Adaptive processes of Staphylococcus aureus isolates during the progression from acute to chronic bone and joint infections in patients

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Summary

Staphylococcus aureus bone and joint infection (BJI) is associated with significant rates of chronicity and relapse. In this study, we investigated how S. aureus is able to adapt to the human environment by comparing isolates from single patients with persisting or relapsing BJIs that were recovered during the initial and recurrent BJI episodes. In vitro and in vivo assays and whole-genome sequencing analyses revealed that the recurrent isolates induced a reduced inflammatory response, formed more biofilms, persisted longer in the intracellular compartments of host bone cells, were less cytotoxic and induced less mortality in a mouse infection model compared with the initial isolates despite the lack of significant changes at the genomic level. These findings suggest that S. aureus BJI chronicization is associated with an in vivo bacterial phenotypical adaptation that leads to decreased virulence and host immune escape, which is linked to increased intraosteoblastic persistence and biofilm formation.

Introduction

Staphylococcus aureus is known as one of the leading pathogens responsible for bone and joint infections (BJIs), particularly in the presence of a prosthetic joint or orthopaedic device (Tande and Patel, 2014; Tong et al., 2015). Despite the combination of adequate surgical treatment and an optimally prolonged course of antimicrobial chemotherapy, these infections are associated with a high rate of relapse and can evolve to a chronic disease (Osmon et al., 2013; Tande and Patel, 2014). Indeed, many clinical observational studies have demonstrated that S. aureus BJIs can persist asymptptomatically before relapse occurs months or years after the initial episode, even in immunocompetent hosts (Greer and Rosenberg, 1993; Bosse et al., 2005; Stevens et al., 2007).

Although the involvement of multidrug-resistant S. aureus strains, including methicillin-resistant S. aureus (MRSA), may contribute to this persistence, the rate of treatment failure remains high, even in cases of methicillin-susceptible S. aureus (MSSA) BJI (Sheehy et al., 2010). Thus, Wieland et al. (2012) and Valour et al. (2014) reported treatment failure rates of 12.2% (5/41) and as high as 24.2% (16/66) in native MSSA BJI respectively.

The physiopathology of the chronicization of BJIs is incompletely understood. Two main bacterial factors have been proposed to explain the persistence of S. aureus BJIs: biofilm formation (Brady et al., 2008; Hall-Stoodley and Stoodley, 2009; Sanchez et al., 2013) and the internalization and persistence of bacteria in osteoblasts (Hudson et al., 1995; Ellington et al., 1999; Jevon et al.,...
1999; Ellington et al., 2003) accompanying bacterial phenotype switching to small-colony variants (von Eiff et al., 1997; Kahl et al., 1998; Proctor et al., 2006; Sendi et al., 2006; von Eiff et al., 2006; Tuchscherr et al., 2010; Löffler et al., 2014). Intracellular and biofilm-embedded bacteria remain protected from both the action of the host immune system and the majority of antimicrobial agents (Conlon, 2014).

We and others have previously shown that S. aureus isolates recovered from populations of patients with chronic BJIs are better adapted to the intracellular compartment than those recovered from different patients with acute BJIs (Kalinka et al., 2014; Valour et al., 2015). However, all of the studies that have suggested the implications of these bacterial mechanisms that allow for the adaptation of S. aureus isolates during the progression from acute to chronic BJIs are supported either by in vitro and in vivo models of staphylococcal long-term infections based on laboratory isolates or by comparisons of two distinct sets of clinical isolates, i.e. isolates recovered from patients presenting with acute BJIs and isolates recovered from patients presenting with recurrent BJIs (Tuchscherr et al., 2010; Kalinka et al., 2014; Valour et al., 2015). To date, no study has compared isolates recovered from the same patient at the time of the initial diagnosis and at the time of chronicization. Here, we selected three such pairs of isolates that were recovered from three patients dealing with chronic recurrent BJIs (Table 1). We compared the isolates of each pair by using a large panel of phenotypic in vitro and in vivo assays and applied genomic comparisons to determine whether S. aureus is able to adapt its phenotype in vivo to a novel environment and subsequently induce chronic infection.

Results

The recurrent S. aureus isolates were stronger producers of mature biofilm

The crystal violet staining method revealed that the recurrent isolates from patients 2 and 3 formed signifi-
cantly more mature biofilms (132±23% and 241±67% respectively) than the initial isolates (100%) after 48 h (P<0.01 for both; Fig. 1). Notably, no difference was observed in the isolates recovered from patient 1.

