The strategies of *Staphylococcus aureus* to develop a chronic infection

Habilitation

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From

Dr. rer.nat. Lorena Tuchscherr

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Gutachter

Internal:

1- **.Prof. Dr. med. Bettina Löffler, Institut für Medizinische Mikrobiologie, UKJ, Jena.**
2- **Prof. Dr. med. Michael Bauer, Klinik für Anästhesiologie und Intensivmedizin, UKJ, Jena.**

External:

1- **Prof. Dr. Andreas Peschel, Institut für Mikrobiologie und Infektionsmedizin, Univ. Tübingen, Tübingen.**

Teaching qualification obtained on 6th July, 2017.

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1. Prof. Dr. Löffler , Prof. Dr Peters und Prof. Dr Sordelli.

2..MTAs und students from Münster: B. Schuhen, K. Broschwig, M. Brück, Marie Hachmeister and Jennifer Geraci.

3. MTAs from Argentina: S. Soldavini and L. Medina

4. Colleges: S. Niemann, V. Hoerr, C. Kreis

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Jena, den 23. June 2016

Dr. Lorena Tuchscherr de Hauschopp

Contents

Contents	1
List of publications	2
1. Introduction	4
1.1 *S. aureus* and Persistence	4
1.2. Objectives of this work	6
2. Results and discussions: Description of main results and papers	7
2.1 Acute phase of infection and host response	7
2.2 In vivo models to study staphylococcal infection	9
2.3 Development and dynamic of small colony variants (SCVs)	12
2.4 Host controlled by *S. aureus* during persistence: mechanisms of immune escape	22
2.5 Loss of capsule during persistence	24
2.6 Approaches for vaccine development and treatment to eliminate *S. aureus*	27
3. Conclusions and overview	32
4. List of abbreviations	35
5. References	36
6. Ehrenwörtliche Erklärung	42
7. Curriculum Vitae	43
8. Complete list of publications	46
9. Oral presentations	49
10. Acknowledgements	50
11. Copies of original publications used for this Habilitation	51

List of publications

The present Habilitation is based on the following original publications. The publications where I am corresponding author are indicated (*).

a. **Tuchscherr L**, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, Bremer-Streck S, Kiehntopf M, Medina E, Kribus M, Raschke M, Pletz M, Peters G, Löffler B. *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. J. Antimicrob Chemother. **2016** Feb;71(2):438-48. **(*)**

b. **Tuchscherr L**, Löffler B. *Staphylococcus aureus* dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection. Curr Genet. **2015** Jun 30.

c. **Tuchscherr L,** Bischoff M, Lattar SM, Noto Llana M, Pförtner H, Niemann S, Geraci J, Van de Vyver H, Fraunholz MJ, Cheung AL, Herrmann M, Völker U, Sordelli DO, Peters G, Löffler B. Sigma Factor SigB Is Crucial to Mediate *Staphylococcus aureus* Adaptation during Chronic Infections. PLoS Pathog. **2015** Apr 29;11(4).

d. Kalinka J, Hachmeister M, Geraci J, Sordelli D, Hansen U, Niemann S, Oetermann S, Peters G, Löffler B, **Tuchscherr L**. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. Int J Med Microbiol. **2014** Nov;304(8):1038-49.

e. Horst SA, Hoerr V, Beineke A, Kreis C, **Tuchscherr L**, Kalinka J, Lehne S, Schleicher I, Köhler G, Fuchs T, Raschke MJ, Rohde M, Peters G, Faber C, Löffler B, Medina E. A novel mouse model of *Staphylococcus aureus* chronic osteomyelitis that closely mimics the human infection: an integrated view of disease pathogenesis. Am J Pathol. **2012** Oct;181(4):1206-14.

f. **Tuchscherr L**, Medina E, Hussain M, Völker W, Heitmann V, Niemann S, Holzinger D, Roth J, Proctor RA, Becker K, Peters G, Löffler B. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med. **2011** Mar;3(3):129-41.

g. **Tuchscherr L**, Löffler B, Buzzola FR, Sordelli DO. *Staphylococcus aureus* adaptation to the host and persistence: role of loss of capsular polysaccharide expression. Future Microbiol. **2010** Dec;5(12):1823-32.

h. **Tuchscherr L**, Heitmann V, Hussain M, Viemann D, Roth J, von Eiff C, Peters G, Becker K, Löffler B. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis. **2010** Oct 1;202(7):1031-40.

i. Grundmeier M, **Tuchscherr L**, Brück M, Viemann D, Roth J, Willscher E, Becker K, Peters G, Löffler B. Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells. J Infect Dis. **2010** Mar 15;201(6):871-80.

j. **Tuchscherr LP**, Buzzola FR, Alvarez LP, Lee JC, Sordelli DO. Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice.Infect Immun. **2008** Dec;76(12):5738-44.

1- Introduction

1.1- *S. aureus* and Persistence

Staphylococcus aureus is a gram-positive coccal bacterium that is a member of the Firmicutes. *S. aureus* is a facultative pathogenic bacterium that can colonize the epithelial surfaces of humans and domestic animals and can also cause different types of severe tissue infections. Approximately 20% of individuals are persistently nasally colonized with *S. aureus*, and 30% of individuals are intermittently colonized [1, 2]. However, other sites can be colonized, including the axillae, groin, and gastrointestinal tract [3]. Colonization provides a reservoir from which bacteria can cause different types of infection [4]. In a study of bacteraemia, blood isolates were identical to nasal insolates in 82% of patients, suggesting that *S. aureus* can occasionally breach epithelial barriers and enter the bloodstream [5].

S. aureus is the main pathogen of osteomyelitis worldwide. Osteomyelitis is a bone infection that can be associated with high levels of inflammation and bone tissue destruction. The infection can sometimes develop into a chronic course and may become extremely difficult to treat with antimicrobials [6].

Osteomyelitis can be categorized into three groups, according to the route used by the infecting bacterium to gain access to the bone [7]. First, in haematogenous osteomyelitis, staphylococci access the bone tissue via the bloodstream. This form of osteomyelitis affects mainly prepubertal children. Second, osteomyelitis develops by way of a contiguous spread from a local infection after trauma, bone surgery, or joint replacement. Finally, osteomyelitis can be peripheral to vascular insufficiency. This form of osteomyelitis occurs mostly in diabetic patients and usually originates from an infected foot ulcer that spreads to the bone. All its forms can present in the acute or chronic phase and in virtually any bone. Osteomyelitis is the most frequent cause of non-traumatic limb amputation, as antimicrobial compounds often fail to clear the infection [6-9].

Despite the availability of effective antimicrobial agents to treat staphylococcal infection, it continues to be a major cause of morbidity and mortality worldwide [10]. Epidemiological studies due to the spread of successful clones continue to be reported from virtually every geographic region [11].

S. aureus is one of the most important pathogens in the healthcare and community setting. The emergence of antimicrobial-resistant strains, especially those that are resistant to methicillin, has been a constant feature. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major public health concern and is responsible for both hospital- and community-associated infections worldwide. It is estimated that MRSA infections within the health care

setting alone affected more than 150,000 patients annually in the European Union, with an additional cost of 380 million Euros [12]. In addition, MRSA has developed as a colonizer and pathogen of domestic animals and is linked with livestock-associated infections [13].

In addition to resistance development, many mechanisms appear to contribute to the success of *S. aureus* as a pathogen. Those mechanisms include commensal colonization, long-term survival on environmental surfaces and diverse mechanisms of immune host evasion, and the armamentarium of virulence determinants, often with redundant functions, are among the most important [14-17]. To establish an infection, *S. aureus* bears a multitude of virulence factors, including adhesive surface proteins (adhesins) and toxic compounds that act in concert, destroy host tissue, and resist the host defence system [10]. Yet, the extent of endowment with virulence factors can vary widely between clinical isolates. Furthermore, the expression of almost all virulence factors is controlled by a complex *S. aureus* regulatory network that allows for rapid adaptation; for example, the quorum-sensing system accessory gene regulator (Agr) enhances the expression of toxins and other secreted cytotoxic factors, and the alternative sigma factor B SigB (σB) modulates stress responses [18, 19]. During bacteraemia or sepsis, the bacteria need to survive within the bloodstream and to defend against immune cells. Notably, secreted pore-forming toxins (e.g., α-hemolysin/α-toxin) cause inflammation and contribute to sepsis development [20]. To settle an infection in host tissue, e.g., bone tissue, bacteria first need to adhere to host structures, such as the extracellular matrix or host cells. For this, *S. aureus* expresses various surface proteins with adhesive functions, such as fibronectin-binding proteins (FnBPs), clumping factors (Clfs), collagen-binding protein (Cna), bone sialoprotein-binding protein (Bbp), the anchorless protein extracellular adhesion protein (Eap), and the extracellular matrix binding protein (Emp) [21]. Up until now, a clear association between a single adhesin and the development of osteomyelitis could not be demonstrated [6]. After initial settling of the infection, the bacteria must adapt to the host tissue for persistence and to escape from the host immune system. For this, *S. aureus* can invade different types of host cells, including osteoblasts [22]. In the past decades, *S. aureus* was increasingly recognized as a facultative intracellular pathogen. *S. aureus* has the ability to invade cells and survive intracellularly for various periods. Many studies were conducted to investigate whether intracellular *S. aureus* contributes to persistent and therapy-refractory infections. *S. aureus* can be internalized and survive in endothelial cells, epithelial cells, fibroblasts, osteoblasts and keratinocytes (as non-professional phagocytic cells). Recently reports even document bacterial survival within professional phagocytes, such as human monocyte-derived macrophages, and some studies have examined *S. aureus* survival in neutrophils [17, 23, 24].

The persistence strategy of *S. aureus* may be the reason for infections that take chronic courses, which can be extremely difficult to eradicate by antibiotic treatment, even though the strains are susceptible to antibiotics in vitro. Currently, the mechanism for persistence in the presence of host defences and antibiotic therapy is not completely understood. The methods of persistence are not clear and include different mechanisms. The persister cells can be formed as dormant cells, e.g., non-dividing cells, such as small colony variants (SCVs) [25]. The SCVs are macroscopically slowly growing, without pigmentation, deficient at producing exotoxins and frequently auxotrophic for menadione, hemine and thymidine [26]. For many years, the appearance of SCVs has been associated with chronic infections [26, 27]. Osteomyelitis is one type of infection that frequently develops to chronicity [28]. The diverse strategies of persistent *S. aureus* include crosstalk between global regulators (*agr, sigB* and *sarA*) and rapid feedback according to the environmental stimulus [29, 30]. This work provides a summary of the complex steps of staphylococcal infection, from acute infection to the development of chronicity. The main objective is to understand the biology of persister staphylococcal cells and offer insights into their role in infection development.

1.2- Objectives of this work:

I investigate the passage of *S. aureus* from an acute and septic infection to a chronic and persisting infection. Different tools were used like fluorescent image, real time PCR, cell culture and animal models. According our results, I focused my research on:

1- To analyse the bacterial regulatory mechanisms and changes in virulence factor expression which are required to establish a chronic infection..
2- To study the *S. aureus* mechanism of persistence in professional vs. non-professional phagocytes.
3- To characterized the formation of staphylococcal small colony variants during the course of infection.
4- To find possible therapeutic targets to avoid the persistence of *S. aureus*.

2- Results and discussion

2.1- Acute phase of infection and host response

Grundmeier M, **Tuchscherr L**, Brück M, Viemann D, Roth J, Willscher E, Becker K, Peters G, Löffler B. Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells. J Infect Dis. 2010 Mar 15;201(6):871-80.

The first step in establishing an infection is bacterial adherence, followed by the invasion of host cells. Many authors have demonstrated that most clinical staphylococcal isolates exhibit a strong invasive phenotype [31]. Epithelial or endothelial cells are the first barrier for *S. aureus* to start the infection. These cells express molecules that attract, bind and activate cells from the immune system to defend against the bacteria. To find out how *S. aureus* can provoke epithelial/endothelial inflammatory reactions, we used microarray, real-time PCR and ELISA analysis. We selected 3 invasive strains (Cowan I, 6850 and ST239) with highly invasive phenotypes for our study. Bacterial internalization was quantified using a flow cytometry invasion assay and revealed equally efficient uptake of the different strains by endothelial cells. These results were confirmed by electron microscopy (Fig. 1). The bacteria were mainly located within phagosomes, and major parts of the endothelial cells were infected.

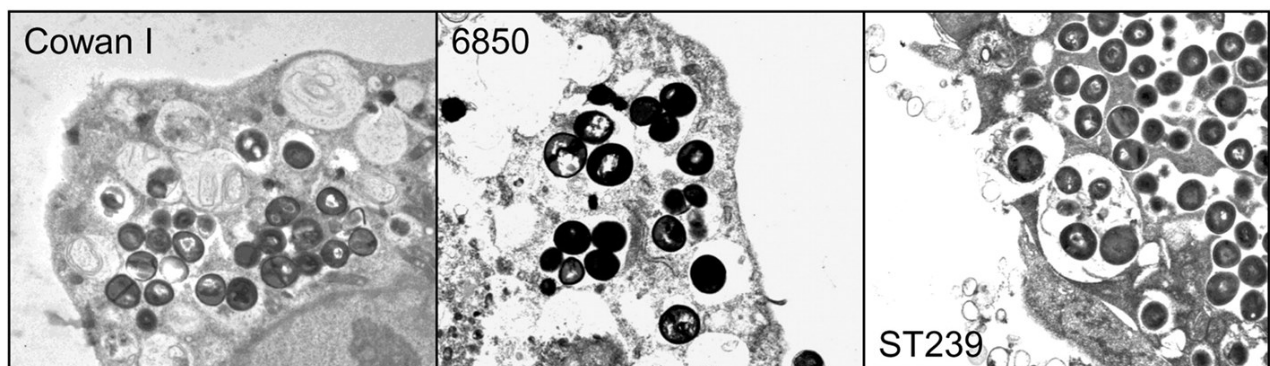


Fig. 1: Electron micrographs of endothelial cells infected with *Staphylococcus aureus*. Confluent human umbilical vein endothelial cells (HUVEC cells) were infected with live *S. aureus* Cowan I, 6850, or ST239 and incubated for 4 h. Cells were then fixed and processed for electron microscopy. All bacterial strains tested were internalized by endothelial cells, and located mainly within phagosomes.

