Bactériologie et Hygiène
Hôpital Trousseau
37044 Tours cedex
France
Phone: 33 234 389 430
Fax : 33 247 478 588
email : n.vandermee@chu-tours.fr

ABSTRACT

Staphylococcus aureus clonal complex 398 is a livestock-associated pathogen that poses a worldwide threat because of its ability to colonize and infect both humans and animals. We used high-resolution whole-genome microarrays, prophage profiling, immune evasion cluster characterization and whole-genome sequencing to investigate the roles of prophages in the emerging human-adapted subpopulation of CC398 that has been associated with invasive infections in humans living in animal-free environments.

We characterized one phage and two prophages specifically harbored by CC398 isolates belonging to the emerging subpopulation. We introduced the phage into permissive prophage-free isolates. We investigated the effects of lysogeny on the host ability to resist further phage infection and transformation, to acquire the capacity to invade human cells, and to express virulence factors encoded by prophages. We report evidence of a defective ϕMR11-like helper prophage, named StauST398-5pro, specifically associated with the emerging non-LA CC398 subpopulation. StauST398-5pro confers substantial protection against horizontal genetic transfer to its host. It interacts with a human-associated β-converting prophage encoding immune-modulating proteins such that virulence genes are expressed during stress situations.

Our findings provide insight into the role of phages in the expression of virulence and in the spread of genetic information among new host-adapted *S. aureus* isolates. We demonstrate that functional prophage elements can condition host specificity and confer new virulence traits on emerging intra-species clones of bacteria.

Keywords: *Staphylococcus aureus*, sequence type 398, prophage, virulence, gene expression, host specificity, transformation, transduction.

53 **INTRODUCTION**

54 Originally detected in pig farmers in Europe (Witte, 2007), *S. aureus* belonging to
55 CC398 has become a worldwide threat associated with livestock, their human contacts and
56 food products (van Belkum, 2008). Microarrays and whole-genome sequencing approaches
57 applied to a large number of CC398 isolates distinguished two clades within the CC398
58 lineage (Price, 2012; Uhleman, 2012; McCarthy, 2011): the classical LA clade, isolates of
59 which have long been responsible for frequent and transient colonization, and rare
60 infections, of farmers and veterinarians (Huijsdens, 2006); and a human clade. This human
61 clade is comprised of two subpopulations: the ancestral human subpopulation, and the
62 emerging human-adapted non-LA CC398 subpopulation that has recently and increasingly
63 been causing invasive infections worldwide in humans living in animal-free environments
64 (Price, 2012; Valentin-Domelier, 2011; Jimenez, 2011; Stegger, 2010), and that readily
65 colonize and spread between humans (Uhleman, 2012).

66 Phages serve as a driving force in bacterial pathogenesis, contributing both to the
67 evolution of bacterial hosts through gene transfer, and to bacterial pathogenesis at the time
68 of infection (Wagner, 2002). *S. aureus* is highly lysogenic. *S. aureus* phages are double-
69 stranded DNA phages belonging to the *Siphoviridae* family (Goerke, 2009). Comparative
70 genomic analyses have revealed substantial diversity of these phages; nevertheless, they
71 also display extensive mosaicism, with genes organized into functional modules that are
72 frequently exchanged between phages (Kahankova, 2010; Xia, 2013). Temperate phages play
73 an important role in the pathogenicity of *S. aureus*. Some carry genes encoding diverse
74 virulence factors including panton-valentine leukocidin, staphylokinase, enterotoxins,
75 chemotaxis-inhibitory proteins, and exfoliative toxins. Other phages have inserted into and

76 consequently interrupt chromosomal virulence genes such as those for β-hemolysin (*hlb*)
77 and lipase (*geh*) (Goerke, 2009).

78 The two clades of the CC398 lineage are characterized by different prophages. LA
79 CC398 isolates commonly carry phages ϕ2 and ϕ6 (Schijffelen, 2010; Hallin, 2011; McCarthy,
80 2011; Hallin, 2011), or a ϕAvβ prophage (Price, 2012). By contrast, isolates belonging to the
81 human clade contain β-converting ϕ3 prophage variants that encode two immune-
82 modulating proteins (Goerke, 2006; McCarthy, 2011; Price, 2012; Uhleman, 2012). These
83 proteins are the staphylococcal complement inhibitor, SCIN, that prevents
84 opsonophagocytosis and killing of *S. aureus* by human neutrophils; and the chemotaxis
85 inhibitory protein of S. aureus, CHIPS. There is now evidence that the emerging
86 subpopulation differs from the ancestral human LA subpopulation by additional prophage
87 features (ϕ1, ϕ2, ϕ5 or ϕ7) relevant to its epidemiology (Uhleman, 2012; McCarthy, 2011).
88 However, this issue has not been thoroughly investigated.

89 We aimed to characterize the prophage elements specifically associated with the
90 *emerging* subpopulation. We applied whole genome analysis techniques to study the
91 prophage content of isolates recovered in livestock environments and from cases of invasive
92 infections in humans in animal-free environments. We studied the contribution of prophage
93 content to phage resistance, competence for transformation and ability to invade human
94 cells. We demonstrate that phages can condition host specificity and confer new virulence
95 traits on pathogenic and host-adapted *S. aureus* strains.

96

97 **MATERIALS AND METHODS**

98 ***Ethics Statement*.** The human isolates from The Netherlands were obtained during
99 prospective surveillance, approved by the medical ethics committee of the Sint Elisabeth
100 Hospital, at Tilburg, The Netherlands (protocol number 0749). All participants provided
101 written informed consent. The strains isolated from animals were obtained by non-invasive
102 sampling during a field study. This study was performed in accordance with the Dutch
103 guidelines and as such did not require the approval of an ethics committee. The farmers
104 gave informed consent and agreed to the collection of samples. The French isolates were
105 obtained from clinical samples obtained during annual surveillance studies that were run
106 according to the French Healthcare recommendations for prevention of infection. Ethical
107 approval of the surveillance programs was obtained at the national level from the Réseau
108 Alerte Investigation Surveillance des Infections Nosocomiales (RAISIN). In accordance with
109 national French legislation and following the national procedure established by RAISIN, the
110 surveillance study was run jointly with the regional surveillance coordinator, the director of
111 the participating healthcare institutions and the physicians responsible for caring for the
112 patients. The director and the physicians provided written consent for participation in the
113 study. All patients or their relatives were individually approached for oral consent for
114 participation in the study.

115 ***Bacterial isolates*.** Twenty-one CC398 isolates were studied (Tab. 1), comprising two
116 populations of isolates: (i) ten isolates representative of the diversity of the LA clade,
117 including nine Dutch and one French isolates, recovered from humans (n=4) or animals
118 (n=6), and belonging to the major *spa*-types t899 (n=2), t011 (n=2), t034 (n=2), t571 (n=2)
119 and t108 (n=2); (ii) eleven isolates recovered over a four-year period in three geographically
120 separated French regions (Valentin-Domelier, 2011) from patients with no LA risk factors

121 and diagnosed with bloodstream infections (n=10) or urinary tract infection (n=1). These
122 isolates belonged to six different *spa*-types: t899 (n=1), t1451 (n=2), t571 (n=5), t6605 (n=1),
123 t5635 (n=1) and t9378 (n=1).