The recurrent S. aureus isolates persisted longer in the osteoblasts despite the same capacities for adhesion and internalization

Despite the same capacities for adhesion and internalization of the initial and recurrent S. aureus isolates (P>0.05; Figs 2A, 2B and S1), we next evaluated whether the isolates were cleared as efficiently by the osteoblasts. Using the murine osteoblast-like cells, the persistence levels of the recurrent isolates obtained from patients 1, 2 and 3 that were observed 72 h post infection were 1.18-, 2.00- and 2.07-fold greater respectively (P<0.01 for all) than those of the initial isolates (Fig. S1). When using human primary osteoblasts, the intracellular bacteria counts 48 h post infection were 1.08-, 1.78- and 4.26-fold greater for the recurrent isolates recovered from patients 1, 2 and 3 respectively, compared with the initial isolates (P=0.105, P=0.01 and P=0.01 respectively; Fig. 2C). Taken together, these data suggest that the recurrent S. aureus isolates tended to persist in the osteoblasts for longer than the initial isolates.

The recurrent S. aureus isolates caused less cellular damage and tended to induce a less osteoblastic inflammatory response

We also determined the cytotoxicities to the MC3T3 cells and human primary osteoblasts that were induced by measuring lactate dehydrogenase (LDH), which is released by damaged cells into the culture supernatant. Regardless of the osteoblasts tested, the relative LDH amounts released from the osteoblasts infected with the recurrent isolates from patients 1, 2 and 3 were significantly lower than those from the cells infected with the initial isolates within each pair (P<0.05, P<0.05 and P<0.01 for the MC3T3 cells (Fig. S1) and P<0.05, P<0.01 and P<0.001 for the human primary cells (Fig. 2D)). These data demonstrate that the recurrent isolates

Table 1. Characteristics of the patients and isolates.

| Patient no. | Sex, age (year) | Site of infection | Duration of symptoms (days)a | Surgical treatment | Duration of antibiotic therapy (days) | Time to failure or relapse (days)b | MLST type | spa type |
|-------------|----------------|------------------|-----------------------------|-------------------|-------------------------------------|-----------------------------------|-----------|---------|
| 1 M, 26     | Tibia osteosynthesis material | 12 | Material removed | 82 | 0 | ST15 (CC15) | 385 |
| 2 M, 80     | Total knee arthroplasty | 3 | Irrigation and debridement | 191 | 10 | ST25 (CC25) | 78 |
| 3 F, 82     | Total hip arthroplasty | 3 | Irrigation and debridement | 98 | 36 | ST15 (CC15) | 84 |

a Evolution delay at the time of diagnosis of the first episode, i.e. time from orthopaedic device implantation to isolation of initial S. aureus strain.
b Time from antibiotic interruption to relapse/failure.

The S. aureus multilocus sequence typing (MLST) and the S. aureus protein A (spa) typing were performed as previously described [41,42]. M, male; F, female; ST, sequence type; MLST, multilocus sequence typing.
were less cytotoxic and less virulent than the initial isolates.

Following these observations, we hypothesized that the recurrent isolates induced a lower cellular activation than the initial isolates. We then analysed the cytokine release profiles in the cellular supernatant by using Luminex™ technology. Although not all of the differences were significant (using Mann–Whitney test between the initial and recurrent isolates for each patient), we observed that the recurrent isolates from all three patients induced less cytokine production for all of the tested cytokines. Depending on the tested cytokine (i.e. TNF-α, IFN-γ, IL-12p70, IL-5, IL-6 and IL-4), the human osteoblasts infected with the recurrent S. aureus isolates secreted cytokines at levels that were 1.07- to 11.79-fold lower than those of the osteoblasts that were infected with the initial isolates (Fig. 3). Similar results were observed when the MC3T3 cells were used (data not shown). These data are indicative of a tendency towards the induction of a reduced inflammatory response by the recurrent S. aureus isolates compared with the initial isolates.

The S. aureus isolates recovered from the recurrent bone and joint infections exhibited lower levels of hemolysin alpha expression

Because it has previously been demonstrated that the secretion of bacterial virulence factors is responsible for cell death and cellular activation, we quantified the secretions of hemolysin alpha (hla) by the initial and recurrent isolates as a marker of staphylococcal virulence expression based on the capacity of hla to induce

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**Fig. 1.** Evaluation of the biofilm-forming abilities of the S. aureus clinical isolates. Quantification of mature biofilm formation 24 h post incubation in BHI + 1% glucose by the crystal violet staining method for the initial and recurrent isolates recovered from three different patients. All of the results are expressed as percentages of the values obtained for the initial strains. The horizontal bars denote the means derived from three independent experiments conducted in quadruplicate. The statistical analyses were performed using the Mann–Whitney test.

**Fig. 2.** Comparison of the adhesion, internalization and persistence levels in osteoblasts and cytotoxicty induced by the initial and recurrent S. aureus isolates by using the gentamicin protection assay. Human primary osteoblasts were infected with S. aureus at mois of 250:1 (for 2 h at 37°C).