In staphylococci, the production of virulence factors is mainly controlled by the accessory gene regulator *(agr*) system, a quorum-sensing mechanism that controls gene expression

according to the bacterial cell density [32]. To quantify the expression of important virulence factors, including adhesins and secreted toxins, reverse-transcription PCR (RT-PCR, Fig. 2) was performed. All the strains expressed adhesins (fibronectin and protein A), but the Cowan I strain failed to express important virulence factors related to the *agr* system, such as α-toxin (hla). In contrast, strains 6850 and ST239 strongly expressed the regulator *agr* and related virulence factors (Fig. 2A and B).

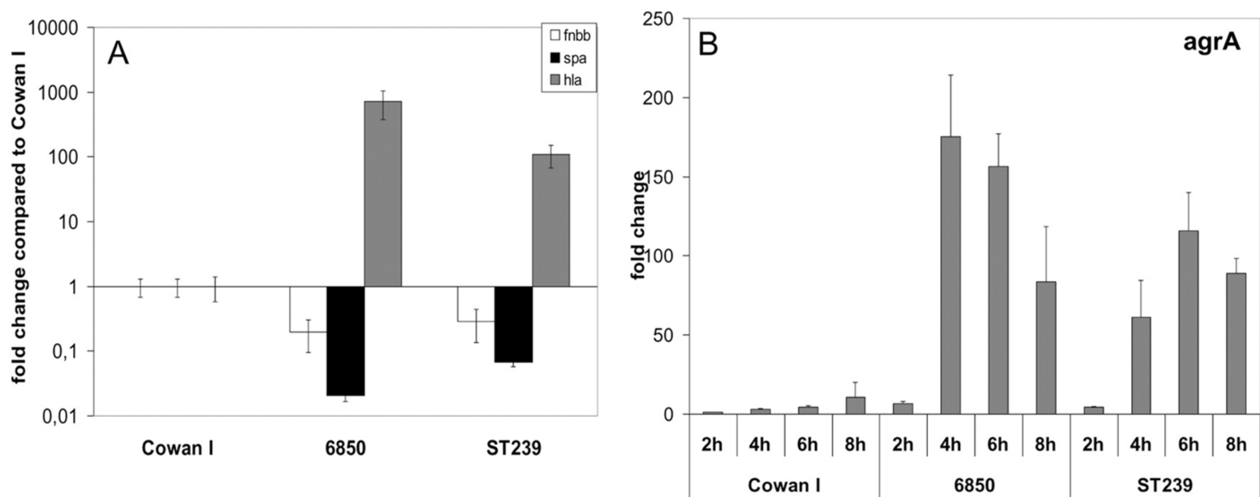


Fig. 2: Differential expression of genes by *Staphylococcus aureus* strains Cowan I, 6850, and ST239. **A**: Results of real-time PCR from *S. aureus* Cowan I, 6850, and ST239. **B:** *S. aureus* Cowan I, 6850, and ST239 were grown for 2, 4, 6, or 8 h, as indicated, and bacterial RNA was extracted and *agr* expression was analysed by real-time PCR.

To determine whether the invasiveness is directly associated with inflammation, we analysed the endothelial gene expression in response to different *S. aureus* strains using microarray analysis. Gene expression analysis revealed that most of the genes were highly upregulated following stimulation with strains 6850 and ST239 compared with uninfected cells, whereas no genes were highly upregulated in response to *S. aureus* Cowan I. Both strains 6850 and ST239 induced the upregulation of genes that are involved in the endothelial immune response, including cytokines, chemokines and adhesion proteins. However, strain Cowan I failed to highly upregulate any genes that could play a role in the antimicrobial response. These results were confirmed by real-time PCR and ELISA [33].

To investigate the clinical relevance of our findings, we analysed 12 clinical isolates for their *agr* expression and divided them into low-*agr*-expressing strains and high-*agr*-expressing strains. As expected, the high-*agr*-expressing strains exhibited high *hla*, haemolytic activity, and proteases and induced strongly enhanced chemokine expression. In contrast, the low-*agr*-expressing strains caused no or only weak chemokine expression as well as virulence factors, similar to Cowan I.

In summary, we found that *S. aureus* strains could cause different types of infections. Isolates that express a multitude of virulence factors induce an extensive immune response and affect many cellular functions. A high level of inflammation might help the host eliminate *S. aureus* but could also produce tissue damage, which favours bacterial spread to deep tissue structures. Strains defective in the *agr* system invade host cells but do not cause a huge inflammatory reaction, which might represent an alternative strategy of *S. aureus* to evade the host immune system and cause chronic and recurrent infections (Fig. 3).

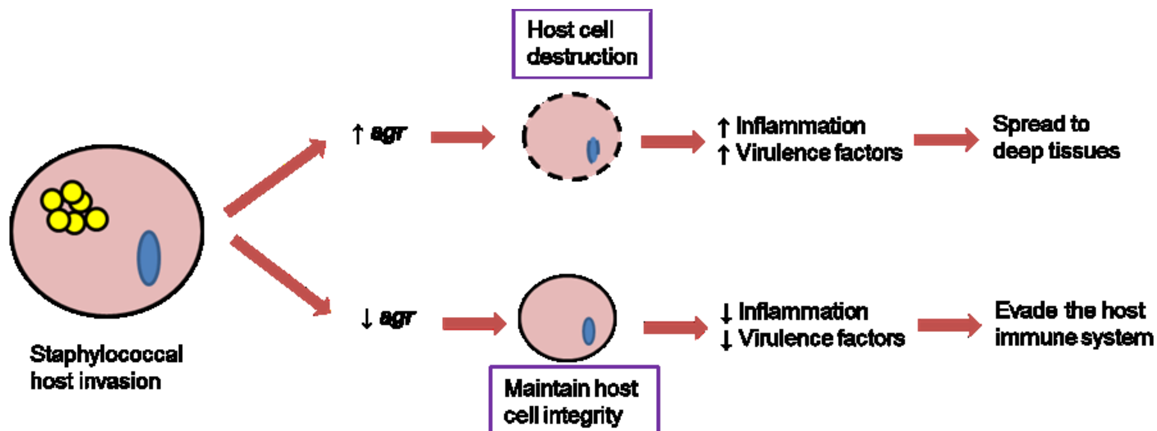


Fig. 3: Summary of events after *S. aureus* host cell invasion. In general, after host cell invasion different courses of infection are possible depending on the bacterial strain and its expression of virulence factors by the agr system. If the ingested staphylococci express a multitude of virulence factors, especially a-toxin, then the bacteria induce proinflammatory and cytotoxic effects within the host cells, which often results in the death of the host cell. If the staphylococci downregulate their virulence factor expression, for example due to a defect in the agr system, then they can persist within the host cells without causing damage; this cell looks morphologically intact, although it contains living bacteria. In this intracellular location the bacteria are likely well protected against the host immune system and against antimicrobial treatments additionally this might be a reservoir for chronic infections.

2.2- In vivo models to study staphylococcal infections

Horst SA, Hoerr V, Beineke A, Kreis C, **Tuchscherr L**, Kalinka J, Lehne S, Schleicher I, Köhler G, Fuchs T, Raschke MJ, Rohde M, Peters G, Faber C, Löffler B, Medina E. A novel mouse model of *Staphylococcus aureus* chronic osteomyelitis that closely mimics the human infection: an integrated view of disease pathogenesis. Am J Pathol. 2012 Oct;181(4):1206-14.

To study the intrinsic steps of *S. aureus* infections, it is necessary to have a model that resembles human pathogenesis and tools to follow *S. aureus* during infection. For this, we established a haematogenous osteomyelitis model in mice to analyse the infection process

from the acute to the chronic stage. The animal studies provide experimental proof for the molecular basis of pathogenesis and the role of the immune system. Additionally, we created a new tool to label bacteria for detection by magnetic resonance imaging (MRI).

The pathogenesis of osteomyelitis remains poorly understood, partly for lack of experimental models that closely mimic human disease. Although diverse animal models of osteomyelitis have been established, they are often lacking the full spectrum of the human disease, including both the acute and chronic phases. To overcome this limitation, we created a novel murine model of osteomyelitis in which bone tissue was infected via the bloodstream. The murine model recapitulates important aspects of acute and chronic stages of osteomyelitis in humans, as demonstrated by histopathology, MRI, X-ray imaging and electron microscopic examination (Fig. 4). Histological analysis of infected bones revealed a massive infiltration of inflammatory cells during the acute phase of the infection. As in the human disease, bone resorption started to become apparent when the infection entered the chronic phase. We could also detect the formation of bone sequestra (necrotic bone) in this murine model (Fig. 5).

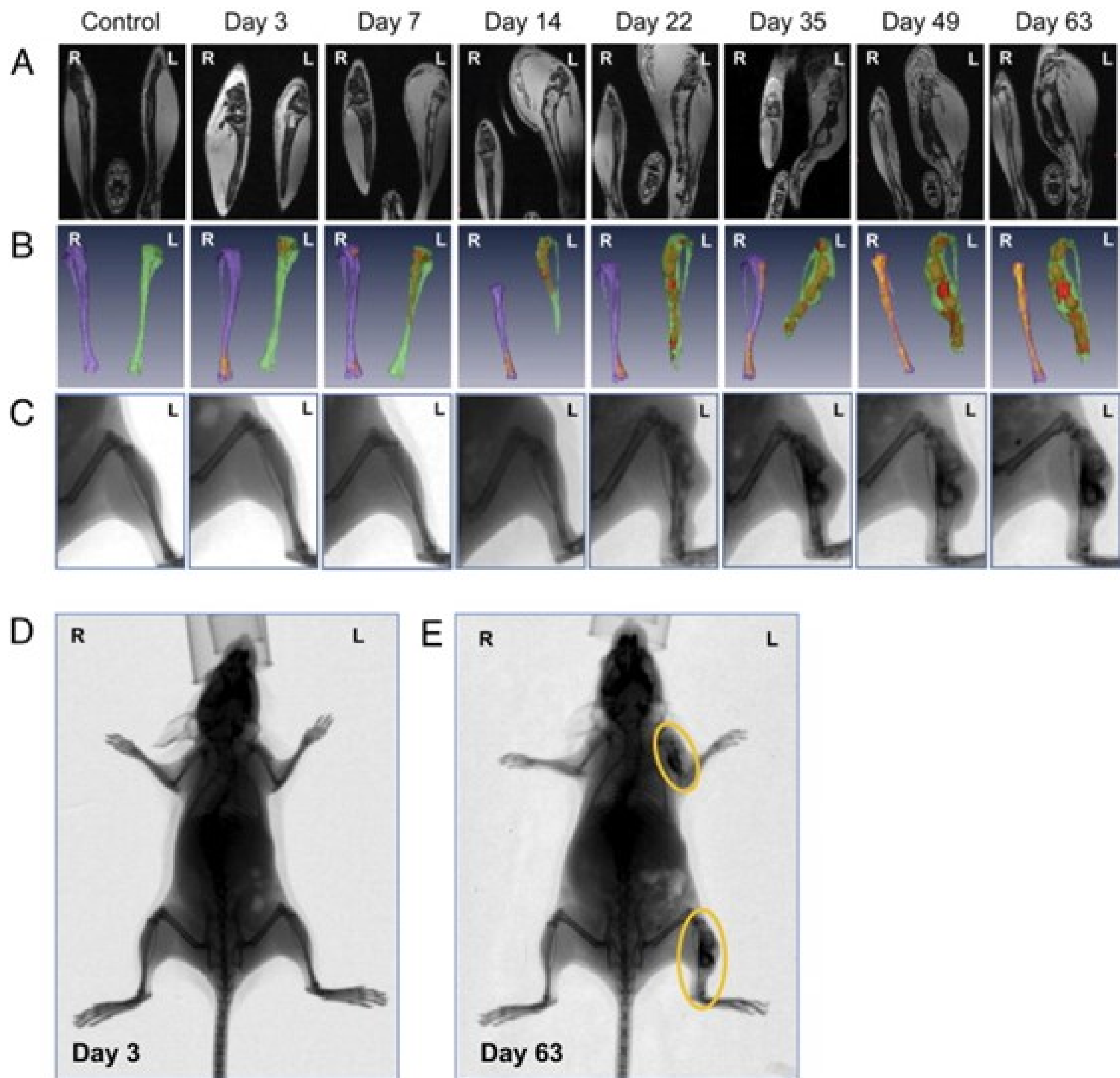


Fig. 4: Sequential MRI and X-ray imaging showing the progression of osteomyelitis in the tibiae of *S. aureus*-infected mice during acute and chronic phases of infection. **A**: MRI scans of the right (R) and left (L) tibiae of an uninfected (control) and a *S. aureus*-infected mouse at progressive times after bacterial inoculation. **B**: Three-dimensional reconstruction of the tibiae and inflammatory lesions after segmentation of MRI. Inflammatory lesions are coloured orange and pink (right) or red and brown (left) according to the inflammatory depth in the bones. The noninflamed area (the area of the bone without signs of inflammation) of the right leg is shown in magenta and the noninflamed area of the left leg is shown in green. **C**: Sequential radiography of a left tibia, showing the progression of bone deformation over time. **D and E**: Whole-body X-ray radiography on day 3 (**D**) and day 63 (**E**) of infection.

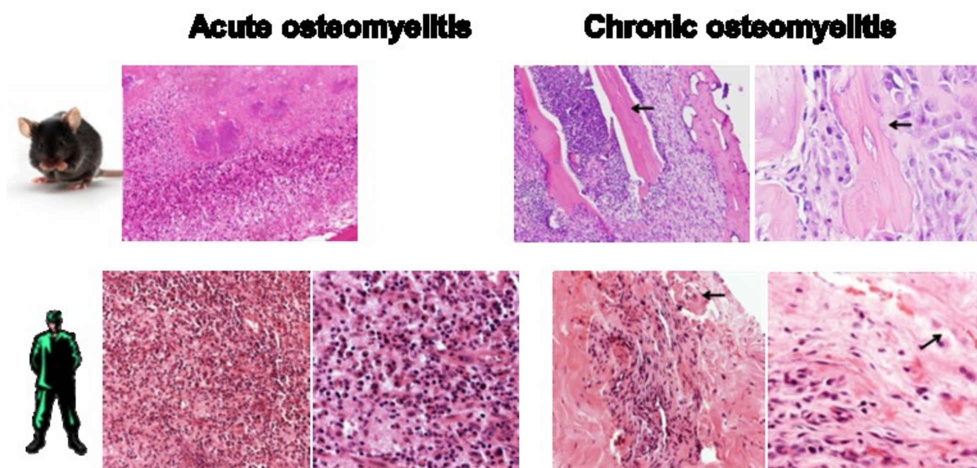


Fig. 5: Histopathological evaluation of mouse and human osteomyelitis during acute and chronic disease stages (H&E stain). In the acute osteomyelitis, massive infiltration of immune cells was detected, whereas in the chronic stage of infection, the resorption lacunae produced by the activity of individual osteoclasts is shown. This pattern is similar in mice and patients.