124 ***DNA microarray experiments.*** The microarray has been described previously
125 (Charbonnier, 2005). Microarrays were produced by *in situ* synthesis of a set of 15,600 60-
126 mer long oligonucleotide probes (Agilent, Palo Alto, CA, USA). The probe set included 8,877
127 probes covering approximately 95 % of all ORFs annotated in strains N315 and Mu50
128 (Kuroda, 2001), MW2 (Baba, 2002), COL (Gill, 2005), NCTC 8325 (Gillaspy, 2006), USA300
129 (Diep, 2006), MRSA252 and MSSA476 (Holden, 2004) and including those on their plasmids.
130 Each gene was covered by one to 12 probes depending on gene length. Each test DNA (test
131 channel) was hybridized against the reference DNA (a mixture of DNA from all four strains
132 used to design the array) in a single hybridization reaction. Fluorescence intensities were
133 extracted using Feature Extraction software (Agilent, version 6.1.1). The signal was analyzed
134 with in-house software that estimates the presence probability (EPP) for the sequence of
135 each oligonucleotide probe, as previously described (Charbonnier, 2005). EPP values ≤1%
136 were extracted and considered to indicate the absence of the sequence.

137 ***Prophage profiling and immune evasion cluster (IEC) characterization.*** A PCR-based
138 assay for 47 prophage genes belonging to nine main phage modules was used (Kahankova,
139 2010). To characterize the IEC of the prophages, PCR was used to test for sequences
140 corresponding to the prophage genes *hlb*, *scn*, *chp*, *sak*, *sep* and *sea* by the method
141 developed by van Wamel (van Wamel, 2006). The primers described in Suppl. File Tab. 2
142 were used to test genomic and phage lysate DNA for *chp* and *scn*.

143 ***Isolation, propagation and characterization of phages.*** The CC398 isolates were
144 each assessed for their potential as a donor strain. Cultures were treated with mitomycin C

145 as described previously (de Gialluly, 2003). The filtered supernatants, corresponding to
146 putative lysates, were stored at −80°C. The lytic activity of the putative lysates was tested
147 against the 27 strains of the international phage typing system (de Gialluly, 2003) and the 21
148 CC398 isolates used as indicator strains. A putative lysate was considered to be a phage
149 suspension if it provided at least 20 clear plaques on at least two indicator strains. Phages
150 were propagated using the non-LA isolate S100. Titrated phage preparations were kept at
151 −80°C. The induced phage preparations were centrifuged on a sucrose density gradient.
152 Phage particles were negatively stained with 2% uranyl acetate, examined in a JEOL 1230
153 transmission electron microscope at an accelerating voltage of 120 kV, and photographed.
154 Morphological types were defined on the basis of phage tail length.

155 ***Whole genome sequencing.*** The genomes of CC398 isolates S100, S124, S124/S100-ϕ
156 were sequenced as previously described (Gizard, 2013), and the prophage contents assessed
157 by searching for conserved phage genes and known phage-encoded virulence genes. The
158 sequences are available through GenBank resources (see Tab. 2).

159 ***Study of the effects of lysogeny on resistance to phages, competence for***
160 ***transformation and invasion of human cells.*** The two prophage-free isolates (S123 and
161 S124) were exposed to the lysate produced by the non-LA isolate S100. Cells surviving after
162 incubation at 30°C for 18 hours were plated on fresh agar plates, incubated at 37°C for 18
163 hours and screened for their prophage content by PCR as described above. A prophage-
164 containing isolate (transductant) was isolated. ***Phage resistance.*** To determine susceptibility
165 to *S. aureus* phages, the 21 CC398 isolates were phage-typed using the international set of
166 phages according to the procedure described previously (de Gialluly, 2003). ***Transformation***
167 ***experiments.*** DNA of pCN38 was prepared from *E. coli* DH5α and *Staphylococcus* RN4220
168 (Lee, 1995; Gizard, 2013). DNA of pMW401 was prepared from *Enterococcus faecalis* as

169 previously described (Beaume, 2010). The media used for overnight culture and subsequent
170 mini-preps (Qiaprep Spin Miniprep Kit, Qiagen) included Luria Bertani Broth (LB Broth,
171 Miller, BD Difco) for *E. coli* DH5α, supplemented with 100 mg/L of ampicillin; Mueller-Hinton
172 broth (MHB; BD Difco) for *S. aureus* RN4220; and Tryptic soy Broth for *E. faecalis* (Soyban-
173 Casein Digest Medium, BD Bacto). Chloramphenicol (10 mg/L) was included as appropriate.
174 Preparation of competent cells and transformation of RN4220, S100, S123, S124, S124/S100-
175 ϕ, S92, S93 and S94 were as described previously (Beaume, 2010). Following
176 electroporation, transformants were plated on Mueller-Hinton Agar, supplemented with
177 chloramphenicol: 10 mg/L for RN4220; 15 mg/L for S100, S123, S124, S124/S100-ϕ; and 20
178 mg/L for S92, S93, S94. The number of transformants obtained per µg of plasmid DNA was
179 calculated. ***Internalization experiments.*** The Cowan strain is internalized by human cells and
180 was therefore used as a positive control. Strain KH11 was used as a non-invasive, negative
181 control. The internalization procedure and the lysostaphin protection assay were based on a
182 published method (Sinha, 1999) with minor differences. Briefly, cultures of the cell line 293
183 were maintained under humidified air with 5 % CO_2 at 37°C in Dulbecco's minimal essential
184 medium (DMEM)-nutrient mixture F-12 (nut mix F-12) containing Glutamax I, a stable
185 glutamine dipeptide, supplemented with 10 % fetal calf serum (FCS), 50 IU of penicillin per
186 mL, and 50 µg of streptomycin per mL. For the lysostaphin assay, 293 cells were plated in
187 poly-ornithine coated plates (1.8 cm diameter) and cultured for 24 hours, such that there
188 were approximately 0.9×10^5 cells/well. Bacterial were harvested from culture, washed,
189 counted and diluted (Sinha, 1999); these bacterial dilutions were added to the plates
190 containing 293 cells at a multiplicity of infection [MOI] of 15:1. The plates were incubated for
191 1 h at 37°C, and the bacterial suspensions were then replaced with lysostaphin medium
192 (DMEM-nut mix F-12, 10 % FCS, 20 µg of lysostaphin per mL). The plates were further

193 incubated for 30 min, and the 293 cells then lysed in 1 mL of sterile distilled H_2O.
194 Appropriate serial dilutions of the cell lysate in PBS were plated on Mueller-Hinton agar or
195 TSA plates, and CFU were counted manually. ***Transcription experiments.*** Gene-specific
196 primers and probes were designed using Primer Express 3.0 (Applied Biosystems) and are
197 shown in Suppl. File Tab. 2. Oligonucleotide primers and probes were used at final
198 concentrations of 0.2 and 0.1 µM, respectively, in a final volume of 10 µL of one-step RT–
199 PCR enzymatic mixture (Invitrogen, Carlsbad, Germany); a Mx3005P system (Agilent) was
200 used as described previously (Beaume, 2010). Results were normalized to those for the *hu*
201 gene, as described previously. These assays provided relative gene expression values for *chp*,
202 *scn* and *sak* in various isolates and in various stress conditions.
203 **Statistical data.** Chi-square tests and Fisher's exact test (two-tailed) were used to test
204 associations; a P value <0.05 was considered significant.