A. The adhesion levels to the human primary osteoblasts were estimated at 2 h post-infection.
B. The invasion capacities were assessed by quantifying the viable intracellular bacterial loads at 3 h post infection after gentamicin treatment for the primary human osteoblasts.
C. The intracellular bacterial persistence capacities were estimated at 48 h post infection. All of the results are expressed as percentages of the values (cfu recovered after host cell lysis) obtained for the initial strains. The horizontal bars denote the means derived from three independent experiments conducted in triplicate.
D. Cytotoxicity was estimated by quantifying LDH release by the infected osteoblasts at 48 h post infection. The horizontal bars denote the means derived from three independent experiments conducted in triplicate. The results are expressed as the differences in LDH release between the infected and uninfected cells (ΔLDH). The statistical analyses were performed by using the Mann–Whitney test.

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The haemolytic activities of the recurrent *S. aureus* isolates of patients 2 and 3 were 2- and 6.5-fold lower respectively than those of the initial ones (*P* < 0.01; Fig. 4A). No hla activity was detected for the isolates recovered from patient 1. Thereafter, whole-genome sequencing (WGS) analysis of this locus sequence was applied to both the initial and the recurrent strains from patient 1 and revealed the presence of a non-synonymous mutation that changed the amino acid at position 107 (*P* → *S*) (Fig. 4B). This non-synonymous substitution (using *S. aureus* strain NCTC8325 as the reference) was located in the triangle region (Gouaux *et al*., 1997; Menestrina *et al*., 2001) within which non-synonymous substitutions affecting the amino acids at positions 110 and 152 are known to diminish the lytic activity of hla and at positions 100, 104 and 108 have also been implicated in cell lysis (Walker and Bayley, 1995).

**The recurrent isolates were less virulent based on two in vivo mouse models**

These *in vitro* data led us to confirm these findings *in vivo* by using two mouse models to compare the virulences of the initial and recurrent isolates that were collected from patient 3. First, the bacterial colony-forming unit (cfu) counts from the blood at 1, 3 and 24 h after the challenge revealed no difference in the bacteraemia level between the isolates recovered from the initial and recurrent BJIs using the acute intraperitoneal infection model. Nevertheless, the recurrent isolates persisted for a longer duration in the intraperitoneal compartment than the initial isolates.
(4,407 ± 1,009 vs 2,117 ± 1,176 cfu ml⁻¹ respectively at 3 h post infection; P < 0.01; Figs 5A and S2). When using the lung infection model, at 24 and 48 h post infection, the bacterial counts in the lungs and spleens of the animals challenged with the initial isolates were significantly greater than those in the organs recovered from the survivals animals infected with recurrent isolates (P < 0.05, except for the counts in the spleen after 24 h, Figs 5B, 5C and S2). Finally, the isolates recovered from the acute initial episode of BJI caused greater mortality than the recurrent isolates as early as 24 h post infection [62% (10/16) vs 0% (0/16), P < 0.01; Fig. 5D]. The data from both animal models

Fig. 5. In vivo models of intraperitoneal infection and lung infection mimicking pneumonia in mice.

Intrapерitoneal infection model. A. CD-1 female mice were intraperitoneally inoculated with 5 × 10⁶ cfus per mouse in 0.5 ml of PBS. After 3 h, the mice were sacrificed, and 4ml of PBS was intraperitoneally injected to recover the surviving bacteria. Bacterial quantification was performed by plate counting. The horizontal bars represent the means. The survival of the mice over 48 h following intratracheal inoculation is expressed as percentages from three independent experiments (each group, n = 8).

C. D. Bacterial loads in the lungs and spleen were estimated by plate counting 48 h post infection. The black lines denote the means. Significant differences were obtained by using the Mann-Whitney test.
suggest that the \textit{S. aureus} isolate recovered from the recurrent infection was less virulent than the isolate collected during the initial infection.

\textit{Genomic comparisons of the initial and recurrent \textit{S. aureus} isolates recovered from the same patients revealed no significant differences}

We were next interested in exploring the molecular mechanisms responsible for \textit{in vivo} and \textit{in vitro} changes observed with the recurrent \textit{S. aureus} isolates. We aimed to determine whether these mechanisms could be related to the presence of specific mutations or the accumulation of mutations in specific genomic regions, including virulence genes, gene expression regulators and regulatory regions. For this purpose, we performed WGS of the three pairs of isolates and performed intrapatient comparisons of the genomes (Table S2). The genome sizes with the pairs were nearly identical with the exception of the isolates from patient 3, in which a plasmid was lost in the recurrent isolate. This plasmid, also recovered in CC15 pairs of patients 1, carries 23 open reading frames (ORFs), among which we identified resistance genes to antibiotics (\textit{bla} operon) and heavy metals (cadmium) and a bacteriocin operon.

Totals of nine, eight and six variants (SNPs and/or insertion–deletions) were identified within the pairs obtained from patients 1, 2 and 3 respectively (Table 2). Only a low number of these variants occurred in coding DNA sequences (CDSs), but all of the impacted CDS and intergenic regions varied across the different pairs. Notably, we did not find any variants that affected the major regulatory systems that are known to control the expression of virulence in \textit{S. aureus} (Table S3).