To summarize these concepts, we have described a novel murine model of *S. aureus* that mimics the natural route of infection in haematogenous osteomyelitis. This model can facilitate the identification of bacterial factors involved in bone tropism and provides an important platform for microbiological and immunological studies [34].

2.3- Development and dynamic of small colony variants (SCVs)

Tuchscherr L, Medina E, Hussain M, Völker W, Heitmann V, Niemann S, Holzinger D, Roth J, Proctor RA, Becker K, Peters G, Löffler B. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med. 2011 Mar;3(3):129-41.

Löffler B, **Tuchscherr L**, Niemann S, Peters G. *Staphylococcus aureus* persistence in non-professional phagocytes. Int J Med Microbiol. 2014 Mar;304(2):170-6. doi: 10.1016/j.ijmm.2013.11.011. Epub 2013 Dec 1. Review.

Tuchscherr L, Bischoff M, Lattar SM, Noto Llana M, Pförtner H, Niemann S, Geraci J, Van de Vyver H, Fraunholz MJ, Cheung AL, Herrmann M, Völker U, Sordelli DO, Peters G, Löffler B. Sigma Factor SigB Is Crucial to Mediate *Staphylococcus aureus* Adaptation during Chronic Infections. PLoS Pathog. 2015 Apr 29;11(4):e1004870

Tuchscherr L, Löffler B. *Staphylococcus aureus* dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection. Curr Genet. 2015 Jun 30.

Although *S. aureus* is primarily considered an extracellular pathogen, recent evidence suggests that this bacterium can invade a variety of nonprofessional phagocytic cells. The intracellularity of *S. aureus* has been implied as immune-evasive strategy, thereby escaping detection by professional phagocytes. During its intracellular life, *S. aureus* can change the phenotype to small colony variants (SCVs) [26, 35-37]. Subpopulations of SCVs have been found in a wide variety of bacterial species [26, 38, 39], but they have been most extensively studied in *S. aureus*. In general, SCVs form small colonies on agar plates (approximately 10 times smaller than the parent strain) due to their slow growth rate and reduced metabolism, which also explains their decreased susceptibility to a variety of antibiotics [40, 41]. They express a changed pattern of virulence factors, including the reduced expression of exotoxins, such as α-haemolysin (α-toxin), and an increased expression of adhesins, such as the fibronectin-binding proteins (FnBPs) [42]. Furthermore, SCVs are frequently auxotrophic for menadione and haemin, compounds involved in the biosynthesis of electron transport chain elements, or thymidine [26, 42, 43].

SCVs recovered from clinical specimens are often not stable and can rapidly revert to their wild-type phenotype when subcultivated [44]. For this reason, most knowledge and laboratory work on SCVs has been obtained with stable site-directed mutants with mutations in the electron transport system that mimic the SCV phenotype, e.g., *hemB* and *menD* mutants [45, 46]. Consequently, data on the development and dynamics of SCVs are largely missing. Further reported mechanisms leading to the formation of SCVs (in vitro and in vivo) include prolonged exposure to subinhibitory concentrations of antibiotics [45, 47, 48] or to exoproducts from other bacteria, e.g., *Pseudomonas aeruginosa* [49]. Moreover, there is growing evidence that the formation of SCVs could also be due to regulatory mechanisms, involving global regulators (e.g., sigB, sarA and agr), Clp ATPases [48, 50, 51] or non-protein-coding RNAs as regulatory molecules [52].

There are many open questions regarding the signals and factors that induce the formation of *S. aureus* SCVs. However, the central question that needs to be addressed first is whether the development of SCVs is only a rare, marginal or laboratory phenomenon (possibly due to gene mutations) or whether the formation of SCVs is an integral part of the normal bacterial life cycle that is required for adaptation and persistence. In the latter case, a dynamic and reversible formation of SCVs has to be assumed. The phenomenon of rapid phenotype

switching of genetically identical cells (bet-hedging strategy) has been described for a variety of other microorganisms [53-55]. Particularly, in a stressful and fluctuating environment, stochastic differentiation into distinct phenotypes can provide a strong advantage that promotes bacterial persistence [25, 56, 57].

To analyse the mechanisms of SCV formation, several in vitro cell cultures and in vivo murine models of *S. aureus* long-term infections were used [58, 59]. The recovered bacteria were analysed for their virulence potential and their dynamic capacity to revert to the wild-type phenotype.

First, we infected A549 cells (cell line ATCC CCL-185 and human lung adenocarcinoma) with *S. aureus* strain 6850 and the nasal isolate 628. After 4 weeks, we could recover from the infected host cells viable colonies of both strains with a high phenotypic diversity. The proportion of SCVs recovered increased up to 90% with increased time of intracellular persistence. We performed the same experiment using primary cells (HUVEC and osteoblasts) and obtained the same result after 1 week (Fig. 6A, B).

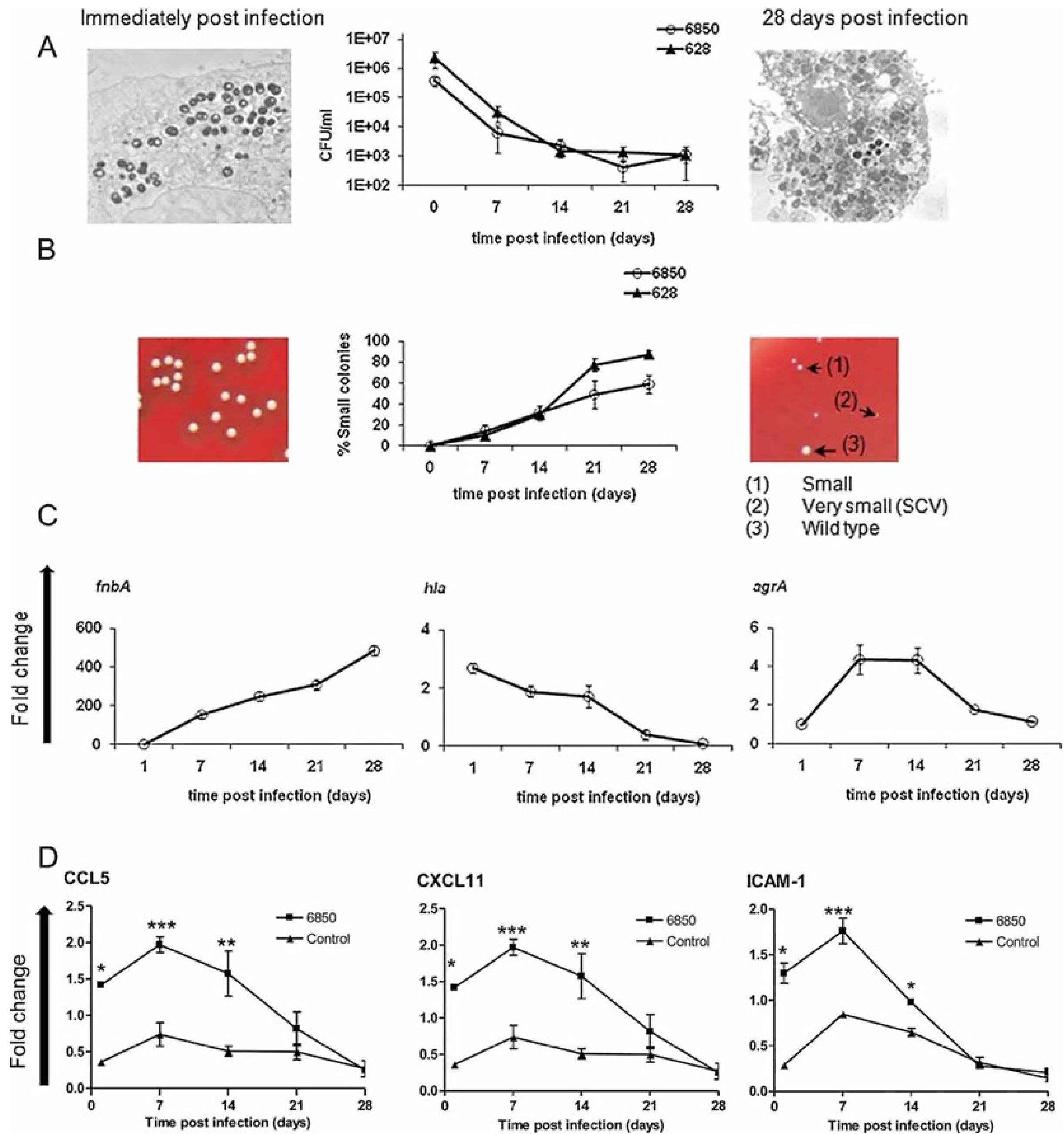


Fig. 6: Staphylococci survive within cultured host cells for 28 days, change phenotypes, and virulence factor expression. **A:** Epithelial cells (A549) were infected with different staphylococcal strains (6850 or 628) and analysed for 28 days. The number of viable intracellular persisting bacteria was determined weekly by lysing host cells, plating the lysates on agar plates and counting the colonies the following day (n = 3, ±SEM). Electron micrographs of infected cells were performed directly after infection and 28 days post-infection showing morphological intact staphylococci within epithelial cells. **B:** Percentage of small and very small (SCV) phenotypes (<5 and <10-fold smaller than those of the wild-type phenotype, respectively) recovered over the 28 days (n = 3, between 30 and 300 colonies examined in each sample, ±SEM). Photographs of recovered colonies were performed directly after and 28 days post-infection showing small and SCV colonies. **C:** Changes in bacterial gene expression (strain 6850) of fibronectin binding protein A (fnbA), α-haemolysin (hla) and agr during the course of infection were determined by real-time PCR (n = 5, ±SD). **D:** Changes in host cell response measured by the expression of CCL5, CXCL11 and ICAM-1 over time following infection with strain 6850 (n = 6). *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 in comparison with values from uninfected cells.

We next determined the expression of virulence factors during phenotypic switching and the innate immune response. Interestingly, we found high expression of FnBPA (*fnbA*) and downregulation of *hla* (α-toxin) and the global accessory gene regulator *agr,* which controls the expression of many secreted virulence factors. In parallel, the host response, measured by the acute inflammatory cytokine levels (CCR5/RANTES or CXCL11/I-ITAC) or ICAM-1/CD54 levels, was attenuated (Fig. 6C, D). The results clearly showed diminished *hla* and *agr* levels over time, suggesting reduced virulence. Consequently, persisting bacteria appear to be as silent as possible to avoid provoking the host immune system. In contrast, *fnbA* levels increased during long-term persistence, indicating that the bacteria become highly adhesive and are rapidly taken up by new host cells once they are released. Similar results were obtained with primary cells (HUVECs and osteoblasts). Furthermore, we analysed this phenomenon in vivo in tissue samples from osteomyelitis mice (see section “model to study persistence”) and in human tissue derived from patients with chronic *S. aureus* infection. In both cases, we observed an increased rate of SCVs during the process of infection (30 days). Additionally, the temporal RNA expression profiles obtained were similar to the alterations obtained in cell culture models. Taken together, the bacteria show common adaptation mechanisms, including the formation of SCVs and downregulation or upregulation of toxins. Next, we analysed the dynamics of this switching process. For this, we analysed SCVs obtained from cell culture, animal models and patient samples before and after subcultivating steps. After 24 h, all SCVs recovered the original wild-type phenotypes, including the cytotoxic activity (related with increased *hla* and *agr* expression), whereas they showed decreased invasiveness (related with decreased *fnb* expression) (Fig. 7).

Taken together, these results demonstrate that *S. aureus* dynamically alters its *agr* and virulence factor expression to bypass innate immune defences and establishes conditions favouring prolonged intracellular persistence. Our research explains that the phenotypic switching of *S. aureus* is indeed an essential feature of the staphylococcal life cycle, and the formation of SCVs in the intracellular milieu produces a chronic infection [58]

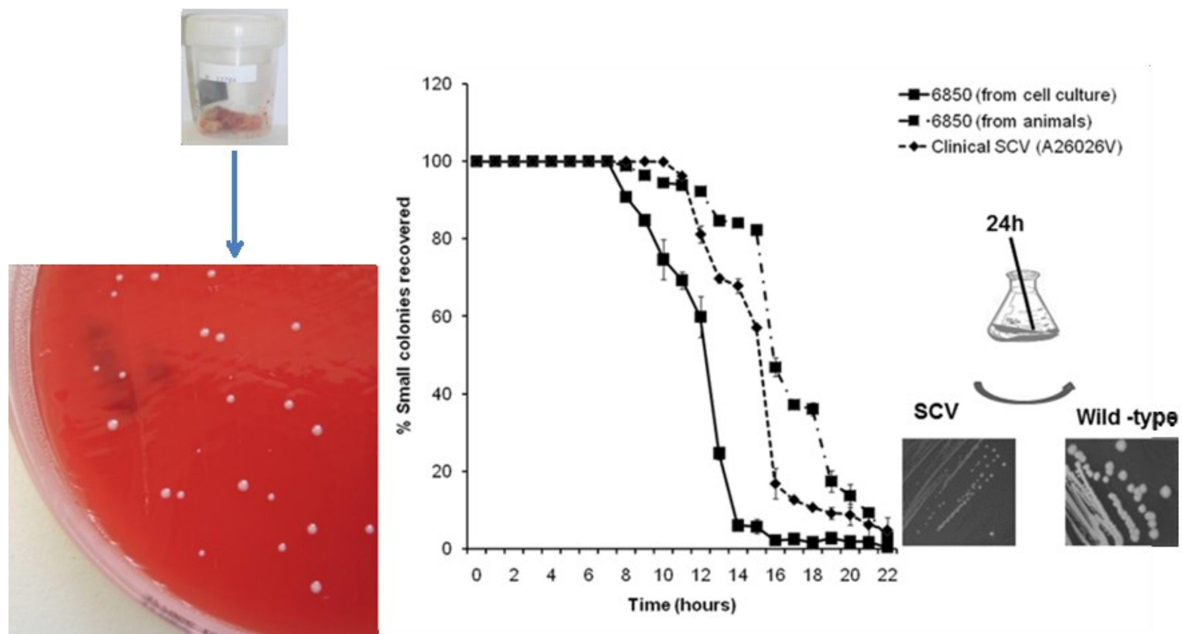


Fig. 7: *S. aureus* obtained from clinical tissues revealed changes in phenotype switching. SCV phenotypes recovered from in vitro (cell culture) chronic infection and in vivo (animals) chronic infection models and from an chronic osteomyelitis patient (A26026V) were subcultivated in BHI at 37°C with shaking. Every hour samples were plated on agar plates to determine the percentage of small and SCV phenotypes.