205 **RESULTS**

206 **1. IDENTIFICATION OF CC398 ISOLATES BELONGING TO THE EMERGING** SUBPOPULATION**.**

207 We studied the prophage content of 21 CC398 isolates to identify those belonging to
208 this *emerging* subpopulation. We searched for isolates carrying a β-converting prophage
209 encoding an immune evasion cluster (IEC) —characteristic of the two subpopulations of the
210 human clade (Price, 2012; Uhleman, 2012; McCarthy, 2011)— and additional prophages
211 elements, believed to distinguish isolates of the ancestral subpopulation from the emerging
212 human subpopulation (Price, 2012). Using three different methods, IEC characterization,
213 prophage profiling and hybridization of genomic DNA with microarrays, the ten non-LA
214 MSSA isolates were all distinguished from the LA clade and confirmed to belong to the
215 human clade**.** An IEC carrying *chp* and *scn* (van Wamel, 2006) was found in all non-LA
216 isolates, and no IEC was found in any of the LA isolates (Tab. 1). LA lysogens showed a
217 diversity of prophage profiles, all of which lacked a Sa3 integrase; and their DNA hybridized
218 with probes representing the L54a phage (Fig. 1). In contrast, the prophage profiles of non-
219 LA isolates were more homogeneous: all prophages in these isolates had genes encoding a
220 Sa3 integrase and F serogroup; and the DNA from all the non-LA isolates hybridized with
221 probes corresponding to a β-converting prophage encoding the putative virulence factor
222 CHIPS. This indicates that all these non-LA isolates contain a β-converting prophage encoding
223 an IEC.

224 Prophage profiling and microarray data revealed two subgroups among the non-LA
225 MSSA isolates (Tab.1; Fig.1). One subgroup comprised six isolates only harboring Sa3
226 prophage elements. Four isolates in the second subgroup harbored additional prophage
227 elements next to the Sa3 prophage elements. These additional elements hybridized with
228 probes for a gene encoding a protein related to SaPI, and probes for phages ϕSLT and ϕETA.

229 Some of these additional prophage elements were similar to those in the LA-isolates.
230 Isolates belonging to the first of these two subgroups were considered to belong to the
231 ancestral subpopulation, and those of the second subgroup to belong to the emerging
232 subpopulation. These subgroups were named A, for ancestral subpopulation, and E, for
233 emerging subpopulation respectively.
234
235 **2. PHAGE ISOLATION.**
236 For further characterization of prophage features specifically associated with the
237 emerging subpopulation represented by isolates of subgroup E, we tested whether the
238 prophage elements identified in their genomes encoded functional or defective prophages.
239 The 21 isolates were treated with mitomycin C and culture filtrates obtained. Fourteen of
240 the 21 filtrates, including those of subgroup E, produced plaques on indicator strains,
241 indicating the presence of infectious particles (Suppl. file Tab. 1). LA-isolate cultures cleared,
242 indicating cell lysis, and thus that the prophages induced were functional. In contrast, cell
243 lysis was not observed in any non-LA isolate cultures following treatment with mitomycin C:
244 even after prolonged incubation, these cultures remained turbid. This indicates that most
245 non-LA cells were able to survive in the presence of their phage particles, suggesting that the
246 lysogens had superinfection immunity of or that phages were defective. One infective lysate
247 was studied further: S100-ϕ obtained from the non-LA isolate of subgroup E S100. The
248 phage was purified and propagated. Electron microscopy analysis (Suppl. file Fig. 1) indicated
249 that the phage belongs to the *Siphoviridae* family: phages of this family have isometric, non-
250 enveloped heads and filamentous cross-banded tails with short terminal fibers. The phage
251 particles in lysate S100-ϕ had 53nm-diameter heads and138nm-long tails (± 10nm).
252

253 **3. LYSOGENIZATION OF PROPHAGE-FREE ISOLATES WITH THE PHAGES PRODUCED BY S100.**

254 A reliable method for studying the consequences of lysogeny is to lysogenize clonal
255 recipient strains devoid of prophage elements with natural phage. Prophage profiling and
256 whole-genome sequencing results demonstrated that the LA isolates S123 and S124 were
257 devoid of prophages, and these isolates were therefore used as prophage-free recipients.
258 The infective lysate S100-ϕ was used to transduce the prophage-free isolates S123 and S124.
259 Lysogenization was only obtained with S124, leading to isolate S124/S100-ϕ. Lysogenization
260 was confirmed by phage profiling and whole-genome sequencing of the transductants.
261 S124/S100-ϕ lysogens were not stable, as the prophage was rapidly lost during bacterial
262 culture.

263

264 **4. CHARACTERIZATION OF PHAGES AND PROPHAGES**

265 **OF THE EMERGING HUMAN-ADAPTED NON-LA ISOLATE S100.**

266 Using whole-genome sequencing, we identified one phage, StauST398-1 in the non-
267 LA S100-ϕ lysate. Whole-genome comparison of StauST398-1 with parental (S124),
268 transductant (S124/S100-ϕ) and donor (S100) isolates revealed that the StauST398-1 phage
269 resulted from the recombination of the prophages, StauST398-4pro and StauST398-5pro, in
270 the S100 donor isolate genome (Fig. 2). This recombination has led to the loss of part of the
271 IEC locus (Fig. 2).

272 The StauST398-4pro genome (accession number KC595278) is a linear, double-
273 stranded DNA molecule of 41.9 Kb and 0.339% G+C. It contains 65 putative coding
274 sequences and was inserted into the *hlb* gene, 824 nucleotides downstream from the ATG
275 codon. Genes for lysogeny, including an integrase (RusA), a repressor protein, and an
276 antirepressor, were found between genes involved in the lytic cycle and replication. No

277 known restriction system was found. However, part of a putative type III restriction-
278 modification system was identified by the annotating tool. The phage genome carries two
279 putative virulence genes, *chp* and *scn*, around 2.5 Kb upstream from the insertion site. The
280 sequence of StauST398-4pro shares approximately 97% identity with phi3, recently
281 described in *S. aureus* strain 71193 (Uhleman, 2012) and 91% with the close neighbor of
282 phiNM3, recently described by Price and colleagues (Price, 2012). The two other phages
283 described by Price and colleagues are much more divergent from StauST398-4pro (<15%
284 identity). PCR with primers specific for StauST398-4pro, amplified fragments of this β-
285 converting ϕ3-prophage from all of the non-LA CC398 isolates but none of the LA isolates
286 (Suppl. File Tab.2).

287 The StauST398-5pro genome (accession number KC595279) is a 40.2 Kb, linear,
288 double-stranded DNA with 0.353% G+C. It carries 55 putative coding sequences, including a
289 putative gene for a superantigen similar to enterotoxin B. It is inserted in *smpB*. It has a
290 modular organization with gene clusters involved in DNA packaging, head and tail
291 morphogenesis, cell lysis, lysogeny, replication and regulation. The lysogeny module was
292 found between the lysis cassette and the replication module, with a repressor protein cI, the
293 repressor cro and an antirepressor that appeared to result from antA/B recombination
294 (Davis, 2002). The transcriptional regulation module comprises a dimeric dUTPase, an
295 integrase activator and a rho terminator factor. This phage lacks an integrase, indicating a
296 lytic-defective prophage. Homology searches identified a putative restriction system.
297 StauST398-5pro shares 75% identity with ϕMR11 (Rashel, 2007) and with ϕETA (Yamaguchi,
298 2000) over more than half of its length. The remaining 15 Kb of the genome showed a highly
299 mosaic structure with small segments (generally <3 Kb) sharing some similarity with ϕPVL,