\textbf{Discussion}

In this study, we investigated whether the prolonged contact of clinical \textit{S. aureus} isolates with human hosts during the progression from acute to chronic BJIs led to bacterial adaptations that modified the virulence phenotype and/or genotype of \textit{S. aureus}. For this, three pairs of isolates collected from three patients with recurrent MSSA

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Patient} & \textbf{Location} & \textbf{Nucleotide position} & \textbf{Variant} & \textbf{Initial isolate} & \textbf{Recurrent isolate} & \textbf{Genomic context} & \textbf{Protein} & \textbf{Changes in protein} \\
\hline
1 & chr & 2124297 & SNP (synonymous) & A & T & Intragenic (locus 2183) & DeoC & None \\
 & chr & 213204 & SNP & A & G & Intergenic & nap & nap \\
 & chr & 2292532 & SNP & C & T & Intergenic & nap & nap \\
 & chr & 2292604 & SNP & T & G & Intergenic & nap & nap \\
 & chr & 2292613 & SNP & T & A & Intergenic & nap & nap \\
 & chr & 2292623 & SNP & G & A & Intergenic & nap & nap \\
 & chr & 2483865 & SNP (non-synonymous) & C & T & Intragenic (locus 3936) & Hypothetical protein & Glu → Lys \\
 & chr & 2484200 & SNP (non-synonymous) & A & G & Intragenic (locus 3936) & Hypothetical protein & Trp → Arg \\
 & chr & 2484215 & SNP (non-synonymous) & T & C & Intragenic (locus 3936) & Hypothetical protein & Thr → Ala \\
2 & chr & 1026283 & SNP & T & C & Intergenic & nap & nap \\
 & chr & 1026318 & SNP & G & A & Intergenic & nap & nap \\
 & chr & 1945070 & SNP & C & A & Intergenic & nap & nap \\
 & chr & 2210965 & SNP & G & A & Intergenic & nap & nap \\
 & chr & 2302808 & SNP & T & A & Intergenic & nap & nap \\
 & chr & 2302809 & SNP & A & T & Intergenic & nap & nap \\
 & chr & 962982 & Indel & T & – & Intragenic (locus 4177) & comK & Frameshift \\
 & chr & 1487044 & SNP (non-synonymous) & G & A & Intragenic (locus 4673) & Hypothetical protein & Pro → Leu \\
3 & chr & 234389 & SNP & G & A & Intergenic & nap & nap \\
 & chr & 255401 & SNP & T & C & Intergenic & nap & nap \\
 & chr & 272577 & Indel & TGATTAA – & Intragenic (locus 3764) & EssC & Frameshift \\
 & chr & 104017 & Indel & TGAT – & Intragenic (locus 1060) & GTP-binding protein, TypA/BipA-like protein & Frameshift \\
 & chr & 1591517 & Indel & – & GACC & Intragenic (locus 1616) & Uridine kinase & Frameshift \\
 & chr & 1839176 & SNP (non-synonymous) & A & G & Intragenic (pseudogene 4150) & Membrane-associated protein & STOP → W \\
\hline
\end{tabular}
\caption{Variants identified for the three couples of initial and recurrent isolates.}
\end{table}

All variants occurring in CDSs were validated by PCR and subsequent Sanger sequencing. When non-synonymous substitutions occurred, the amino acids are indicated using the three-letters IUPAC abbreviations. nap, not applicable; –, absence; chr, chromosomal
BJI at the time of the initial diagnosis and the time of relapse were compared in various in vitro and in vivo models.

In vivo, biofilm formation is classically associated with treatment failure, notably in the case of foreign material-related infections (McConoughhey et al., 2014). Here, we demonstrated that the isolates collected from patients 2 and 3 during the chronic phase of BJI exhibited an increased capacity to form mature biofilm compared with the respective strains collected during the initial phase of infection. For patient 1, the capacity to form mature biofilms did not vary between the acute and recurrent isolates, which suggests that the adaptations for the chronicity of *S. aureus* depend on the host and/or isolate. Interestingly, although all three patients presented orthopaedic device-associated infections, only patient 1 had undergone material removal at the time of the initial infection. This observation may suggest that the persistence of material could play a role in the adaptive response of *S. aureus* towards increased biofilm formation. Studies comparing pairs of isolates collected from patients with recurrent BJIs with and without abiotic materials are required.

Several authors have suggested that the life of *S. aureus* in the intracellular compartment is part of the long-term infection process (von Eiff et al., 2006). Our data revealed no differences between the initial and recurrent isolates in terms of their abilities to adhere to and invade osteoblasts. These results are in line with those reported by Kalinka et al. (2014), who compared a set of *S. aureus* isolates collected from patients suffering from acute infection with isolates collected from other patients who experienced BJI relapses. We demonstrated that the isolates recovered from recurrent BJIs exhibited the following characteristics compared with the isolates collected from the same patients during the acute phase: (i) they persisted intracellularly for much longer, (ii) they induced a lower level of cellular death and (iii) they induced less severe host cellular activation as reflected by cytokine release. This is in line with the data that we also obtained for biofilm data and suggest that a phenotypic in vivo adaptation of the initial isolates occurs during their ‘stay’ in the host cells. Interestingly, these original data are in agreement with our previous findings based on hospital-acquired and community-acquired MRSA isolates and MSSA isolates from acute or chronic BJIs, which demonstrated that interactions between osteoblasts and *S. aureus* are a major physiopathological mechanism in the clinical presentation of BJIs (Rasigade et al., 2013; Valour et al., 2015).