Due to the rapid switching process between wild-type and SCV strains, regulatory mechanisms that control the expression of almost all virulence factors could be involved in the phenotype switching mechanism [29, 30] . The main global *S. aureus* regulators include the accessory gene regulator (*agr*), the staphylococcal accessory regulator A (*sarA*), and the alternative sigma B factor (*sigB*) (Fig. 8). To test the function of the global regulatory systems in the course from acute to chronic infection, we created several knockout strains with mutations in global regulators. We generated single, double and triple mutants for the defined factors [29, 30, 59].

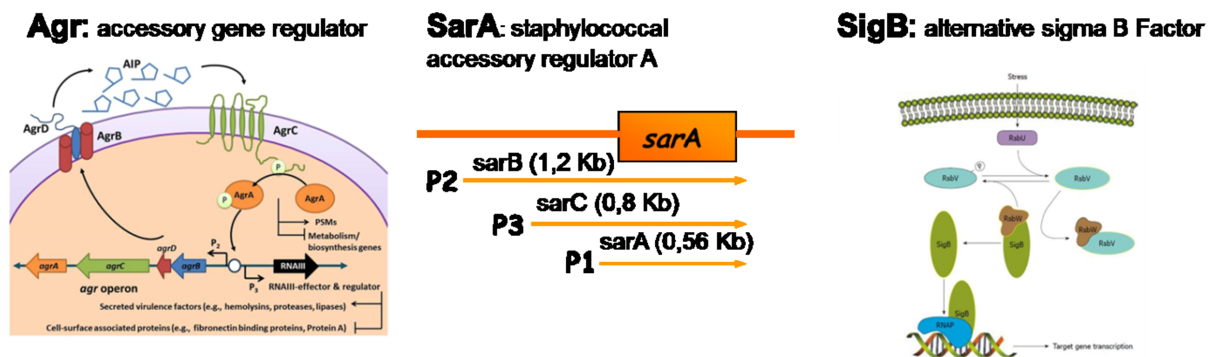


Fig. 8: General description of global regulators in *Staphylococcus aureus*. The main global regulators of *S. aureus* are *agr* (accessory gene regulator), *sarA* (staphylococcal accessory regulator A) and *sigB* (alternative sigma factor B). The interaction between all global regulators determines the expression of virulence factors and adhesins during the course of infection.

All the strains were characterized by LC-MS/MS to provide an overview of the levels of virulence factor expression in each strain. The data show that the *sarA* mutant and, even more, the double- and the triple-mutants released a drastically reduced number of virulence factors in comparison with the WT strain. As secreted virulence factors are particularly directed against professional phagocytes, we tested the effect of bacterial supernatants on neutrophils (PMNs) isolated from humans and mice (Fig. 9). These results suggest that the *agr* and *sarA* systems are required to mount an aggressive and cytotoxic phenotype during acute infection, while sigB appears to be less important for virulence.

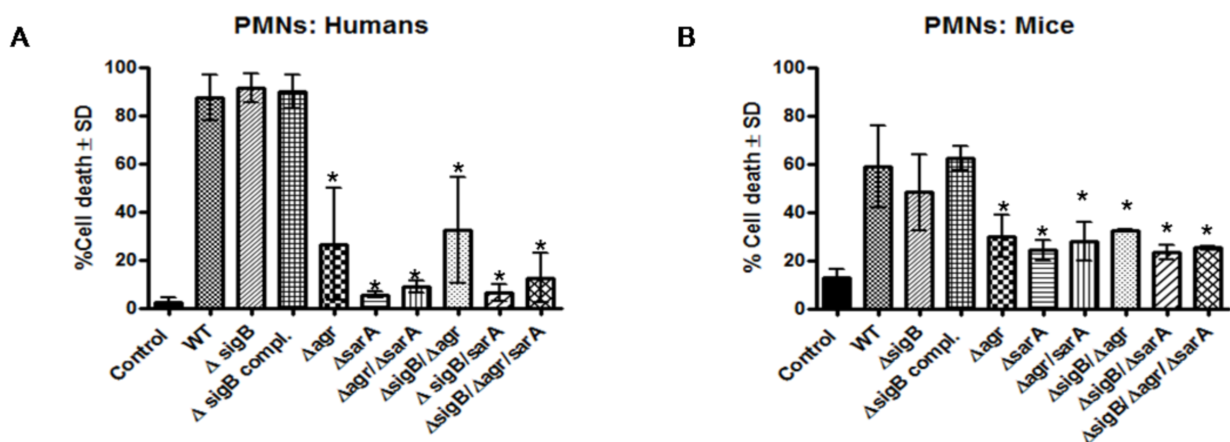


Fig. 9: The combined action of *agr* and *sarA* is required for inflammation and cytotoxicity in the acute stage of infection. Cytotoxicity experiments and analysis of host inflammatory responses were performed in polymorphonuclear cells (PMNs) using wild-type strain LS1 and derivate mutants. **A and B:** PMNs were freshly isolated form human blood (**A**) and bone marrow of Balb/C mice (**B**) and 1×106/0.5 ml cells were incubated with 5% v/v of bacterial supernatants for 1 h. Cells were then washed, stained with annexin V and propidium iodide and cell death was measured by flow cytometry.

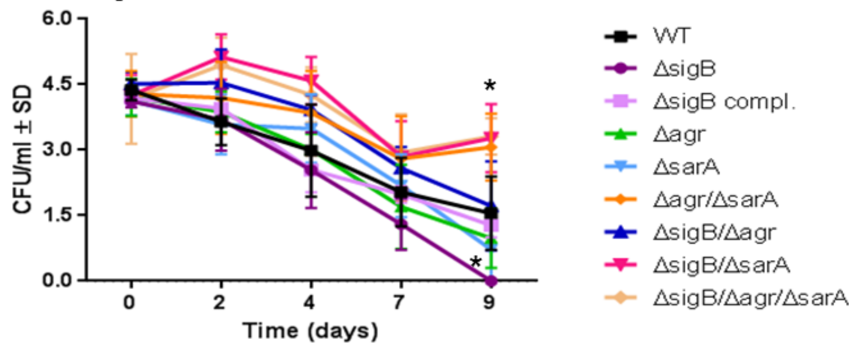
To analyse the function and interplay of global regulatory systems in the course from acute to chronic infection, we infected osteoblast and endothelial cell cultures with wild-type and the knockout strains with mutation in global regulators and analysed their ability to persist intracellularly for 9 days. In general, the number of intracellular bacteria was decreased during the whole infection course (Fig. 10A), but considerable differences between the strains appeared after 9 days (Fig. 10B). The *agr*/*sarA* and *sigB/sarA* double mutants as well as the triple mutant were able to persist within the intracellular location at significantly higher

numbers (up to 100-fold) than the corresponding wild-type strain. In contrast, the *sigB* mutants were completely cleared from the host cells within 7 – 9 days, but this effect could be fully reversed by the complementation of sigB (Fig. 10A)

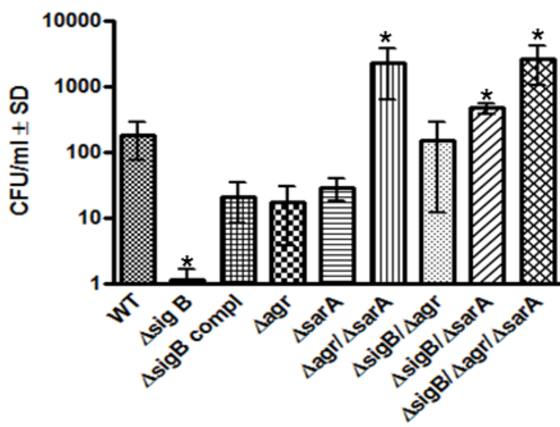
Interestingly, in the present study, we found that all *sigB* mutants completely failed to develop SCV phenotypes after 7 days of intracellular persistence (Fig. 10C). By analysing the recovered colonies from the *sigB* mutants, we observed much less phenotypic diversity than in the wild-type and other mutants, as the plates revealed only uniformly large, white colonies. These effects could be reversed by complementation of the *sigB* mutations with an intact *sigB* operon, thus proving a clear and specific connection between the bacterial ability to form dynamic SCVs and the SigB system. These results demonstrate the relationship between the dynamic formation of SCVs and crosstalk of global regulators. Yet, SigB promotes bacterial persistence and is highly associated with the bacterial ability to form SCVs and the adaptation process.

Taken together, our results show that the *agr* system, which upregulates the expression of toxins and secreted virulence factors, such as alpha-hemolysin (Hla), is required in the acute phase of the infection to defend the bacteria against invading immune cells, whereas *agr* needs to be silenced during chronic infection to allow bacterial persistence. Downregulation of *agr* and related toxins enables the bacteria to silently persist within the intracellular location without provoking the host immune response and without killing the host cells. In contrast, the expression of SigB plays a crucial role during the persistence and dynamic formation of SCVs.

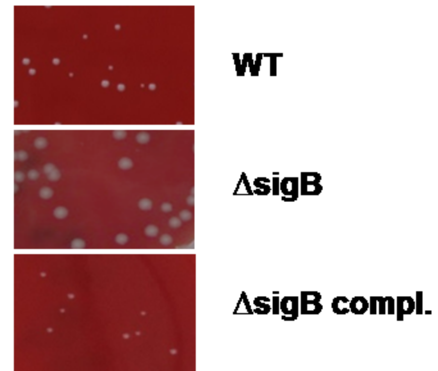
A: CFU/ml during the infection course



B: CFU/ml after 9 days



D: SCVs after 7 days



C: %SCVs after 9 days

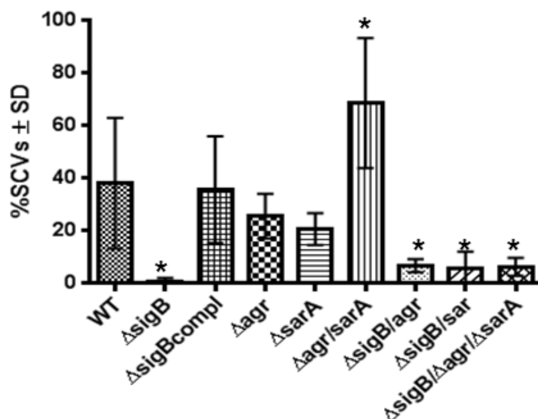


Fig. 10: Intracellular persistence of LS1 and mutants after infection of osteoblasts. **A:** Cultured osteoblasts were infected with *S. aureus* strain LS1 (WT), mutants of LS1 or complemented mutants and infected cells were analysed for up to 9 days. The number of viable intracellular persisting bacteria was determined every 2 days by lysing host cells, plating the lysates on blood agar plates and counting the colonies on the following day. **B:** The results after 9 days are shown separately. The results shown are from osteoblast infection experiments, however, similar results were obtained after infection of endothelial cells. **C:** The percentage of small and very small (SCV) phenotypes (<5 and <10-fold smaller than those of the wild-type phenotypes, respectively) recovered were determined after 7-9

days p.i. The values of all experiments represent the mean ± SD of at least three independent experiments performed in duplicates. * P≤0.05 ANOVA test comparing the effects induced by the wild-type strains and the corresponding mutants. **D:** Photographs of recovered colonies, 7 days post infection of endothelial cells with strains LS1, LS1ΔsigB or LS1ΔsigB compl.

Summarizing our results (Fig. 11), we developed a schema for chronic infection courses. We found that *S. aureus* can persist within host cells at low numbers for several weeks. Intracellularly, the bacteria change their phenotype and their expression of virulence factors to avoid activation of the host immune system. Within their host cells, the bacteria are most likely well-protected against the host immune system and against most antimicrobial treatments. However, these phenotypes are very dynamic; they can rapidly regain full virulence when they leave the intracellular environment and can efficiently cause a new episode of infection. One of the main factors involved in this phenomenon is *sigB*, which is involved in the formation of SCVs. This dynamic switching between aggressive and hiding phenotypes could be an explanation for chronic infections, which are mostly resistant against antimicrobial treatments [29, 30, 59].

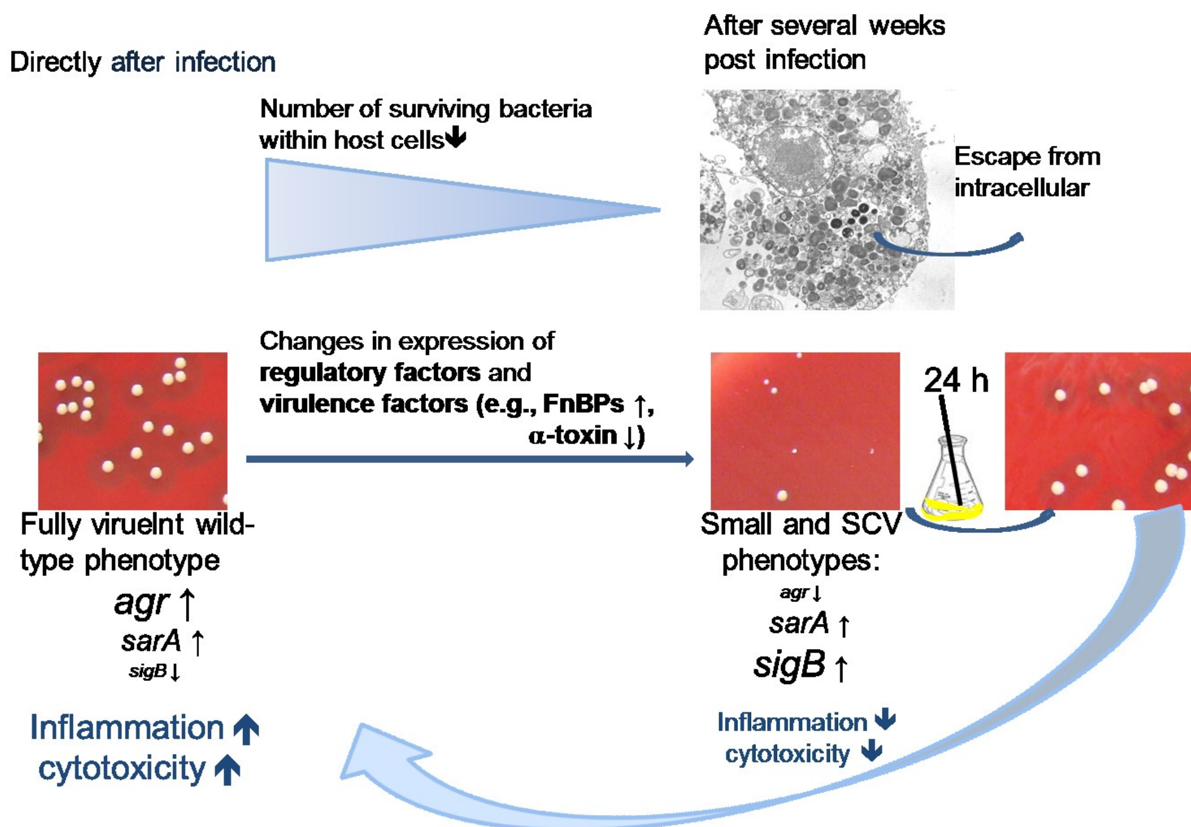


Fig. 11: Summary of chronic staphylococcal infections. *S. aureus* can persist within host cells at low numbers for several weeks. Intracellular bacteria change their phenotype and their

expression of virulence factors to avoid activation of the host immune system. Within host cells the bacteria are likely protected against the host immune system and against most antimicrobial treatments. However, these phenotypes are very dynamic, they can rapidly regain full virulence when they leave the intracellular environment and can efficiently cause a new episode of an infection. This dynamic switching between aggressive and obscure phenotypes could be an explanation for chronic infections, which are largely resistant to antimicrobial treatments.