300 ϕETA, ϕ13 or ϕROSA (Yamaguchi, 2000; Kaneko, 1998; Iandolo, 2002). PCR tests with
301 specific primers detected StauST398-5pro only in isolates of subgroup E (Suppl. File Tab.3).
302 The StauST398-1pro genome (accession number JX013863) is 45.2 Kb, 0.345% G+C,
303 and contains 72 putative protein-coding genes. The 5-35Kb region of this phage genome is
304 homologous to ϕETA (Yamaguchi, 2000) and has two 5 Kb regions of similarity with the
305 genome extremities of ϕNM3 (Bae, 2006; Baba, 2008). The StauST398-1pro genome
306 encodes a dUTPase and a putative endonuclease (*nuc*) also present in ϕNM3. The phage
307 replicon is similar to *S. aureus* phage ϕ80 (Christie, 2010). It contains the IEC with only *scn*
308 but not *chp,* which is 3 Kb upstream from the *attL* site in StauST398-4 pro. The recombinant
309 phage StauST398-1 was longer than the prophage StauST398-1pro in the S124 genome,
310 resulting from a partial deletion of the prophage during the insertion. To evaluate the
311 integrity of the IEC during phage excision and insertion, phage lysate S100-ϕ and genomic
312 DNA from isolates S100, S124 and S124/S100-ϕ were subjected to PCR to test for the
313 presence of *chp* and *scn*. Both genes were present in StauST398-1, whereas only *scn* was
314 detected in StauST398-1pro. This indicates that *chp* was lost upon integration of StauST398-
315 1 into the S124 genome.
316
317 **5. IMPACT OF LYSOGENY ON PHAGE RESISTANCE, TRANSFORMATION AND INVASION OF HUMAN CELLS.**
318 To investigate whether lysogeny in CC398 isolates of subgroup E affected various
319 host characteristics, we tested lysogenic and non-lysogenic *S. aureus* for (i) resistance to
320 infection by a large panel of *S. aureus* phages, (ii) transformation by vectors propagated in *S.*
321 *aureus* or other hosts, and (iii) epithelial cell invasiveness.
322 ***5.1. Phage resistance.*** The prophage-free isolates S123 and S124 were highly
323 susceptible to phage infections; by contrast, all LA *and* non-LA lysogens were resistant to

324 numerous, diverse *S. aureus* phages (Suppl. file Tab. 1). Lysogenization of the prophage-free
325 isolate S124 with S100-ϕ lysate, containing the recombinant phage StauST398-1, resulted in
326 resistance to a broad range of phages, similar to isolates lysogenized with the ϕ42 prophage
327 that encodes a powerful restriction modification (RM) system (Dempsey, 2005).

328 ***5.2. Transformation experiments***. The efficiency with which the following strains
329 could be transformed was determined: the parental strains (S123 and S124), transductant
330 strain (S124/S100-ϕ), non-LA donor S100 of subgroup E (harboring StauST398-4pro and
331 StauST398-5pro) strains, three non-LA isolates of subgroup A (that only harbor StauST398-
332 4pro), and *S. aureus* RN4220, a laboratory strain lacking a RM system (Nair, 2011) (Tab. 2).
333 The prophage-free isolates S123 and S124 were transformed efficiently by both RN4220 and
334 *E. coli* plasmid DNA. Plasmid DNA from *Enterococcus faecalis*, a potential source of the *van*
335 operon, was also taken up by these two isolates at a frequency similar to that for *S. aureus*
336 RN4220. By contrast, the naturally lysogenic isolates S1 and S100 were resistant to
337 transformation with these plasmid DNAs. The efficiency of transformation of the S124
338 prophage-free isolate was substantially reduced by lysogenization with the non-LA infective
339 lysate. Note that the reduction of transformation efficiency upon lysogenization may have
340 been partially masked because of the instability of lysogeny observed in S124/S100-ϕ. The
341 isolates of subgroup A, that harbor the IEC-containing prophage StauST398-4pro and lack
342 StauST398-5pro, were efficiently transformed with plasmid DNA (RN4220 or *E. coli* DH5); this
343 indicates a weaker protection against transformation by a broad range of foreign DNAs than
344 observed with the isolates of subgroup E that harbor the two prophages (StauST398-4pro
345 and StauST398-5pro).

346 ***5.3. Invasion experiments***. *S. aureus* is not an obligatory intracellular pathogen.
347 However, the intrinsic virulence of isolates is associated with their ability to reside in the

348 cytoplasm of host epithelial or endothelial cells, particularly in chronic infections (Sinha,
349 1999; Voyich, 2005). Invasion of epithelial cells by representative isolates (the parental (S123
350 and S124) and donor (S100) strains, LA isolates (S1 and S130), non-LA isolates (S89 and S94),
351 and two control strains (Cowan and KH11) was evaluated. Note that the transductant
352 S124/S100-ϕ could not be studied, because of rapid loss of the phage during the
353 experiments. All the LA and non-LA lysogens were 3-4 fold more invasive than the Cowan
354 strain (Fig. 3), a highly invasive strain used as a positive control. The prophage-free isolates
355 S123 and S124 were not able to invade cells; however, lysogenization conferred invasiveness
356 on these strains.

357 ***Transcription experiments.*** RNA was isolated during exponential growth and
358 stationary phase to test for the expression of virulence-related genes of the IEC locus (*chp,*
359 *sak* and *scn*). The following non-LA isolates were studied: S81 carrying StauST398-4pro and
360 StauST398-5pro (subgroup E), S94 and S89 only carrying StauST398-4pro. The unstable
361 transductant S124/S100-ϕ was not studied. In all three isolates, the expression of virulence-
362 related genes was strongly induced during stress, particularly exposure to concentrations of
363 antibiotics below the minimum inhibitory concentration (MIC) (Fig. 4). This is consistent with
364 the general increase in gene expression during stress, particularly during infection (Beaume,
365 2010). Virulence-related mRNAs were similarly abundant during stationary and exponential
366 growth phases in both S94 and S89, two isolates that only harbour StauST398-4pro;
367 however, these mRNAs were more abundant in S81, the isolate carrying StauST398-4pro and
368 StauST398-5pro, during the stationary phase than the exponential growth phase.

369 **DISCUSSION**

370 Using a novel approach, different to those used previously (Price, 2012; Uhleman,
371 2012; McCarthy, 2011), we report prophage features specifically associated with the CC398
372 isolates that have recently been causing an increasing number of invasive infections
373 worldwide in humans living in animal-free environments (Stegger, 2010; Valentin-Domelier,
374 2011; Jimenez, 2011) and that readily colonize humans and spread between them (Uhleman,
375 2012). We used a variety of methods, including genomic microarray analysis, phage content
376 profiling, immune-evasion cluster typing and genome sequencing, to detect and analyze
377 these genetic elements. We report evidence for a helper prophage, not previously
378 described, that interacts with the well-recognized human-associated β-converting prophage,
379 encodes a restriction modification system, and influences the ability of CC398 isolates to
380 colonize and infect humans.

381 We used genotypic methods allowing subpopulations to be distinguished within an
382 apparently homogeneous population of *S. aureus* isolates. It has limitations. One is the way
383 we used of microarrays, which allowed analysis of only known or identified target
384 sequences. Also the choice of strains included in the analysis may have affected the findings,
385 and the number of strains studied was limited. We are currently investigated a larger
386 collection of strains. We were nevertheless able to identify particular phenotypes specific to
387 groups of strains and at least partly relate these phenotypes to the genome content of
388 representative isolates used for full genome sequencing.

389 The characterization of prophages from naturally lysogenic non-LA isolates provided
390 evidence of two distinct subgroups among MSSA isolates responsible for human invasive
391 infections. The first subgroup includes isolates that only carry a Sa3 prophage (named
392 StauST398-4pro). The second subgroup comprises isolates carrying this same Sa3 prophage,

393 but also a ϕMR11-like prophage (named StauST398-5pro). StauST398-4pro is an IEC β-
394 converting Sa3 prophage that has been described in isolates responsible for human
395 infections in LA- and non-LA environments (Stegger, 2010; Valentin-Domelier, 2011;
396 Jimenez, 2011), but StauST398-5pro has not previously been detected in isolates responsible
397 for human infections in a LA environment. Therefore, we suggest that isolates only harboring
398 StauST398-4pro belong the ancestral human LA subpopulation, and that StauST398-5pro is
399 specific to isolates responsible for the more recent human invasive infections in a non-LA
400 environment, i.e. isolates of the emerging human-adapted non-LA CC398 subpopulation.