Furthermore, two in vivo models were used to test one of the three pairs of clinical isolates and highlighted the diminished virulence of the isolate recovered from the recurrent BJI compared with the initial isolate. The intraperitoneal infection model revealed that the recurrent isolate persisted longer in the peritoneum. By using this model, Cohn (1962) demonstrated that the reduction of viable bacteria in the intraperitoneal cavity is related to phagocytosis and the intracellular killing of bacteria caused by the local attraction of leucocytes to the peritoneum. In this model, the greater persistence of the recurrent isolates observed in our model was possibly related to a diminished ability to stimulate the immune system and be recognized by immune cells. The lung infection model that mimics pneumonia definitively demonstrated that the recurrent isolates were less virulent based on the bacterial counts in the lung and spleen and the mortality rates.

To our best knowledge, this is the first study to demonstrate the adaptive phenotypes of isolates recovered from the same patients during the progression of BJI. One of the limits of the present study was the low number of tested isolates, which was a result of the difficulty of obtaining isolates responsible for both the initial and chronic phases of infection from the same patients. Experiments with more pairs of isolates should be performed to reinforce our conclusions. Despite these limitations, our data clearly highlight the adaptations of *S. aureus* during the course of the chronicization of BJIs. The variation in the intensity of this adaptation across the pairs of isolates is likely related to intraindividual bacterial variability, the variable delay between the initial and recurrent infections and the differential management of the patient (as illustrated for patient 1 with material removal).

Taken together, these in vivo and in vitro data reveal that prolonged contact of *S. aureus* with the human microenvironment induces profound and stable bacterial adaptations that lead to a decrease in virulence. These adaptations can occur because of environmental pressures within the bone, such as inflammatory responses, hypoxia, nutrient deficiency, osmolarity, low pH, intracellular factors, biofilm and antibiotic therapies. Notably, despite antibiotic treatment over several months, all of the recurrent isolates harboured the same antibiotic susceptibility profiles and minimum inhibitory concentrations than initial isolates. These data suggest that the bacterial adaptive phenomenon does not affect and is not affected by the level of antibiotic resistance.

In addition to BJI, only a few studies have reported *S. aureus* adaptations over the course of human chronic infectious diseases. One such study applied comparative genome sequencing to three *S. aureus* isolates that were obtained sequentially over 26 months from the airway of a cystic fibrosis patient and revealed variations in phage content and genetic polymorphisms in genes that influence antibiotic resistance and the global regulation of virulence (McAdam et al., 2011). The identified genetic factors within the patient (as illustrated for patient 1 with material removal).
variations correlated with differences in growth rate, haemolytic activity and antibiotic sensitivity, which implies a profound effect on the ecology of S. aureus. Specifically, a high rate of often non-synonymous mutations and multiple independent indel polymorphisms affected the activity of SigB, which is a staphylococcal alternate transcription factor that is involved in the stress response. Similar observations were also reported by Tuchscherr et al. (2015) in BJI animal models, which suggests that SigB could be a key player in adaptation in chronic infections. However, the genomic comparisons between the initial and recurrent isolates conducted in our study revealed no significant changes in the core genome. Specifically, no mutations were observed in SigB or its operon or in any of the main regulators of S. aureus. Additionally, our results suggest that the adaptation to chronicity in the context of BJIs is not a result of the accumulation of specific or shared mutations in any other genes associated with the expression of virulence.

Interestingly, the number of identified mutations was limited, which excludes the hypothesis of the selection of a hypermutator subpopulation that might have allowed for rapid genetic adaptation. Moreover, in the three pairs of isolates, the non-synonymous substitutions and indels in the protein-coding regions affected different pathways. In view of the diversity and/or lack of predicted functions of the genes that were affected in the three pairs, it is highly unlikely that these mutations correspond to a phenotypic convergence that allows for a switch to a chronic bacterial pattern. Nevertheless, only experimental validation of the influences of these genetic changes on the chronicity phenotype in isogenic isolates will allow us to reach a definitive conclusion.

In light of our results, an alternative scenario is that in the context of BJIs, the phenotypic changes that led to the decreased virulence of the isolates involved in recurrence/relapse might have been a result of regulation at the transcriptomic/proteomic levels. This hypothesis is supported by recent observations reported by our group that revealed the roles of non-coding RNA in bacterial persistence and decreases in virulence (Romilly et al., 2014). Nevertheless, further studies investigating the roles of the host response and/or bacterial epigenetic factors in the regulation of virulence in the context of chronic BJIs are underway.