2.4- Host controled by *S. aureus* during persistence: mechanisms of immune escape

Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, von Eiff C, Peters G, Becker K, Löffler B. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis. 2010 Oct 1;202(7):1031-40. doi: 10.1086/656047.

Kalinka J, Hachmeister M, Geraci J, Sordelli D, Hansen U, Niemann S, Oetermann S, Peters G, Löffler B, **Tuchscherr L**. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. Int J Med Microbiol. 2014 Nov;304(8):1038-49.

During the course of infection, the host and pathogen dynamically interact with each other. On one hand, the host has a high capacity to kill the bacteria, but on the other hand, *S. aureus* can be hidden intracellularly, and the host apparently fails to notice the presence of the microorganisms. The acute phase is largely determined by the expression of multiple bacterial factors (e.g., surface bound adhesins and exotoxins) that enable *S. aureus* to adhere to host structures, destroy tissue, and invade various types of host cells [29, 33]. Cellular invasion can be followed by intracellular bacterial persistence. However, the host organism has a sophisticated defence system against bacterial infections. The immune response involves multiple cytokines that activate professional cells to very rapidly eliminate all microorganisms [33]. Conversely, *S. aureus* can successfully evade the host response.

As we described before, one of the mechanisms that *S. aureus* uses to avoid elimination is phenotype switching to SCVs. To determine how the host reacts to staphylococcal persistence, we analysed the host response after bacterial infection. For our study, endothelial cells were infected with highly virulent wild-type strains (6850), SCV-phenotype strains (IIb13 and JB1) and the complemented mutant (IIb13 with restored hemB, KM4). The host response was analysed by microarray and real-time PCR [60].

All the strains revealed high cell invasiveness. Infection of endothelial cells with wild-type phenotypes or SCVs caused considerable differences in gene expression. Most genes of chemokines and innate immune factors were upregulated after infection with wild-type strains compared to uninfected control cells. In contrast, SCV-infected endothelial cells showed reduced gene expression. Thus, infection with a complemented SCV strain (KM4) resulted in a similar pattern of chemokine expression as wild-type strains. The results were confirmed by real-time PCR and ELISA (Fig. 12). Moreover, the inflammatory response was analysed for 4 days. The wild-type and complemented mutant showed high chemokine expression during the first two days. However, 3 days after infection, chemokine release decreased to the level of uninfected control cells. As expected, the SCV mutant IIb13 did not cause a considerable increase in chemokine expression at any time point measured [60].

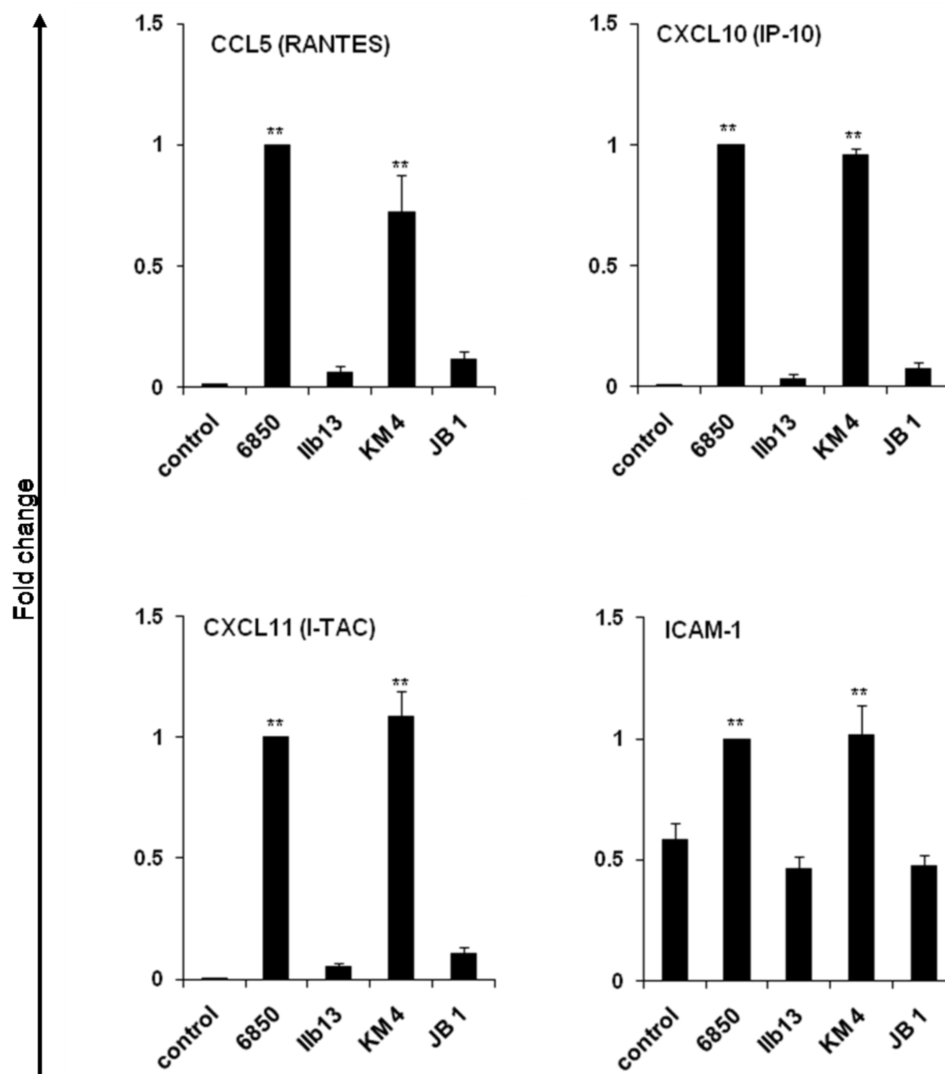


Fig. 11: Confirmation of endothelial gene expression by real-time PCR. RNA was extracted from endothelial cells infected with different *Staphylococcus aureus* strains (6850, IIB13, KM4 and JB1) for microarray analysis, and results were confirmed by real-time PCR. Real-time PCR of 4 genes (CCL5, CXCL10, CXCL11 and ICAM-1) was performed. Results demonstrate the relative increase in gene expression, compared to the unstimulated cells.

Data shown are the mean ± standard error of 3 independent experiments performed in duplicate. Results are compared with uninfected control cells. **P $\leqslant$ .01.

Our results demonstrate that when located intracellularly, SCVs largely avoid activation of the host innate defence system and that they do not kill the host cells during persistence. This finding can be explained by the downregulation of important virulence factors in SCVs, e.g., α-toxin and proteases, which contribute to inflammation and tissue destruction [29, 33].

Another important aspect of host-pathogen co-evolution is to investigate whether staphylococcal strains have special pre-established characteristics according to the type of infection [61]. To determine whether staphylococcal virulence factors are associated with osteomyelitis and contribute to a chronic course of infection, different clinical isolates were analysed. The comparison between 10 isolates from acute osteomyelitis, 10 isolates from chronic osteomyelitis, 10 isolates from sepsis and 10 isolates from nasal colonization demonstrated that the chronic osteomyelitis isolates showed high host cell invasion rates, low cytotoxicity and the ability to persist and adapt within osteoblasts. Furthermore, isolates from both acute and chronic osteomyelitis strongly produced biofilm and induced high levels of host cell inflammation, which may explain the tissue destruction and bone deformation observed as typical complications of long-lasting bone infections [61]. In this way, we can assume that some strains are adapted to defined host tissue, and these characteristics are already fixed in the genome.

Taken together, we can conclude that *S. aureus* evades the host response by switching phenotypes to form SCVs, which are more highly invasive and prevent the host immune response. Nevertheless, some virulence factors are fixed in the genome depending on the type of infection [60, 61].

2.5- Loss of capsule: One mechanism of persistence

> **Tuchscherr L**, Löffler B, Buzzola FR, Sordelli DO. *Staphylococcus aureus* adaptation to the host and persistence: role of loss of capsular polysaccharide expression. Future Microbial. 2010 Dec;5(12):1823-32. doi: 10.2217/fmb.10.147.

S. aureus is a versatile microorganism that can use different mechanisms to survive and evade the host response. During long-term persistence, we can distinguish different steps, including the dynamic adaptation to the tissue (the formation of SCVs, as described before)

and, later on, the final adaptation where the bacteria loses genes that are not necessary in its present environment. An example of this is the loss of the capsule, which is associated with chronic infection. The high plasticity of the *S. aureus* genome makes this genus highly adaptive to environmental changes, which leads to significant phenotypic diversification of *S. aureus* clinical isolates [62]. Capsules increase the bacterial virulence by contributing to phagocytosis resistance [63]. Staphylococcal capsule production was first described in 1931 by Gilbert. Because capsule detection methods were crude (India ink negative staining, colony morphology on agar plates and in serum-soft agar, and lack of cell-associated clumping factor), only a few strains of *S. aureus* were recognized as capsule-positive [64]. These highly encapsulated strains (typified by strains M and Smith diffuse) produced mucoid colonies and showed strong resistance to phagocytosis and virulence in a mouse model. In 1982, Karakawa and Vann proposed a new capsular polysaccharide-typing test using adsorbed rabbit antiserum [65]. By this new method, most of the staphylococcal isolates were found to be encapsulated, and eight capsular serotypes were described. The strongly encapsulated strains M and Smith diffuse were named serotypes 1 and 2, respectively. These serotypes are rarely found among clinical isolates. The other serotypes produce non-mucoid colonies on solid medium, and some authors have defined them as microencapsulated to distinguish them from the atypical mucoid strains [62, 66].

Eleven capsular serotypes have been described, but most clinical isolates of *S. aureus* (isolated from humans or animals) belong to capsular types (CP) 5 or 8. The prevalence of CP5 or CP8 is dependent on the geographical area [66, 67].

Strains that do not react with antibodies to serotypes 1, 2, 5 or 8 are referred to as non-typeable (NT) [62, 68]. In this context, a prevalent *S. aureus* clone in bovines of Argentina with subclinical mastitis exhibited a deletion of almost the entire *cap* cluster and was named NT. The deletion was associated with the presence of an insertion element, IScap [68]. In those strains, 63 bp of the 3´ end of the *capP* gene remained in place, confirming that the *cap* gene cluster that was initially present in the genome was deleted. It is important to note that subclinical mastitis is a chronic condition involving long-term survival of the bacteria in the infected udder [68, 69].

Not all clinically relevant *S. aureus* isolates from humans produce CP5 or CP8 [12,21]. Furthermore, loss of CP5(8) expression has been associated with the persistence of *S. aureus* in the infected host. This hypothesis has been supported by different experimental and clinical studies. Studies in a mouse model of mastitis have shown that an isogenic mutant lacking CP expression persisted in higher numbers and for a longer time in the mammary glands than their capsulated counterparts [70]. Moreover, clinical studies testing

staphylococcal osteomyelitis strains from different patients showed a higher proportion of NT *S. aureus* in patients with chronic osteomyelitis [71].

To enter the intracellular environment, bacteria have evolved different strategies, such as the expression of adhesins and downregulation of different regulators and virulence factors, including CP5(8) microcapsules. Previous studies have demonstrated that the expression of CP5(8) interfered with bacterial adhesion by masking adhesins [72]. Furthermore, *agr* inactivation can be advantageous to *S. aureus* for intracellular survival. Indeed, an increase in the capacity for cell invasion occurs after the inhibition of the *S. aureus agr* system, wherein the expression of cell wall proteins is elevated [73]. Subsequently, due to their slow growth rate, SCV populations are not able to reach quorum sensing conditions to activate the *agr* system. Additionally, the *agr* system was inactive in SCVs isolated from the lungs of cystic fibrosis patients [26, 74].

Taken together, the coordinated expression of *S. aureus* virulence factors appears to be critical for the development of an infection. *S. aureus* can change its lifestyle between “adherent” and “aggressive” in response to bacterial density sensed by the *agr* quorum-sensing system [18]. Then, *S. aureus* can access the intracellular milieu by exposing high levels of adhesins because of the loss of the capsule. Inside the cells, the microevolution continues through the formation of SCVs. The microevolution from the “aggressive” to the “adherent” phenotype is explained in Fig. 12. Antibodies to CP5(8) opsonize encapsulated *S. aureus* (“aggressive phenotype’”) and favour its subsequent removal by professional phagocytes. At the expected rate for a point mutation, stable mutants that do not express CP5(8) (non-capsulated) emerge and are selected (“adhesive phenotype”). These mutants can expose their adhesins to gain access to the intracellular milieu. In this new environment, the intracellular stress conditions select the formation of SCVs [72].