401 Lysogeny is a symbiotic strategy in which the prophage has a stable niche in the host
402 genome, and protects its host against attack by other phages (Goerke, 2009), through
403 co-immunity, CRISPR interference (Barrangou, 2007; Marraffini, 2008) or expression of RM
404 activities that inhibit interspecies and even intraspecies gene transfers (Barrangou, 2007;
405 Park, 1999). This may be particularly relevant for the CC398 lineage. Our sequencing findings
406 clearly indicate that CC398 does not harbor any genes for a type IV RM system similar to
407 those previously described (Corvaglia, 2010). In addition, the 296 bp fragment of the *sauI-*
408 *hsdSI* gene, a reliable discriminative marker of *S. aureus* CC398 (Stegger, 2011), is present
409 and strictly conserved in our isolates. However, our transformation and transduction
410 experiments clearly indicate that the presence of this *sauI-hsdSI* gene, a component of a
411 type I RM system, is not associated with protection against uptake of foreign DNA (Waldron,
412 2006; Corvaglia, 2010).

413 We demonstrate that CC398 prophages, like most described *S. aureus* prophages,
414 enabled their hosts to exclude other phages and exogenous DNA. However, the protection
415 conferred by these prophages varied: the protection conferred by StauST398-4pro prophage
416 was however limited, but if StauST398-5pro prophage was present in the non-LA isolate, the

417 protection was strong. *In silico* analysis of complete genomes from naturally lysogenic non-
418 LA CC398 did not identify any CRISPR or CRISPR-associated genes. Prophage sequences
419 encoding a putative RM system were identified in StauST398-5pro. Thus, the emerging
420 CC398 isolates, all of which harbor StauST398-5pro, have a prophage-encoded RM system
421 conferring strong protection against horizontal genetic transfer, similar to that described for
422 the ϕ42 prophage (Dempsey, 2005). This protection may confer a selective advantage over
423 competing organisms in their environment.

424 When prophages are fully functional, lysogeny is an Achilles heel for the bacteria;
425 they are vulnerable to environmental factors that cause sublethal DNA damage and
426 subsequent prophage induction. Prophage repressors are inactivated during the SOS
427 response, allowing the phage lytic cycle to proceed (Davis, 2002; Ubeda, 2005). Non-LA
428 isolates were able to produce phage particles, but only following recombination between
429 two defective prophages: the IEC (*chp-scn*) β-converting *S. aureus* prophage variant that has
430 previously been reported to be defective (van Wamel, 2006), and StauST398-5pro, the
431 genome of which does not contain an integrase gene. If integrated prophages become
432 defective, SOS-induced prophages do not lyse cells such that the host cells become
433 insensitive to the bactericidal effects of DNA-damaging antibiotics. This situation is thus
434 similar to that of cells that are not lysogens. We observed this phenomenon during the non-
435 LA phage lytic cycle. Therefore, in the human environment, where the SOS response is
436 frequently activated (Ubeda, 2005), natural lysogens belonging to the *emerging*
437 subpopulation and harboring the defective prophages StauST398-4pro and StauST398-5pro
438 may not be less vulnerable.

439 Prophages can contribute directly to bacterial pathogenesis at the time of infection
440 (Sumby, 2003). The expression of temperate prophage genes, that are generally repressed,

441 may be induced during stress and/or induction of the SOS system, because the repressor, cI,
442 is inactivated by RecA. This can result in increased transcription of virulence factors, as has
443 been demonstrated for staphylokinase production in *S. aureus* strains carrying β-converting
444 phages with the *sak* gene (Rooijakkers, 2006). We demonstrated that the non-LA prophage
445 StauST398-5pro encodes a putative superantigen similar to enterotoxin B and the non-LA β-
446 converting prophage StauST398-4pro encodes and expresses two human-specific virulence
447 genes (*chp* and *scn*) (van Wamel, 2006). *S. aureus* enters the body through breaches in the
448 skin or mucous membranes, and is then immediately confronted by the host innate defense
449 system. To counteract innate immunity, *S. aureus* expresses immune-modulating proteins
450 encoded by β-converting prophages, that facilitate long-term colonization in humans
451 (Goerke, 2006; Rooijakkers, 2006): the staphylococcal complement inhibitor (SCIN) prevents
452 opsonophagocytosis and killing of *S. aureus* by human neutrophils, the chemotaxis inhibitory
453 protein of *S. aureus* (CHIPS), the bacterial plasminogen activator (SAK) and the superantigens
454 SEA and SEB, that down-regulate chemokine receptors on monocytes. Our transcription
455 experiments indicate that non-LA CC398 lysogens may benefit from the production of SEB,
456 CHIPS and SCIN (products of *seb*, *chp* and *scn* genes, respectively) when exposed to
457 conditions that favor prophage induction.

458 In the genome of StauST398-5pro, we identified a transcription repressor and
459 antirepressor resulting from antA/B recombination, characteristic of a helper prophage
460 (Davis, 2002). The association between a helper-phage and a satellite prophage allows the
461 production proteins encoded by the satellite phage that may escape lysogenic repression
462 because of interactions between repressors and antirepressors. In *S. aureus*, the association
463 between a β-converting phage and a helper phage has been shown to result in the
464 expression of prophage-encoded virulence genes, such as *sea* and *sak*, during stress (e.g.

465 DNA damage) and lysogeny (Coleman, 1989; Carroll, 1993). Therefore, lysogeny associating
466 the ϕMR11-like helper prophage StauST398-5pro (encoding *seb*) and the satellite
467 β-converting prophage StauST398-4pro (encoding *chp* and *scn*) may facilitate the expression
468 of prophage virulence genes, notably *chp* and *scn*, both when the prophage is induced and
469 during lysogeny.

470 Lysogenization of *S. aureus* with CC398-phages conferred increased invasiveness. It
471 seems unlikely that phage-encoded lysins affect human epithelial cells, as their target
472 structures are not found in eukaryotic cells. However, phage endopeptidases, for example
473 lysostaphin which is capable of degrading some human proteins, may affect mammalian
474 tissues (Park, 1999). This possibility could be investigated with an animal model of
475 endocarditis for example, as CC398 isolates have been associated with a human case of
476 endocarditis in a highly immuno-compromised patient (Schijffelen, 2010). However, the two
477 subgroups of isolates recovered from human invasive infections (those harboring StauST398-
478 4pro and those harboring StauST398-4pro and StauST398-5pro) did not differ in their ability
479 to invade human cells, suggesting that the StauST3985-pro prophage is not involved.

480 The prophages StauST398-4pro and StauST398-5pro were inserted at different loci in
481 the genomes of non-LA isolates, but it all cases inactivated a bacterial virulence gene:
482 StauST398-4pro in *hlb* and StauST398-5pro in *smpB.* Phages are potentially unstable and
483 bacterial isolates tend to loose their phages. Thus, the maintenance of a β-converting
484 prophage in the host chromosome indicates that this phage is beneficial or even essential.
485 Such prophages may complement strain physiology and allow survival in the potentially
486 hostile environmental conditions during colonization and infection (Smeltzer, 1994; Wagner,
487 2002). Studying *S. aureus* and diabetic feet, Lavigne recently associated the disruption of a
488 staphylococcal virulence gene by a ROSA-like prophage with the colonizing status of

489 *S. aureus* isolates (Messad, 2012). The authors demonstrated that lysogeny resulted in the
490 human immune response being limited thereby favoring resilient and stable colonization.
491 StauST398-4pro and StauST398-5pro in CC398 isolates may have a similar effect; virulence
492 may be reduced by prophage integration, favoring colonization, and thus bloodstream
493 infection (del Rio, 2009).