To conclude, the present study using clinical isolates demonstrated that S. aureus possesses a plethora of resources for adapting to the novel stressful environment encountered during the infection of humans. Our results demonstrate that S. aureus isolates involved in BJI are able to modify in vivo their phenotype including intraosteoablatic behaviours and ability to form biofilm to constitute bacterial reservoir, leading to chronicity and relapse. Our study encourages the use and development of therapeutic strategies targeting intracellular and biofilm-embedded bacteria to avoid or eradicate chronic BJIs.

**Experimental procedures**

**Ethics statement**

All animal work was approved by the Comité d’Evaluation Commun au Centre Léon Bérard, à l’Animalerie de transit de l’ENS, au PBES et au laboratoire P4 (CECCAPP) under identification no. ENS_2014_027. The clinical portion of this study was conducted with the approval of the French South-East ethics committee (no. CAL2013-018 and no. CAL2011-21 for collections of human primary osteoblasts and clinical isolates respectively). In accordance with the French legislation, written informed patient consent was not required for any part of the study.

**Patient selection**

Three patients with persisting or relapsing MSSA BJIs were included in this study. The data related to clinical characteristics, time to relapse or failure of treatment and surgical and antibiotic treatments are summarized in Table S1. Antimicrobial susceptibility tests were performed by using the disc diffusion method or the E-test® method for glycopeptides, penicillin G and oxacillin (Table S1) and revealed no differences between the initial and recurrent strains in any patient.

**Biofilm formation**

Mature biofilm formation was quantified by using the tissue culture plate method with crystal violet as previously described (Valour et al., 2013). This method allows for the semiquantitative measurement of mature biofilms (Stepanovic et al., 2007) (Supporting Information).

**Infection of osteoblasts with S. aureus**

Murine osteoblast-like cells [MC3T3, CRL-2593™ purchased from LGC Standards (Teddington, UK)] and human primary osteoblasts were infected (2 h, 37°C) at multiplicities of infection (MOI) of 50:1 and 250:1 respectively to allow for the adhesion and internalization of the bacteria. Next, we used the gentamicin protection assay to select the intracellular bacteria as previously described (Trouillet et al., 2011). Cytotoxicity, inflammatory responses, bacterial adhesion, internalization and intracellular persistence were then evaluated (Supporting Information).

**Hemolysin alpha production**

Hemolysin alpha production was determined by using rabbit erythrocytes as described by Qiu et al. (2010) with modification. Briefly, S. aureus isolates were cultured in brain–heart infusion (BHI; 37°C, 48 h). The bacterial samples were centrifuged (5 min, 3000 r.p.m.), and 200 μl of the culture supernatant at serial dilution was mixed with rabbit erythrocytes (2% PBS). After incubation for 60 min at 37°C, the samples were centrifuged (2000 r.p.m., 5 min), and the OD450 values of the supernatants were measured.
Mouse intraperitoneal and lungs infection models

Female CD-1 mice (8 to 10-week old) were obtained from Charles River Laboratories (Wilmington, USA). Groups of six mice were intraperitoneally inoculated with $5 \times 10^6$ cfu per mouse in 0.5 ml of PBS. After 1 and 3 h, the mice were sacrificed, and 4 ml of PBS was intraperitoneally injected to recover the surviving bacteria. Blood samples were also collected via intracardiac puncture into heparinized tubes. The bacterial quantification was performed by plate counting.

Six-week-old RJOrI:SWISS mice (20–24 g) were purchased from Janvier Laboratories (Le-Genest-Saint-Ise, France). Pneumonia was induced after inoculation of $75 \mu$l of a bacterial suspension adjusted to $10^9$ cfu ml$^{-1}$ as previously described (Roquilly et al., 2010) (Supporting Information).

Genome sequencing and comparative genomics

The DNA extractions were performed by using the QIACube™ automated system (Qiagen) according to the manufacturer’s recommendations. The libraries were prepared according to the Ion Xpress™ Plus genomic DNA fragment library preparation protocol. The libraries were sequenced on an Ion 318™ Chip v2 by using an Ion PGM™ system. Details concerning assembly and annotation, SNP analysis in intragenic as well as intergenic regions and detection of the presence/absence of ORFs are available in Supporting Information.

Statistical analyses

Differences in the quantitative data between the initial and relapse-associated isolates were analysed independently for each patient with the two-tailed Mann–Whitney U-test. Because of the exploratory nature of the work, no correction for multiple hypothesis testing was applied.

Accession numbers

This whole-genome shotgun sequencing project has been deposited in DNA Data Bank of Japan/European Molecular Biology Laboratory/Genbank (DDBJ/EMBL/GenBank) under genome accession IDs CP012970–CP012980.

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Conflict of interest

All authors declare no conflict of interest.