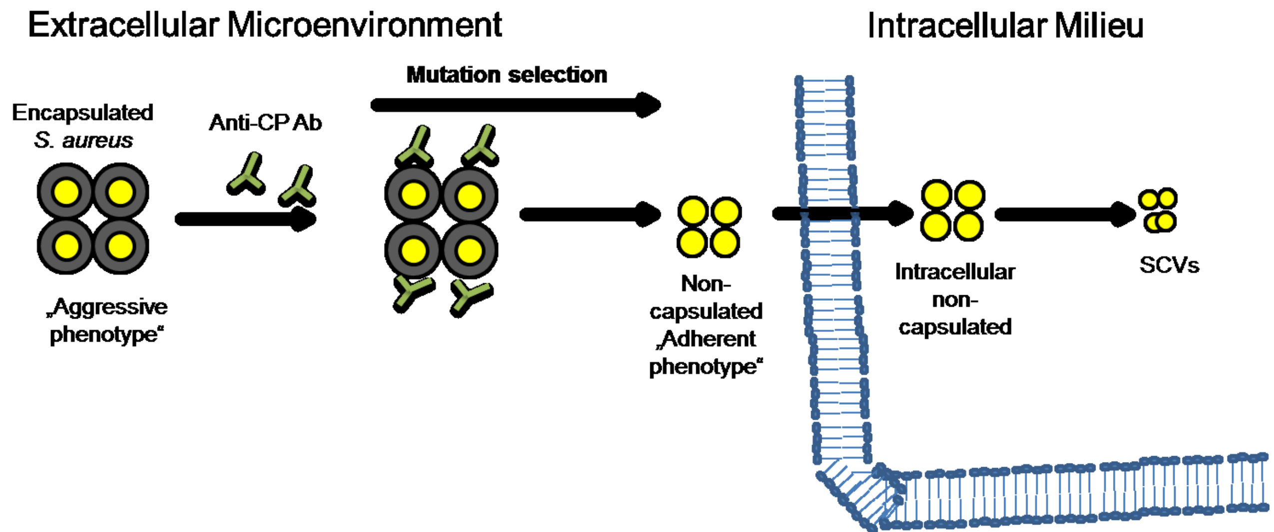


Fig. 12: Microevolution of *S. aureus* from the "aggressive" to the "adherent" phenotype in vivo. Antibodies to CP5(8) opsonize capsulated *S. aureus* ('aggressive phenotype') and lead to its subsequent removal by professional phagocytes. At the expected rate for a point mutation, stable mutants that do not express CP5(8) (nontypeable [NT] variants) emerge and are selected ('adhesive phenotype'). If enough time elapses, total selection of a NT, stable *S. aureus* occurs in the chronically infected host. These NT staphylococci are more efficiently internalized. If loss of CP5(8) occurs due to a mutation in a regulatory system, concomitant loss of other factors such as α-hemolysin also occurs, and such *S. aureus* variants are better adapted to the intracellular environment. NT variants would precede the emergence of non-capsulated SCVs (from left to right, upper portion of the figure).

2.6- Approaches for vaccine development and treatment to eliminate *S. aureus*

Tuchscherr LP, Buzzola FR, Alvarez LP, Lee JC, Sordelli DO. Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice.Infect Immun. 2008 Dec;76(12):5738-44.

L. Tuchscherr, C.A. Kreis, V. Hoerr , L. Flint, M. Hachmeister, J. Geraci, S. Bremer Streck, M. Kiehntopf, E. Medina, M. Kribus, M. Raschke, M. Pletz, G. Peters, B. Löffler. *Staphylococcus aureus* develops increased resistance against antibiotics by forming dynamic small colony variants during chronic osteomyelitis. J Antimicrob Chemother. 2016 Feb;71(2):438-48.

Chronic staphylococcal infections are very difficult to eradicate. The reason for this problem is the highly sophisticated adaptation mechanisms of *S. aureus* to the intracellular

environment and the ability of this bacteria to manipulate innate and adaptive immune responses. Additionally, *S. aureus* is endowed with a multitude of virulence factors. Up until now, many therapeutic strategies have failed because they were designed to focus on one virulence factor as a possible target to design a vaccination or treatment. To prevent staphylococcal infections and avoid the development of chronic infections, a better understanding of staphylococcal infection strategies is needed. A successful therapy should take different aspects in consideration: 1- the phase of infection (acute/chronic), 2- the localization of *S. aureus* (intracellular/extracellular), 3- the metabolic stage of the bacteria (active/dormant) and 4- the possible protection provided by biofilm (biofilm). According to the steps of staphylococcus infection, different approaches were studied (Fig. 13).

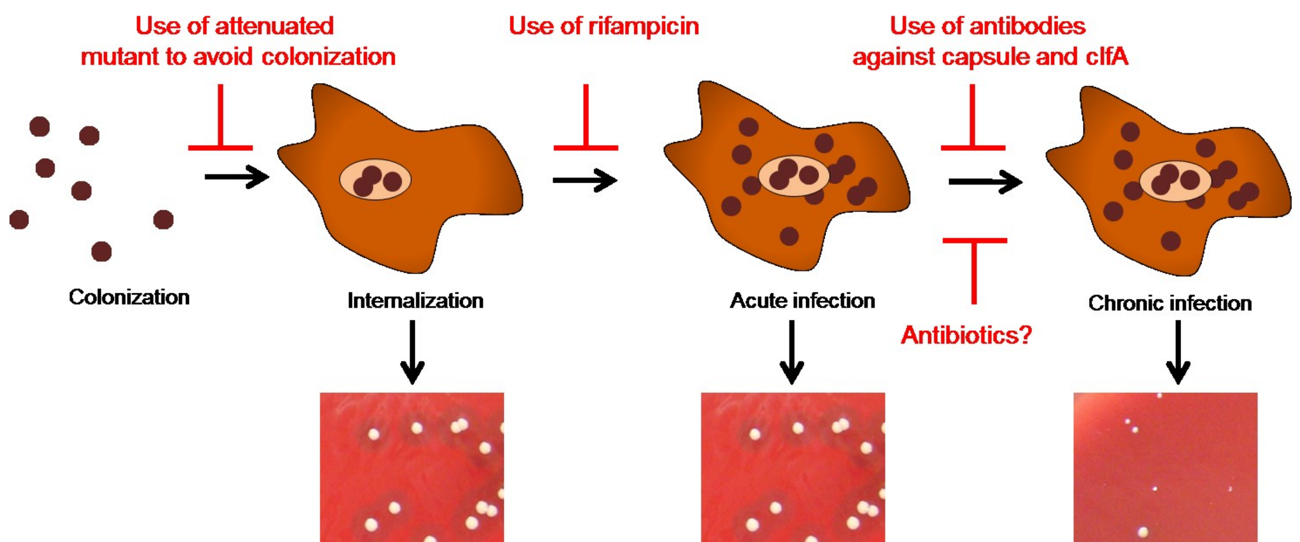


Fig. 13: Summary of different approaches investigated to eradicate *S. aureus*. During the course of infection, there are many stages to interrupt the development of chronic infection. The first step is colonization which can be avoided through pre-treatment with attenuated mutants. The second stage is internalization of *S. aureus* by host cells. Upon internalization of *S. aureus* the use of active intracellular antimicrobials such as rifampicin can clear the bacteria. The last stage is a phenotype switch from the aggressive wild type to a non-aggressive phenotype such as SCVs. The use of antibodies against factors (capsule and clfA) essential for staphylococcal cells may prevent this phenotype switching. A new generation of antibiotics may be required to eliminate the non-metabolic and persistent phenotype like SCVs.

The first approach was directly designed to prevent the passage of *S. aureus* from acute to chronic infection. We found that during the chronic phase, capsule expression is downregulated and adhesins are exposed [72]. One of the most important adhesins is clumping factor (clfs), a cell wall-anchored *S. aureus* surface protein. Using our mastitis murine model [70], we tested antibodies against capsular polysaccharide and clumping

factor. Clinical trials that targeted the capsule or clumping factor A (ClfA) failed to protect the recipients against staphylococcal infections [75-77]. We passively immunized lactating mice with rabbit antibodies to *S. aureus* capsular polysaccharide (CP) serotype 5 (CP5) or CP8 or with monoclonal antibodies to ClfA. Mice immunized with antibodies to CP5 or CP8 or with ClfA had significantly reduced tissue bacterial burdens 4 days after intramammary challenge with encapsulated *S. aureus* strains. After several passages in mice passively immunized with CP-specific antiserum, increasing numbers of stable uncapsulated variants of *S. aureus* were cultured from the infected mammary glands. Furthermore, small colony variants (SCVs) were recovered from the infected mammary glands after several passages in mice passively immunized with CP-specific antiserum. A combination of antibodies was effective at eliminating the *S. aureus* from the mammary glands (Fig. 14A). More importantly, passive immunization with antibodies to both CP and ClfA fully inhibited the emergence of uncapsulated "escape mutants" and significantly reduced the appearance of SCVs (Fig. 14B). To conclude, a vaccine formulation comprising CP conjugates plus a surface-associated protein, adhesin, might be more effective than either antigen alone for the prevention of chronic *S. aureus* infection [69].

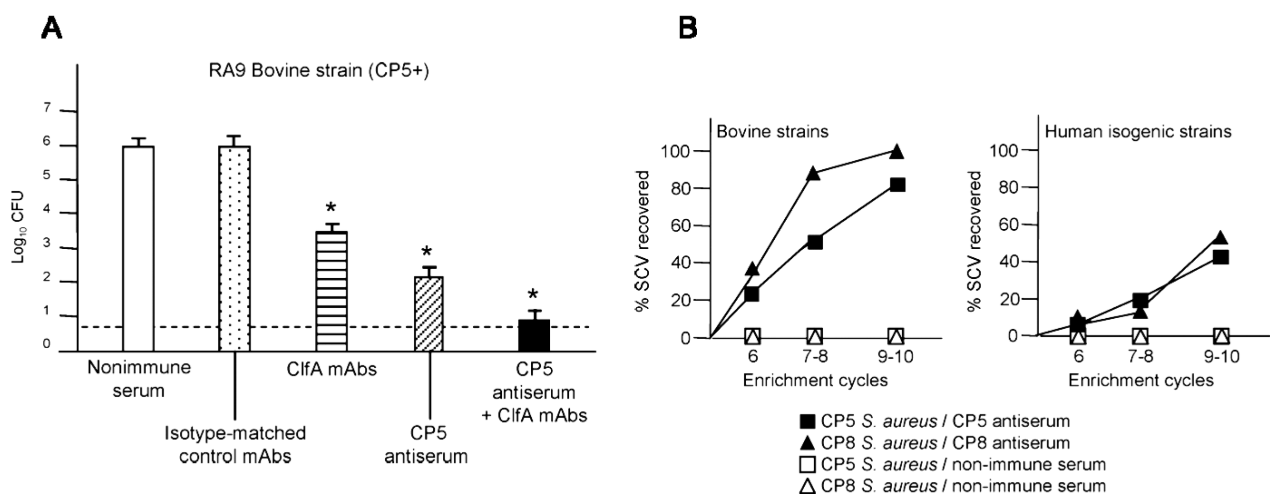


Fig. 14: A: Passive immunization with antibodies to CP5 and ClfA reduced the intra-mammary bacterial load 96 h after intra-mammary challenge. **B:** SCVs recovered from the mammary glands of passively immunized mice during the enrichment experiment.

The second approach was to study the effect of antimicrobial treatment against *S. aureus* in chronic infection courses. As *S. aureus* can invade host cells and adapts to the intracellular environment by forming SCVs, it is likely that they become more tolerant to antibiotics due to

their reduced metabolism. To analyse the efficacy of antibiotics in the acute and chronic stages of bone infections, we performed our established long-term cell culture model in osteoblasts and our murine haematogenous osteomyelitis model. Antibiotics that were tested include β-lactams, fluoroquinolones, vancomycin, linezolid, daptomycin, fosfomycin, gentamicin, rifampicin and clindamycin. Cell culture infection experiments revealed that all tested antibiotics reduced the bacterial numbers within infected osteoblasts when treatment was started immediately. In contrast, we found that some antimicrobial compounds (β-lactams, daptomycin, fosfomycin and clindamycin) lost activity against chronically infecting bacteria when treatment was started only 7 days post infection (Fig. 15). β-lactams, daptomycin and fosfomycin are cell-wall-active antibiotics. These compounds are highly effective bactericidal antibiotics against fast-growing bacteria, but they apparently rapidly lose activity when the bacteria slow down their growth rate, which could limit their efficacy during chronic infections and against SCVs (phenotypic antibiotic resistance/tolerance). Additionally, clindamycin, which inhibits protein biosynthesis, lost activity against persisting bacteria, which can be explained by a reduced metabolism in persisting bacteria and the bacteriostatic mode of action. In contrast, other protein biosynthesis inhibitors (gentamicin and rifampicin) were still active against persisting bacteria; this might be due to their bactericidal effect, which is apparently still effective on SCVs. The bactericidal and/or bacteriolytic effects of moxifloxacin and vancomycin also resulted in high activity against persisting bacteria at the serum concentrations tested.