494 Concordant with recent studies, our findings support the existence of three
495 subpopulations in the CC398 lineage: an ancestral clade that originated in humans (here
496 represented by subgroup A), a lineage composed predominantly of livestock-associated
497 isolates (Price, 2012), and one that emerged recently by clonal expansion in humans (here,
498 subgroup E) (Uhleman, 2012). Indeed, hybridization of genomic DNA with microarrays
499 revealed no LA-prophage remnants in the genomes of the first subgroup of non-LA isolates,
500 consistent with this subgroup being part of the human ancestral clade, and not the classical
501 livestock-associated clade. In contrast, our data demonstrate that subgroup E of non-LA
502 isolates and the LA isolates share some prophage elements, suggesting an animal origin of
503 the newly described prophage StauST398-5pro. Further investigations are required to
504 confirm these findings.

505

506 **CONCLUSION**

507 By studying genomes using microarrays, and determining prophage content and
508 lysogeny characteristics within the CC398 lineage, we describe important features of the
509 human-adapted emerging non-LA CC398 subpopulation. The association of a pre-existing β-
510 converting-defective phage and a newly infecting phage possessing the characteristics of a
511 helper phage, has resulted in pathogen diversification via gene transfer, and contributes to
512 bacterial pathogenesis at the time of infection. We clearly demonstrate that prophage

513 content can influence the phenotypes that contribute to genome plasticity (notably
514 transformation and transduction) and to the invasiveness of *S. aureus*. These prophages in
515 the bacterial host provide protection against horizontal genetic transfer, and also contribute
516 to virulence and adaptation of the host bacterium.
517

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539 **REFERENCES**

540 - Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K. et al., 2002. Genome and
541 virulence determinants of high virulence community-acquired MRSA. Lancet. 359, 1819-
542 27.

543 - Baba, T., Bae, T., Schneewind, O., Takeuchi, F., Hiramatsu, K., 2008. Genome sequence of
544 *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal
545 genomes: polymorphism and evolution of two major pathogenicity islands. J. Bacteriol.
546 190, 300-10.

547 - Bae, T., Baba, T., Hiramatsu, K., Schneewing, O., 2006. Prophages of *Staphylococcus*
548 *aureus* Newman and their contribution to virulence. Mol. Microbiol. 62, 1035-47.

549 - Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P. et al.,2007. CRISPR
550 provides acquired resistance against viruses in prokaryotes. Science. 315, 1709-12.

551 - Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P. et al.,2010. Cartography of
552 methicillin-resistant *Staphylococcus aureus* transcripts: detection, orientation and
553 temporal expression during growth phase and stress conditions. PLoS One. 5, e10725.

554 - Carroll, J.D., Cafferkey, M.T., Coleman, D.C., 1993. Serotype F double- and triple-
555 converting phage insertionally inactivate the *Staphylococcus aureus* beta-toxin
556 determinant by a common molecular mechanism. FEMS. Microbiol. Lett. 106, 147-55.

557 - Charbonnier, Y., Gettler, B., Francois, P., Bento, M., Renzoni, A. et al., 2005. A generic
558 approach for the design of whole-genome oligoarrays, validated for genomotyping,
559 deletion mapping and gene expression analysis on *Staphylococcus aureus*. BMC. Genom.
560 6, p. 95.

561 - Christie, G.E., Matthews, A.M., King, D.G., Lane, K.D., Olivarez, N.P. et al., 2010. The
562 complete genomes of *Staphylococcus aureus* bacteriophages 80 and 80α--implications
563 for the specificity of SaPI mobilization. Virology. 407, 381-90.
564 - Coleman, D.C., Sullivan, D.J., Russell, R.J., Arbuthnott, J.P., Carey, B.F., Pomeroy, H.M.,
565 1989. *Staphylococcus aureus* bacteriophages mediating lysogenic conversion of beta-
566 lysin, staphylokinase and enterotoxin A: molecular mechanism of triphe conversion. J.
567 Gen. Microbiol. 135, 1679-97.
568 - Corvaglia, A.R., François, P., Hernandez, D., Perron, K., Linder, P. et al., 2010. A type III-
569 like restriction endonuclease functions as a major barrier to horizontal gene transfer in
570 clinical *Staphylococcus aureus* strains. Proc. Natl. Acad. Sci. U S A. 107, 11954-58.
571 - Davis, B.M., Kimsey, H.H., Kane, A.V., Waldor, M.K., 2002. A satellite phage-encoded
572 antirepressor induces repressor aggregation and cholera toxin gene transfer. EMBO. J.
573 21, 4240-49.
574 - de Gialluly, C., Loulergue, J., Bruant, G., Mereghetti, L., Massuard, S. et al. ,2003.
575 Identification of new phages to type *Staphylococcus aureus* strains and comparison with
576 a genotypic method. J. Hosp. Infect. 55, 61-7.
577 - del Rio, A., Cervera, C., Moreno, A., Moreillon, P., Miró, J.M. 2009. Patients at risk of
578 complications of *Staphylococcus aureus* bloodstream infection. Clin. Infect. Dis. 48 Suppl
579 4, S246-53.
580 - Dempsey, R.M., Carroll, D., Kong, H., Higgins, L., Keane, C.T. et al.. 2005. Sau42I, a BcgI-
581 like restriction-modification system encoded by the *Staphylococcus aureus* quadruple-
582 converting phage Φ42. Microbiol. 151, 1301-11.

583 - Diep, B.B., Gill, S.R., Chang, R.F., Phan, T.H., Chen, J.H. et al. 2006. Complete genome
584 sequence of USA300, an epidemic clone of community-acquired meticillin-resistant
585 *Staphylococcus aureus*. Lancet. 367,731-9.
586 - Gill, S.R., Fouts, D.E., Archer, G.L., Mongodin, E.F., Deboy, R.T., Ravel, J. et al., 2005.
587 Insights on evolution of virulence and resistance from the complete genome analysis of
588 an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing
589 methicillin-resistant *Staphylococcus epidermidis* strain. J. Bacteriol. 187, 2426-38.
590 - Gillaspy, A.F., Worrell, V., Orvis, J., Roe, B.A., Dyer, D.W. et al. 2006. Gram-positive
591 pathogens. 2nd edition. Edited by Fischetti, V.A., Novick, R.P., Ferretti, F., Portnoy, D.A.,
592 Rood, J. Washington, DC, ASM Press. pp. 381-412.
593 - Gizard, Y., Zbinden, A., Schrenzel, J., François, P. 2013. Whole-Genome Sequences of
594 *Streptococcus tigurinus* Type strain AZ 3a and *S. tigurinus*, a strain causing prosthetic
595 joint infection. Genome Announc. 1(2). doi:pii: e00210-12. 10.1128/genomeA.00210-12.
596 - Goerke, C., Kraning, K., Stern, M., Döring, G., Botzenhart, K. et al., 2004. Increased
597 frequency of genomic alterations in *Staphylococcus aureus* during chronic infection is in
598 part due to phage mobilization. J. Infect. Dis. 189, 724-34.
599 - Goerke, C., Wirtz, C., Flückiger, U., Wolz, C., 2006. Extensive phage dynamics in
600 *Staphylococcus aureus* contributes to adaptation to the human host during infection.
601 Mol. Microbiol. 61, 1673-85.
602 - Goerke, C., Pantucek, R., Holtfreter, S., Schulte, B., Zink, M. et al., 2009. Diversity of
603 prophages in dominant *Staphylococcus aureus* clonal lineages. J. Bacteriol. 191, 3462-68.
604 - Hallin, M., De Mendonca, R., Denis, O., Lefort, A., El Garch, F. et al. 2011. Diversity of
605 accessory genome of human and livestock-associated ST398 methicillin resistant
606 *Staphylococcus aureus* strains. Infcet. Genet. Evol. 11,290-9.