References

Bosse, M.J., Gruber, H.E., and Ramp, W.K. (2005) Internalization of bacteria by osteoblasts in a patient with recurrent, long-term osteomyelitis: a case report. J Bone Joint Surg Am 87: 1343–1347.

Brady, R.A., Leid, J.G., Calhoun, J.H., Costerton, J.W., and Shirtliff, M.E. (2008) Osteomyelitis and the role of biofilms in chronic infection. FEMS Immunol Med Microbiol 52: 13–22.

Cohn, Z.A. (1962) Determinants of infection in the peritoneal cavity: I. response to and fate of Staphylococcus aureus and Staphylococcus albus in the mouse. Yale J Biol Med 35: 12–28.

Conlon, B.P. (2014) Staphylococcus aureus chronic and relapsing infections: evidence of a role for persister cells: an investigation of persister cells, their formation and their role in S. aureus disease. Bioessays 36: 991–996.

Ellington, J.K., Harris, M., Webb, L., Smith, B., Smith, T., Tan, K., and Hudson, M. (2003) Intracellular Staphylococcus aureus: a mechanism for the indolence of osteomyelitis. J Bone Joint Surg Br 85: 918–921.

Ellington, J.K., Reilly, S.S., Ramp, W.K., Smeltzer, M.S., Kellam, J.F., and Hudson, M.C. (1999) Mechanisms of Staphylococcus aureus invasion of cultured osteoblasts. Microb Pathog 26: 317–323.

Gouaux, E., Hobaugh, M., and Song, L. (1997) Alpha-hemolysin, gamma-hemolysin, and leukocidin from Staphylococcus aureus: distant in sequence but similar in structure. Protein Sci 6: 2631–2635.

Greer, R.B., and Rosenberg, A.E. (1993) Case records of the Massachusetts General Hospital. Weekly clinical pathologi- cal exercises. Case 6-1993. A 69-year-old woman with a sclerotic lesion of the femur and pulmonary nodules. N Engl J Med 328: 422–428.

Hall-Stoodley, L., and Stoodley, P. (2009) Evolving concepts in biofilm infections. Cell Microbiol 11: 1034–1043.

Hudson, M.C., Ramp, W.K., Nicholson, N.C., Williams, A.S., and Nousiainen, M.T. (1995) Internalization of Staphylocococcus aureus by cultured osteoblasts. Microb Pathog 19: 409–419.

Jevon, M., Guo, C., Ma, B., Mordan, N., Nair, S.P., Harris, M., et al. (1999) Mechanisms of internalization of Staphylococcus aureus by cultured human osteoblasts. Infect Immun 67: 2677–2681.

Kahl, B., Herrmann, M., Everding, A.S., Koch, H.G., Becker, K., Harms, E., et al. (1998) Persistent infection with small colony variant strains of Staphylococcus aureus in patients with cystic fibrosis. J Infect Dis 177: 1023–1029.

Kalinka, J., Rachmeister, M., Geraci, J., Sordelli, D., Hansen, U., Niemann, S., et al. (2014) Staphylococcus aureus isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. Int J Med Microbiol 304: 1038–1049.

Loffler, B., Tuchscherr, L., Niemann, S., and Peters, G. (2014) Staphylococcus aureus persistence in non-professional phagocytes. Int J Med Microbiol 304: 170–176.

McAdam, P.R., Holmes, A., Templeton, K.E., and Fitzgerald, J.R. (2011) Adaptive evolution of Staphylococcus aureus during chronic endobronchial infection of a cystic fibrosis patient. PLoS One 6 e24301.
McConoughy, S.J., Howlin, R., Granger, J.F., Manring, M.M., Calhoun, J.H., Shirluff, M., et al. (2014) Biofilms in periprosthetic orthopedic infections. *Future Microbiol* 9: 987–1007.

Menestrina, G., Serra, M.D., and Prevost, G. (2001) Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. *Toxicon* 39: 1661–1672.

Osmon, D.R., Berbari, E.F., Berendt, A.R., Lew, D., Zimmerli, W., Steckelberg, J.M., et al. (2013) Diagnosis and management of prosthetic joint infection: clinical practice guidelines by the Infectious Diseases Society of America. *Clin Infect Dis* 56: e1–e25.

Proctor, R.A., von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Hermann, M., and Peters, G. (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* 4: 295–305.

Qiu, J., Wang, D., Xiang, H., Feng, H., Jiang, Y., Xia, L., et al. (2010) Subinhibitory concentrations of thymol reduce enterotoxins A and B and alpha-hemolysin production in *Staphylococcus aureus* isolates. *PLoS One* 5 e9736.

Rasigade, J.P., Trouillet-Assant, S., Ferry, T., Diep, B.A., Sapin, A., Lhoste, Y., et al. (2013) PSMs of hypervirulent *Staphylococcus aureus* act as intracellular toxins that kill infected osteoblasts. *PLoS One* 8 e63176.