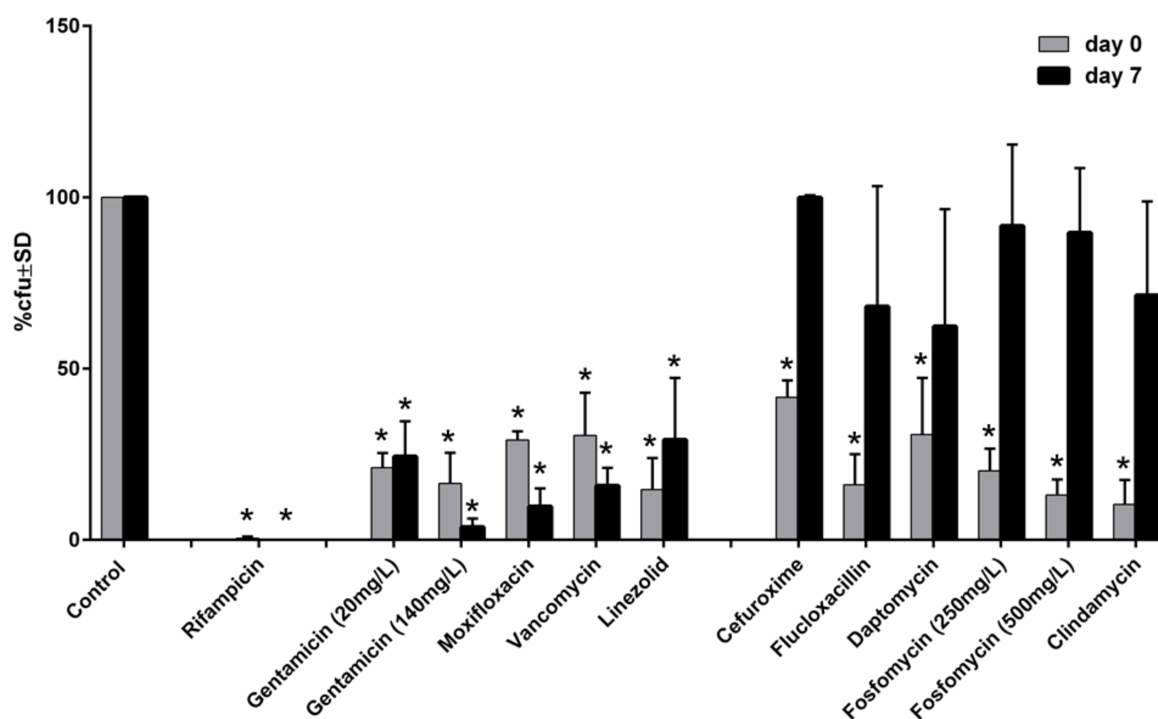


Fig. 15: Intracellular activity of antibiotics against *S. aureus* persisting in osteoblasts. Osteoblasts were infected with *S. aureus* 6850 followed by treatment with antibiotics for 48 h directly after infection or at 7 days post-infection. The number of surviving bacteria were determined by plating and are shown in relation to control (untreated, 100%; 4.36×108±8×106 cfu/mL) cells. Statistical analysis was performed by ANOVA comparing bacterial numbers in untreated control cells with those in treated cells at the two timepoints (n≥3; ±SD). *P≤0.05.

In our study, we further tested treatment efficacy in our haematogenous osteomyelitis model, which develops to chronicity and closely mimics human infection. The subcutaneous application of defined antibiotics (rifampicin, gentamicin, cefuroxime) for 5 days resulted in serum levels that were similar to the concentrations reached in humans. To evaluate the success of treatment, we determined the bacterial load in the bones and used MRI to quantify the areas of inflammation and bone deformation. In the acute phase of infection (treatment 5 days post infection.), we detected a significant reduction in bacteria after treatment with rifampicin, whereas during chronic infection (treatment after 6 weeks post infection), none of the tested antibiotics reduced the numbers of the infecting bacteria (Fig. 16). Furthermore, we observed that low concentrations of gentamicin, moxifloxacin and clindamycin enhanced the formation/ selection of SCVs, which could promote chronic infection.

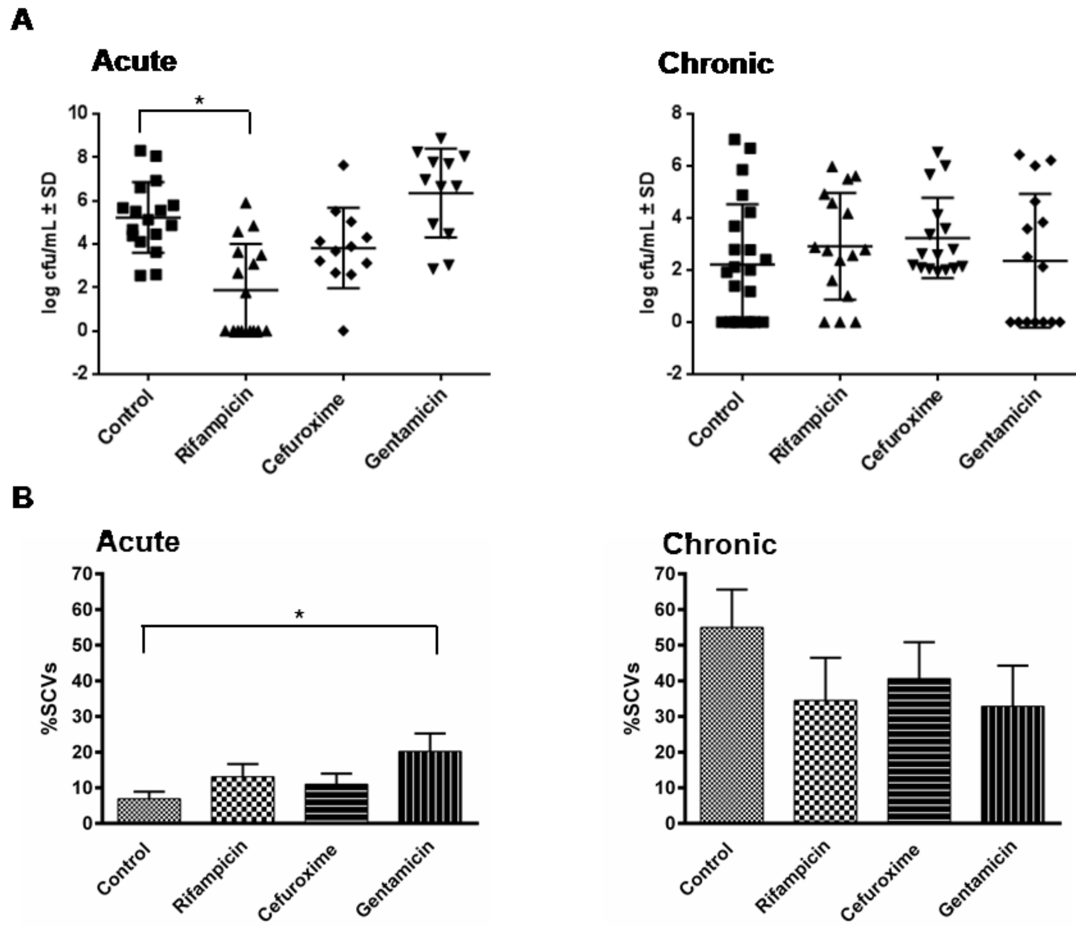


Fig. 16: Treatment of a haematogenous osteomyelitis model in mice with rifampicin, gentamicin and cefuroxime. **A:** Bacterial loads within the tibiae were analysed after treatment in the acute and chronic stages of infection by plating host tissue and counting the number of recovered colonies. **B:** The percentage of SCVs among the recovered colonies was evaluated for each antibiotic in the acute and chronic stages of infection.

Taken together, we found that the low metabolism of SCVs formed during chronic infection impede the action of many commonly used antibiotics.

3- Conclusions and overview

In our work, we investigated the biology of staphylococcal infections, especially the intracellular persistence mechanisms. Using different animal and cellular models, we identified essential factors that are required for successful acute infection and strategies that the microorganism uses to establish a chronic infection and escape from the host response.

As model systems, we established long-term cell culture systems using different types of host cells and a chronic osteomyelitis model in mice that closely mimics the human disease.

During the acute phase, *S. aureus* uses an arsenal of virulence factors to invade and destroy host cells. We found that many strains induce a huge inflammatory response, especially enhancing the expression of genes involved in innate immunity. Furthermore, we analysed the crosstalk of different global bacterial regulators during acute and chronic infections that mediate the expression of almost all virulence factors. According to our data, all the *agr*- and/or *sarA*-mediated factors are required for an acute infection. Agr/sarA-defective strains are not able to fight against the immune host response, and they are rapidly cleared from the host.

An acute infection can develop into a chronic or recurrent infection that becomes extremely difficult to treat. The transition between acute infection and a successful chronic/persistent infection involves many bacterial survival strategies. We found that the development of small colony variants (SCVs) is an essential part of intracellular long-term persistence. We described that SCVs largely avoid activation of the host response, which enables them to survive intracellularly for long periods. Most importantly, the development of persistent SCV phenotypes is a highly dynamic process, as SCVs can rapidly revert back to their fully virulent wild-type phenotypes and start a new course of infection. This bacterial phenotype switching is an integral part of the infection process that enables the bacteria to hide inside host cells. This event is largely mediated by the crosstalk of global regulators. By generating and testing mutants for defined *S. aureus* regulatory factors, we could demonstrate that the stress factor SigB is indispensable for long-term bacterial persistence and the development of SCVs.

In addition to dynamic persistence mechanisms that are largely mediated by regulatory mechanisms, we also studied changes in the staphylococcal genome that are related to long-term persistence. The flexibility of the staphylococcal genome makes this genus highly adaptive to environmental changes, which leads to significant phenotypic diversification of staphylococcal clinical isolates. Analysis of clinical isolates from patients with chronic infection showed an increased rate of a non-capsulated phenotype. The loss of the capsule allows the exposure of adhesins necessary to invade tissues.

Consequently, characteristic features for persisting bacteria are the formation of dynamic SCV-phenotypes, which is dependent on the stress factor SigB, the downregulation of secreted virulence factors (e.g., toxins) and the loss of the capsule. Using these mechanisms, the bacteria enter a stage of dormancy that enables them to escape from the immune response and establish a chronic infection. This bacterial population is difficult to eradicate with conventional antimicrobial treatments. Our antibiotic treatment experiments in chronic infection models revealed that many commonly used antibiotics lost activity against chronically infecting bacteria. Additionally, we described one strategy for a possible

staphylococcal vaccine. We found that the combination of antibodies against the capsule and clumping factor is an effective treatment to eliminate *S. aureus* and to prevent the appearance of SCVs.

In summary, our research explains *S. aureus* mechanisms that enable the shift from an acute and highly inflammatory infection to a persistent state that is extremely difficult to treat. Many factors are involved in this process, such as global regulators, changes in virulence factor expression, and loss of the capsule. A deeper understanding of these processes provides possible candidate molecules to design better therapeutic approaches.

4- List of Abbreviations:

S. aureus	*Staphylococcus aureus*
Agr	*accessory gene regulator*
sarA	*staphylococcal accessory regulator*
sigB	*alternative sigma factor B*
hla	*alpha hemolysin, α-toxin, α-hemolysin*
fnbA/B	*fibronectin binding protein A/B*
emp	*extracellular matrix protein*
eap	*extracellular adherence protein*
clfA/B	*clumping factor A/B*
CP(5)8	*capsule protein type 5 and 8*
NT	*non-typeable (strains that do not react with antibodies to capsule types 1, 2, 5 or 8)*
LC-MS/MS	*liquid chromatography–mass spectrometry*
WT	*wild-type*
MRI	*magnetic resonance imaging*
MRSA	*methicillin-resistant Staphylococcus aureus*
MSSA	*methicillin-sensitive Staphylococcus aureus*
Cna	*collagen-binding protein*
Bbp	*bone sialoprotein-binding protein*
SCVs	*small colony variants*
ELISA	*Enzyme Linked Immunosorbent Assay*
PCR	*polymerase chain reaction*
RT-PCR	*Reverse-transcription PCR*
H&E staining	*Hematoxylin and eosin staining*
hemB	*hemin auxotroph strain*
menD	*Menadione auxotroph strain*
Thy or TD SCV	*Thymidine auxotroph strain*
Clp	*molecular chaperone*
RANTES/CCL-5	*chemotactic for T cells, eosinophils, and basophils,*
CXCL-11/I-TAC	*Interferon-inducible T-cell alpha chemoattractant*
ICAM/CD54	*cell surface glycoprotein*
PMN	*granulocyte polymorphonuclear neutrophil*

5- References

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6- Ehrenwörtliche Erklärung

Ich erkläre hiermit, daß mir die aktuelle Habilitationsordnung der Friedrich-Schiller-Universität Jena bekannt ist.

Ferner erkläre ich, daß ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich/unentgeltlich geholfen:

1. Prof. Dr. Löffler , Prof. Dr Peters und Prof. Dr Sordelli.

2..MTAs und students from Münster: B. Schuhen, K. Broschwig, M. Brück, Marie Hachmeister and Jennifer Geraci.

3. MTAs from Argentina: S. Soldavini and L. Medina

4. Colleges: S. Niemann, V. Hoerr, C. Kreis

Weitere Personen waren an der inhaltlich-materiellen Erstellung der Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Arbeit stehen.

Die Arbeit wurde bisher weder im In- noch Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Ich versichere, daß ich nach bestem Wissen die reine Wahrheit gesagt und nichts verschwiegen habe.

Jena, den 23. June 2016

Lorena Tuchscherr

7- Curriculum Vitae

PERSONAL DATA

Name: Lorena P. N. Tuchscherr de Hauschopp (born: Tuchscherr)
Date of Birth: June 02, 1976.
Place of Birth: Buenos Aires City, Argentina.
Marital status: Married
Children: 2
Citizenship: Argentine.
Country of residence: Germany

Business address: Institute of Medical Microbiology
Erlanger Allee 101
(07747) Jena
Germany
Lorena.tuchscherrdehauschopp@med.uni-jena.de
Phone: +49 36419-393628

EDUCATION

MSc, Biology, 2002
Buenos Aires University, School of Sciences. Buenos Aires, Argentina.

PhD, Biology, 2007
Buenos Aires University, School of Sciences. Buenos Aires, Argentina.
Thesis: Loss of capsule expression of *Staphylococcus aureus* in chronic infection: causative factors and pathological significance of this finding. Dr. Daniel O. Sordelli, Advisor.

Post doc, Biology, 2007 to 2014. University of Münster, Germany

LANGUAGES

- Spanish (native language).
- English
- German

PROFESSIONAL ACTIVITIES

Present Position

- Head of research on *S. aureus* pathogenesis. Institut fur Medizinische Mikrobiologie, Jena, Universitätsklinikum Jena, Germany. From August 2014 to present.

Past Positions

- Laboratory Technician. General Bacteriology Laboratory. Enrique Tornú General Hospital, City of Buenos Aires. From 1996 to 2001.
- Research Assistant. Department of Physiology, School of Medicine, University of Buenos Aires. From 1999 to 2001
- Laboratory Technician. Mycobacteriology Laboratory. Enrique Tornú General Hospital, City of Buenos Aires. From 2002 to 2003..
- Research Assistant. Department of Microbiology, Parasitology and Immunology, School of Medicine, University of Buenos Aires. From 2001 to 2007.
- Training in Harvard University from 20th march of 2004 to 15th October of 2004.
- Institut fur Medizinische Mikrobiologie, Münster Universitätsklinikum Münster, Germany. From november 2007 to 2014.
- Institut fur Medizinische Mikrobiologie, Jena Universitätsklinikum Jena, Germany. From August 2014 to present.