607 - Huijsdens, X.W., van Dijke, B.J., Spalburg, E., van Santen-Verheuvel, M.G., Heck, M.E. et
608 al., 2006. Community-acquired MRSA and pig-farming. Ann. Clin. Microbiol. Antimicrob.
609 5, 26.
610 - Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P. et al. 2004. Complete
611 genomes of two clinical Staphylococcus aureus strains: evidence for rapide evolution of
612 virulence and drug resistance. Proc. Natl. Acad. Sci. U S A. 101,9786-91.
613 - Iandolo, J.J., Worrell, V., Groicher, K.H., Qian, Y., Tian, R., Kenton, S. et al., 2002.
614 Comparative analysis of the genomes of the temperate bacteriophages ϕ11, ϕ12 and
615 ϕ13 of *Staphylococcus aureus* 8325. Gene. 289, 109-18.
616 - Jimenez, J.N., Velez, L.A., Mediavilla, J.R., Ocampo, A.M., Vanegas, J.M. et al., 2011.
617 Livestock-associated methicillin-susceptible *Staphylococcus aureus* ST398 infection in
618 women, Colombia. Emerg. Infect. Dis. 17, 1970-71.
619 - Kahankova, J., Pantucek, R., Goerke, C., Ruzickova, V., Holochova, P. et al., 2010.
620 Multilocus PCR typing strategy for differentiation of *Staphylococcus aureus* siphoviruses
621 reflecting their modular genome structure. Environ. Microbiol. 12, 2527-38.
622 - Kaneko, J., Kimura T., Narita S., Tomita, T., Kamio, Y., 1998. Complete nucleotide
623 sequence and molecular characterization of the temperate staphylococcal bacteriophage
624 ϕSLT carrying Panton-Valentine leukocidin genes. Gene. 215, 57-67.
625 - Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H. et al., 2001. Whole genome
626 sequencing of meticillin-resistant *Staphylococcus aureus.* Lancet. 357, 1225-40.
627 - Lee JC., 1995. Electrotransformation of staphylococci. Methods Mol. Biol. 47, 209-216.
628 - Marraffini, L.A., and Sontheimer, E.J., 2008. CRISPR interference limits horizontal gene
629 transfer in staphylococci by targeting DNA. Science. 322, 1843-45.

630 - McCarthy, A.J., Witney, A.A., Gould, K.A., Moodley, A., Guardabassi L. et al., 2011. The
631 distribution of MGEs in MRSA CC398 is associated with both host and country. Genome
632 Biol. Evol. 3, 1164-74.
633 - Messad, N., Lina, G., Prasjnar, T.K., Renshaw, S.A., Vandenesch, F. et al. 2012. Insertion
634 d'un phage dérégulant la virulence de *Staphylococcus aureus.* Abstr. 32ième Réunion
635 Interdisciplinaire de Chimiothérapie Anti-Infectieuse. O-15.
636 - Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M. et al., 2011. Whole-genome
637 sequencing of *Staphylococcus aureus* strain RN4220, a key laboratory strain used in
638 virulence research, identifies mutations that affect not only virulence factors but also the
639 fitness of the strain. J. Bacteriol. 193, 2332-35.
640 - Park, P.W., Senior, R.M., Griffin, G.L., Broekelmann, T.J., Mudd, M.S., Mecham, R.P.,
641 1999. Binding and degradation of elastin by the staphylolytic enzyme lysostaphin. Int. J.
642 Biochem. Cell. Biol. 27, 139–146.
643 - Price, L.B., Stegger, M., Hasman, H., Aziz, M., Larsen, J. et al., 2012. *Staphylococcus*
644 *aureus* CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock.
645 MBio. 3, e00305-11.
646 - Rashel, M., Uchiyama, J., Ujihara, T., Uehara, Y., Kuramoto, S. et al., 2007. Efficient
647 elimination of multidrug-resistant *Staphylococcus aureus* by cloned lysin derived from
648 bacteriophage ϕMR11. J. Infect. Dis. 196, 1237-47.
649 - Rooijakkers, S.H., Ruyken, M., van Roon, J., van Kessel, K.P., van Strijp, J.A. el al., 2006.
650 Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus*
651 *aureus*. Cell. Microbiol. 8, 1282-93.

652 - Schijffelen, M.J., Boel, C.H., van Strijp, JA., 2010. Whole genome analysis of a livestock-
653 associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of
654 humen endocarditis. BMC. Genom. 11, 376.
655 - Sinha, B., François, P.P., Nüsse, O., Foti, M., Hartford, O.M. et al., 1999. Fibronectin-
656 binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin
657 alpha5beta1. Cell. Microbiol. 1, 101-17.
658 - Smeltzer, M.S., Hart, M.E., Iandolo J.J., 1994. The effect of lysogeny on the genomic
659 organization of *Staphylococcus aureus*. Gene. 138, 51-7.
660 - Stegger, M., Lindsay, J.A., Sorum, M., Gould, K.A., Skov, R., 2010. Genetic diversity in
661 CC398 methicillin-resistant *Staphylococcus aureus* isolates of different geographical
662 origin. Clin. Microbiol. Infect. 16,1017-19.
663 - Stegger, M., Lindsay, J.A., Moodley, A., Skov, R., Broens, E.M. et al. 2011. Rapid PCR
664 detection of *Staphylococcus aureus* clonal complex 398 by targeting the restriction-
665 modification system carrying sau1-hsdS1. J Clin. Microbiol. 49,732-4.
666 - Sumby, P. and Waldor, M.K., 2003. Transcription of the toxin genes present within the
667 staphylococcal phage phiSa3ms is intimately linked with the phage's life cycle. J.
668 Bacteriol. 185, 6841-51.
669 - Ubeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R.P., Penades, J.R., 2005. Antibiotic-
670 induced SOS response promotes horizontal dissemination of pathogenicity island-
671 encoded virulence factors in staphylococci. Mol. Microbiol. 56, 836-44.
672 - Uhleman, A.C., Porcella, S., Trivedi, S., Sullivan, S., Hafer, C. et al., 2012. Identification of
673 a highly transmissible animal-independent *Staphylococcus aureus* ST398 clone with
674 distinct genomic and cell adhesion properties. Mbio. 3, e00027-12.

675 - Valentin-Domelier, A.S., Girard, M., Bertrand, X., Violette, J., François, P. et al., 2011.
676 Methicillin-susceptible ST398 *Staphylococcus aureus* responsible for bloodstream
677 infections: an emerging human-adapted subclone ? PloS One. 6, e28369.
678 - van Belkum, A., Melles, D.C., Peeters, J.K., van Leeuwen, W.B., van Duijkeren, E. et al.,
679 2008. Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in
680 pigs and humans. Emerg. Infect. Dis. 14, 479-83.
681 - van Wamel, W.J., Rooijakkers, S.H., Ruyken, M., van Kessel, K.P., van Strijp, J.A., 2006.
682 The innate immune modulators staphylococcal complement inhibitor and chemotaxis
683 inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting
684 bacteriophages. J. Bacteriol. 188, 1310-15.
685 - Voyich, J.M., Braughton, K.R., Sturdevant, D.E., Whitney, A.R., Porcella, S.F. et al., 2005.
686 Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human
687 neutrophils. J. Immunol. 175, 3907-19.
688 - Wagner, P.L., Waldor, M.K., 2002. Bacteriophage control of bacterial virulence. Infect.
689 Immun. 70, 3985-93.
690 - Waldron, D.E., Lindsay, J.A., 2006. Sau1: a novel lineage-specific type I restriction-
691 modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and
692 between *S. aureus* isolates of different lineages. J. Bacteriol. 188, 5578-85.
693 - Witte, W., Strommenger, B., Stanek, C., Cuny, C., 2007. Methicillin-resistant
694 *Staphylococcus aureus* ST398 in humans and animals, Central Europe. Emerg. Infect. Dis.
695 13, 255-58.
696 - Xia, G., Wolz, C. 2013. Phages of *Staphylococcus aureus* and their impact on host
697 evolution. Infect. Genet. Evol. pii: S1567-1348(13)00171-8. doi:
698 10.1016/j.meegid.2013.04.022.