Romilly, C., Lays, C., Tomasini, A., Caldelari, I., Benito, Y., Hammann, P., et al. (2014) A non-coding RNA promotes bacterial persistence and decreases virulence by regulating a regulator in *Staphylococcus aureus*. *PLoS Pathog* 10 e1003979.

Roquilly, A., Gautreau, L., Segain, J.P., de Coppet, P., Sebille, V., Jacqueline, C., et al. (2010) CpG-ODN and MPLA prevent mortality in a murine model of post-hemorrhage-*Staphylococcus aureus* pneumonia. *PLoS One* 5 e13228.

Sanchez, C.J., Jr., Ward, C.L., Romano, D.R., Hurtgen, B.J., Hardy, S.K., Woodbury, R.L., et al. (2013) *Staphylococcus aureus* biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption in vitro. *BMC Musculoskelet Disord* 14: 187.

Sendi, P., Rohrbach, M., Graber, P., Frei, R., Ochsner, P.E., and Zimmerli, W. (2006) *Staphylococcus aureus* small colony variants in prosthetic joint infection. *Clin Infect Dis* 43: 961–967.

Sheehy, S.H., Atkins, B.A., Bejon, P., Byren, I., Wyllie, D., Athanasou, N.A., et al. (2010) The microbiology of chronic osteomyelitis: prevalence of resistance to common empirical anti-microbial regimens. *J Infect* 60: 338–343.

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

Stevens, O.E., Seibl, J.M., Chen, Y.H., Dickeman, R.D., Noel, J., and Kattner, K.A. (2007) Reactivation of dormant lumbar methicillin-resistant *Staphylococcus aureus* osteomyelitis after 12 years. *J Clin Neurosci* 14: 585–589.

Tande, A.J., and Patel, R. (2014) Prosthetic joint infection. *Clin Microbiol Rev* 27: 302–345.

Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L., and Fowler, V.G., Jr. (2015) *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* 28: 603–661.

Trouillet, S., Rasigade, J.P., Lhoste, Y., Ferry, T., Vandenesch, F., Etienne, J., and Laurent, F. (2011) A novel flow cytometry-based assay for the quantification of *Staphylococcus aureus* adhesion to and invasion of eukaryotic cells. *J Microbiol Methods* 86: 145–149.

Tuchscherr, L., Bischoff, M., Lattar, S.M., Noto Llana, M., Pförtner, H., Niemann, S., et al. (2015) Sigma factor SigB is crucial to mediate *Staphylococcus aureus* adaptation during chronic infections. *PLoS Pathog* 11 e1004870.

Trouillet, S., Heitmann, V., Hussain, M., Viemmann, D., Roth, J., von Eiff, C., et al. (2010) *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. *J Infect Dis* 202: 1031–1040.

Valour, F., Bouaziz, A., Karantos, J., Ader, F., Lustig, S., Laurent, F., et al. (2014) Determinants of methicillin-susceptible *Staphylococcus aureus* native bone and joint infection treatment failure: a retrospective cohort study. *BMC Infect Dis* 14: 443.

Valour, F., Rasigade, J.P., Trouillet-Assant, S., Gagnaire, J., Bouaziz, A., Karantos, J., et al. (2015) Delta-toxin production deficiency in *Staphylococcus aureus*: a diagnostic marker of bone and joint infection chronicity linked with osteoblast invasion and biofilm formation. *Clin Microbiol Infect* 21(6) 568.e1–568.e11.

Valour, F., Trouillet-Assant, S., Rasigade, J.P., Lustig, S., Chanard, E., Meugnier, H., et al. (2013) *Staphylococcus epidermidis* in orthopedic device infections: the role of bacterial internalization in human osteoblasts and biofilm formation. *PLoS One* 8 e67240.

von Eiff, C., Bettin, D., Proctor, R.A., Rolauffs, B., Lindner, N., Winkelmann, W., and Peters, G. (1997) Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. *Clin Infect Dis* 25: 1250–1251.

von Eiff, C., Peters, G., and Becker, K. (2006) The small colony variant (SCV) concept – the role of staphylococcal SCVs in persistent infections. *Injury* 37(Suppl 2): S26–S33.

Walker, B., and Bayley, H. (1995) Key residues for membrane binding, oligomerization, and pore forming activity of staphylococcal alpha-hemolysin identified by cysteine scanning mutagenesis and targeted chemical modification. *J Biol Chem* 270: 23065–23071.

Wiebel, B.W., Marcantoni, J.R., Bommarito, K.M., Warren, D.K., and Marschall, J. (2012) A retrospective comparison of ceftriaxone versus oxacillin for osteoarticular infections due to methicillin-susceptible *Staphylococcus aureus*. *Clin Infect Dis* 54: 585–590.

**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Comparison of the adhesion, internalization and persistence levels in osteoblasts and biofilm formation. *J Clin Infect Dis* 58: 2025–2035.

**Fig. S2.** In vivo models of intraperitoneal infection and lung infection mimicking pneumonia in mice.

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