Teaching experience

- Head of laboratory training for medical students. Department of Microbiology, Parasitology and Immunology, School of Medicine, University of Buenos Aires. From 2001 to 2007.
- Assistant of laboratory trainning for medical students. Institut fur Medizinische Mikrobiologie, Münster Universitätsklinikum Münster, Germany. From november 2007 to 2014.
- Teaching assistant in the Department of Microbiology, Molecular Medicine. School of Biology, University Jena (2015- present).

Teaching courses

26-06-2015 Präsentieren mit Prezi-nicht ohne eine Metapher, Friedrich-Schiller-Universität Jena

03-07-2015 Teaching in English, Friedrich-Schiller-Universität Jena

22-07-2015 Lehrqualifikation Basic, Friedrich-Schiller-Universität Jena

Experience in research training and role as thesis advisor assistance

- 2010-2013 Julia Kalinka, phD: "Host-pathogen interactions in *S. aureus* osteomyelitis"
- 2011-2015 Vijaya Mysore, phD: "*Staphylococcus aureus* SCVs use intracellular persistence in human macrophages as a strategy evade the innate immune response"
- 2012-2013 Christine Muth, Bachelor : "Competition between wt and different mutant on general regulators from *S. aureus* (sigB, agr, SarA)"
- 2012-2013 Elvira Vögel, Master : "Untersuchung zum Einfluss der Stringenten Antwort auf die intrazelluläre Persistenz von *Staphylococcus aureus*"
- 2012 Dr. Caroline Kreis, medical research training: "*Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis"
- 2012 Lisa Flint, phD: "SCV-Induktion durch die Gabe verschiedener Antibiotika als Ursache für die Entwicklung chronischer und Therapie-refraktärer *S.aureus*-Infektionen"
- 2012 Sandra Diekmann, Bachelor: "Influence of tryptophan on SCV formation in *S. aureus*"
- 2013-2014 Anika Blümke, Bachelor : "Phäno- und genotypische Adaptionsmechanismen in *Staphylococcus aureus* Osteomyelitis"
- 2012-2014 Hannah F. Preugschas, Bachelor : "Labeling *S. aureus* with iron oxide nano particles with rhodamine"
- 2013 Marie Hachmeister, Master: "Studien zu Infektionsstrategien von *Staphylococcus aureus* in einem chronischen hämatogenen Osteomyelitismodell in der Maus"
- 2013-2016 Jennifer Geraci, pHD: " The role of adhesins and biofilm formation in chronic *Staphylococcus aureus* infections"
- 2015 Rebekka Salzmann, Bachelor: "Test of new potential antimicrobial drugs against S.aureus: BM-38"
- 2015-2018 Anke Siegmund, pHD: "Detecting and deciphering *Staphylococcus aureus* strategies that cause chronic and persistent infections" (Transregio)
- 2015-2018 Christine Pöllath, pHD: "*Staphylococcus aureus* pathogenesis: from

sepsis to hematogenous chronic bone infections” (Staphbone, CSCC)

- 2015-2016 Christian Fritzsch, Master: “Persistence of *Staphylococcus aureus* within osteocytes and osteoblasts”
- 2016-2019 Vandana Pradeep, pHD: “Metabolic cross talk between host and *S. aureus* during chronic osteomyelitis” (ILRS, microbial communications)
- 2016-2019 Marina García-Moreno, pHD: “*Staphylococcus aureus* pathogenesis: The role of different host cell types during the passage from sepsis to chronic osteomyelitis” (CSCC)

FELLOWSHIPS ,AWARDS and Grants

2000-2001	University of Buenos aires Research Fellowship
2001-2002	Carrillo-Oñativia Research Fellowship, Health Department, Argentina
2003-2006	University of Buenos Aires Doctoral Fellowship
2004	International Union for Microbiological Societies. To visit Dr. jean C. Lee's laboratory, The Chaning Laboratory, Brigham and Women's Hospital Harvard University
2007-2009	CONICET Internal Post-doctoral Fellowship
2007-2010	2007-2010 Skin staph Post doc position
2010-2012	2010-2012 Transregio/IZKF
2012-2024	SFB
2012	IMF
2016-2019	ILRS, Microbial Communications, HKI
2016-2019	CSCC, Center of sepsis, Jena

Jena, 22nd July , 2016

8- Complete list of publications:

The following list of publications is ordered chronologically. The corresponding author is indicated (*). The impact factor (IF) is from the year of publication. In all the publications from 2016 where the IF is still not available, the IF from 2015 was inidcated. The weighted impact factor (gIF) was calculated taking into account the impact factor of journal in the year of publication of the work and the average impact factor for present year (2016 or2015). Information on the average impact factor was found on the website of the isi web of science.

1: Goldmann O, **Tuchscherr L**, Rohde M, Medina E. α-Hemolysin enhances *Staphylococcus aureus* internalization and survival within mast cells by modulating the expression of β1 integrin. Cell Microbiol. 2016 Jun;18(6):807-19. (IF_{2014} 4.915; gIF_{2014} 1.50)

2: **Tuchscherr** L, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, Bremer-Streck S, Kiehntopf M, Medina E, Kribus M, Raschke M, Pletz M, Peters G, Löffler B. *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. J Antimicrob Chemother. 2016 Feb;71(2):438-48. (IF_{2014} 5.313; gIF_{2014} 2.06)

3: Tuchscherr L, Löffler B. *Staphylococcus aureus* dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection. Curr Genet. 2016 Feb;62(1):15-7. (IF_{2014} 6,723; gIF_{2014} 3.04)

4: Grüner M, **Tuchscherr** L, Löffler B, Gonnissen D, Riehemann K, Staniford MC, Kynast U, Strassert CA. Selective Inactivation of Resistant Gram-Positive Pathogens with a Light-Driven Hybrid Nanomaterial. ACS Appl Mater Interfaces. 2015 Sep 23;7(37):20965-71. (IF_{2014} 2.682; gIF_{2014} 1.09)

5: **Tuchscherr** L, Bischoff M, Lattar SM, Noto Llana M, Pförtner H, Niemann S, Geraci J, Van de Vyver H, Fraunholz MJ, Cheung AL, Herrmann M, Völker U, Sordelli DO, Peters G, Löffler B. Sigma Factor SigB Is Crucial to Mediate *Staphylococcus aureus* Adaptation during Chronic Infections. PLoS Pathog. 2015 Apr 29;11(4):e1004870. (IF_{2014} 7.562; gIF_{2014} 3.01)

6: Klinger-Strobel M, Lautenschläger C, Fischer D, Mainz JG, Bruns T, **Tuchscherr** L, Pletz MW, Makarewicz O. Aspects of pulmonary drug delivery strategies for infections in cystic fibrosis--where do we stand? Expert Opin Drug Deliv. 2015 Aug;12(8):1351-74. (IF_{2014} 4.84; gIF_{2014} 2.05)

7: Ring J, Hoerr V, **Tuchscherr** L, Kuhlmann MT, Löffler B, Faber C. MRI visualization of Staphyloccocus aureus-induced infective endocarditis in mice. PLoS One. 2014 Sep 17;9(9):e107179. (IF$_{2014}$ 3.234; gIF$_{2014}$ 4.41)

8: Kalinka J, Hachmeister M, Geraci J, Sordelli D, Hansen U, Niemann S, Oetermann S, Peters G, Löffler B, **Tuchscherr** L. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. Int J Med Microbiol. 2014 Nov;304(8):1038-49. (IF$_{2014}$ 3.614; gIF$_{2014}$ 1.44)

9: Löffler B, **Tuchscherr** L, Niemann S, Peters G. *Staphylococcus aureus* persistence in non-professional phagocytes. Int J Med Microbiol. 2014 Mar;304(2):170-6. (IF$_{2014}$ 3.614; gIF$_{2014}$ 1.44)

10: Kneidl J, Mysore V, Geraci J, **Tuchscherr** L, Löffler B, Holzinger D, Roth J, Barczyk-Kahlert K. Soluble CD163 masks fibronectin-binding protein A-mediated inflammatory activation of *Staphylococcus aureus* infected monocytes. Cell Microbiol. 2014 Mar;16(3):364-77. (IF$_{2014}$ 4.915; gIF$_{2014}$ 1.50)

11: Hoerr V, **Tuchscherr** L, Hüve J, Nippe N, Loser K, Glyvuk N, Tsytsyura Y, Holtkamp M, Sunderkötter C, Karst U, Klingauf J, Peters G, Löffler B, Faber C. Bacteria tracking by in vivo magnetic resonance imaging. BMC Biol. 2013 May 28;11:63. (IF$_{2013}$ 7.431; gIF$_{2013}$ 5.19)

12: Horst SA, Hoerr V, Beineke A, Kreis C, **Tuchscherr** L, Kalinka J, Lehne S, Schleicher I, Köhler G, Fuchs T, Raschke MJ, Rohde M, Peters G, Faber C, Löffler B, Medina E. A novel mouse model of *Staphylococcus aureus* chronic osteomyelitis that closely mimics the human infection: an integrated view of disease pathogenesis. Am J Pathol. 2012 Oct;181(4):1206-14. (IF$_{2012}$ 4.410; gIF$_{2012}$ 2.263)

13: Niemann S, Ehrhardt C, Medina E, Warnking K, **Tuchscherr** L, Heitmann V, Ludwig S, Peters G, Löffler B. Combined action of influenza virus and *Staphylococcus aureus* panton-valentine leukocidin provokes severe lung epithelium damage. J Infect Dis. 2012 Oct 1;206(7):1138-48. (IF$_{201}$ 5.848; gIF$_{2012}$ 2.02)

14: Lattar SM, **Tuchscherr** LP, Centrón D, Becker K, Predari SC, Buzzola FR, Robinson DA, Sordelli DO. Molecular fingerprinting of *Staphylococcus aureus* isolated from patients with osteomyelitis in Argentina and clonal distribution of the cap5(8) genes and of other selected virulence genes. Eur J Clin Microbiol Infect Dis. 2012 Oct;31(10):2559-66. (IF$_{2012}$ 3.024; gIF$_{2012}$ 1.26)

15: Barbagelata MS, Alvarez L, Gordiola M, **Tuchscherr** L, von Eiff C, Becker K, Sordelli D, Buzzola F. Auxotrophic mutant of *Staphylococcus aureus* interferes with nasal colonization by the wild type. Microbes Infect. 2011 Nov;13(12-13):1081-90. (IF_{2011} 3.101; gIF_{2011} 1.04)

16: **Tuchscherr** L, Medina E, Hussain M, Völker W, Heitmann V, Niemann S, Holzinger D, Roth J, Proctor RA, Becker K, Peters G, Löffler B. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med. 2011 Mar;3(3):129-41. (IF_{2011} 10.333; gIF_{2011} 4.64)

17: **Tuchscherr** L, Löffler B, Buzzola FR, Sordelli DO. *Staphylococcus aureus* adaptation to the host and persistence: role of loss of capsular polysaccharide expression. Future Microbiol. 2010 Dec;5(12):1823-32. (IF_{2010} 2.755; gIF_{2010} 1.16)

18: **Tuchscherr** L, Heitmann V, Hussain M, Viemann D, Roth J, von Eiff C, Peters G, Becker K, Löffler B. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis. 2010 Oct 1;202(7):1031-40. (IF_{2010} 5.961; gIF_{2010} 2.09)

19: Grundmeier M, **Tuchscherr** L, Brück M, Viemann D, Roth J, Willscher E, Becker K, Peters G, Löffler B. Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells. J Infect Dis. 2010 Mar 15;201(6):871-80. (IF_{2010} 5.961; gIF_{2010} 2.09)

20: Pardo L, Machado V, Mollerach M, Mota MI, **Tuchscherr** LP, Gadea P, Gardella N, Sordelli DO, Vola M, Schelotto F, Varela G. Characteristics of Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) Strains Isolated from Skin and Soft-Tissue Infections in Uruguay. Int J Microbiol. 2009;2009:472126. (IF_{2009} N/A; gIF_{2009} N/A)

21: Lattar SM, **Tuchscherr** LP, Caccuri RL, Centrón D, Becker K, Alonso CA, Barberis C, Miranda G, Buzzola FR, von Eiff C, Sordelli DO. Capsule expression and genotypic differences among *Staphylococcus aureus* isolates from patients with chronic or acute osteomyelitis. Infect Immun. 2009 May;77(5):1968-75. (IF_{2009} 4.205; gIF_{2009} 1.53)

22: **Tuchscherr** LP, Buzzola FR, Alvarez LP, Lee JC, Sordelli DO. Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. Infect Immun. 2008 Dec;76(12):5738-44. (IF_{2008} 3.451; gIF_{2008} 1.24)

23: **Tuchscherr** LP, Gomez MI, Buzzola FR, Calvinho LF, Lee JC, Sordelli DO. Characterization of a new variant of IS257 that has displaced the capsule genes within bovine isolates of *Staphylococcus aureus*. Infect Immun. 2007 Nov;75(11):5483-8. (IF_{2007} 3.484; gIF_{2007} 1.34)

24: Buzzola FR, Alvarez LP, **Tuchscherr** LP, Barbagelata MS, Lattar SM, Calvinho L, Sordelli DO. Differential abilities of capsulated and noncapsulated *Staphylococcus aureus* isolates from diverse agr groups to invade mammary epithelial cells. Infect Immun. 2007 Feb;75(2):886-91. (IF_{2007} 3.484; gIF_{2007} 1.34)

25: **Tuchscherr** LP, Buzzola FR, Alvarez LP, Caccuri RL, Lee JC, Sordelli DO. Capsule-negative *Staphylococcus aureus* induces chronic experimental mastitis in mice. Infect Immun. 2005 Dec;73(12):7932-7. (IF_{2005} 3.225; gIF_{2005} 1.40)

26: Villar HE, **Tuchscherr** L, Arena M, Longo L, Laurino G, Hoffman M. [Inhibitory and bactericidal activity and selection capacity of mutants resistant and tolerant to vancomycin in *Staphylococcus aureus* strains]. Enferm Infecc Microbiol Clin. 1999 Nov;17(9):454-7. (IF_{1999} N/A; gIF_{1999} N/A)

9- Oral presentations:

"*Staphylococcus aureus* long-term persistence and the relation with chronic infections" 3rd. Immunology meeting, Münster, 2012.

"How can we study staphylococcal infection?" IZKF Münster, Annual Meeting Gut Havichhorst, Münster , 2012

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11- Copies of original publications used for this work

The publications are ordered chronologically from the first to the last one. The impact factors are described on the section 8.