699 - Yamaguchi, T., Hayashi, T., Takami, H., Nakasone, K., Ohnishi, M. et al., 2000. Phage
700 conversion of exfoliative toxin A production in *Staphylococcus aureus*. Mol. Microbiol.
701 38, 694-705.

702 **FIGURE LEGENDS**

703 **Figure 1.**

704 Microarray analysis of LA and non-LA CC398 isolates. Clusters of hybridization probes that
705 distinguish LA from non-LA *S. aureus* CC398. Black dots show probes displaying positive
706 fluorescent signals.

707

708 **Figure 2.**

709 Schematic representation of the recombination between StauST398-4pro and StauST398-
710 5pro that led to StauST398-1. Note that most of the sequence originates from StauST398-5
711 and is flanked by StauST398-4 extremities. This recombination resulted in the partial
712 deletion of the IEC locus.

713

714 **Figure 3.**

715 Epithelial cell invasion experiments**.** Invasiveness of *S. aureus* strains in 293 cells was
716 determined relative to that of the positive control strain (Cowan). Total internalized bacteria
717 were enumerated by counting colony forming units following lysostaphin treatment. The
718 values are means ± SEM of four independent experiments.

719

720 **Figure 4.**

721 Expression of phage-encoded virulence genes following various stresses**.** Three lysogenic
722 strains representative of the non-LA CC398 isolates [S94, subgroup A, harboring Stau398-
723 4pro; S81, subgroup E, harboring Stau398-4pro and Stau398-5pro, and the MRSA S89] were
724 subjected to stresses during exponential growth or the stationary phase, including sublethal
725 concentrations of oxacillin (OXA), erythromycin (ERY), tetracycline (TETRA) or pestanal.

726 Levels of *chp* mRNA (left panel) and *scn* mRNA (right panel) were assessed in strains S94

727 (white bars), S89 (grey bars) and S81 (black bars) by RT-qPCR. Results are mean values for

728 two independent measurements performed in triplicate (mean ± range).

Table 1.

Isolation context, antibiotic resistance, *spa*-type, phage susceptibility, phage profiling and IEC-typing of the 21 CC398 isolates and transductant S124/S100 ϕ.

	Characteristics of CC398 isolates																					
	LA[1]										non-LA[1]											
											subgroup A							subgroup E				
	S123	S124	S132	S131	S1	S129	S125	S126	S134	S130	S92	S94	S93	S106	S103	S91	S89	S105	S96	S81	S100	S124 /S100-ϕ[2]
Hcol / Hinf / A[3]	A	A	A	A	Hcol	Hcol	A	Hcol	A	Hcol	Hinf	Hinf	Hinf	Hinf	Hinf	Hinf	Hinf	Hinf	Hinf	Hinf	Hinf	
Spa-type	11	34	34	11	571	108	108	571	899	899	5635	571	1451	571	571	571	899	1451	6605	571	9378	34
Antibiotic resistance[4]	Tet Ery	Tet	oxa Tet KT Ery	oxa Tet	oxa Tet GnT Ery	oxa Tet	Tet Ery	oxa Tet	oxa Tet	oxa Tet KT Ery	Ery	Ery	Ery	Ery	Ery		oxa Tet		Ery	Ery		Tet
Susceptibility to phages	++	++	no	no	no	no	no	no	+	no	no	no	no	no	no	no	no	no	no	no	no	no
Prophage profiling[5] lysogeny			int6 ant1b	int2 ant5	int2 ant1b	int2	int2	int6	int2	int6 ant1b	int3 ant4b	int3 ant4b	int3 ant4b	int3 ant4b	int3 ant4b	int3 ant4b	int3 ant1b	int3 ant4b	int3 ant4b	int3 ant4b	int3 ant4b	int3 ant4b
replication			dnaD2b	dnaD2b	dnaD2b polA	polA	polA	ant5 polA	polA	dnaC1 polA	dnaD1b	dnaD1b	dnaD1b	dnaD1b	dnaD1b	dnaD1b	dnaD2b	dnaC1 dnaD1b	dnaC1 dnaD1b	dnaC1 dnaD1b	dnaC1 dnaD1b	dnaC1 dnaD1b
regulation of transcription			dut2	dut2	dut3			dut2		dut2							dut3	dut2	dut2	dut2	dut2	dut2
morphogenesis subtype					Bb					Bb	Fa	Fa	Fa	Fa	Fa	Fa	Fa Be	Fa Bb	Fa Bb	Fa Bb	Fa Bb	Fa Bb
phage type	F	A F	A	A	A B	A	A F	A	A	A B F	F	F	F	F	F	F	F	F	F	F	F	F
lytic module			ami2	ami2	ami1	ami2	ami2	ami1	ami2	ami2 ami3	ami2	ami2	ami2	ami2	ami2	ami2	ami1 ami3	ami2 ami4	ami2 ami4	ami2 ami4	ami2 ami4	ami2 ami4
IEC (genes present)	no	no	no	no	no	no	no	no	no	no	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn*)	+ (*chp scn sak*)	+ (*chp scn*)	+ (*chp scn*)

[1]LA: livestock-associated, non-LA: from an animal-free environment, [2]transductant obtained with infective lysate S100-ϕ used to transduce the prophage-free isolate S124. [3]Hcol: human colonizing isolate, Hinf: clinical isolate recovered from an invasive infection, A: animal colonizing isolate, [4]oxa: oxacillin, Tet: tetracyclin, Ery: erythromycin, K: kanamycin, T: tobramycin, [5]The

genomic modules were classified on the basis of the genes for integrase (10 types), anti-repressor (five types), replication proteins *polA*, *dnaC* and *dnaD* (four types), dUTPase (four types), portal protein (eight types), tail appendices (four types) and endolysin (four types) corresponding to the integrase locus, lysogeny control region, and modules for DNA replication, transcription regulation, packaging, tail appendices and lysis respectively.

743 **Table 2.**

744 Transformation efficiencies (number of transformants/µg of plasmid DNA)

745

Recipient isolate				Transformation efficiency according to transforming DNA (number of transformants/µg of plasmid DNA)[1]		
	Origin	Prophage content	GenBank Accession numbers	*E. coli*DH5α	RN4220	*E. faecalis*[3]
RN4220				107050	172543	2-11
S123	LA	No		4175	1011	1-4
S124	LA	no		5048	818	ND
S100	human	StauST398-4pro + StauST398-5pro	KC595278 and KC595279	2	<1	0
S124/S100-ϕ [2]		StauST398-1pro	JX013863	401	41	ND
S92	human	StauST398-4pro		90	14	2
S93	human	StauST398-4pro		90	24	ND
S94	human	StauST398-4pro		463	30	1

746

747 [1] Values (mean of 2 experiments) represent the number of transformants per µg of plasmid DNA, normalized to

748 10^{10}electroporated cells.

749 [2] Results affected by the instability of StauST398-1

750 [3] Values obtained for 2 experiments.

751

752

753

754