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Staphylococcus aureus isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation

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ABSTRACT

Osteomyelitis is a severe inflammatory disease of the bone that is mainly caused by *Staphylococcus aureus*. Particularly, bone infections are difficult to treat and can develop into a chronic course with a high relapsing rate despite of antimicrobial treatments. The complex interaction of staphylococci with osseous tissue and the bacterial ability to invade host cells are thought to determine the severity of infection. Yet, defined bacterial virulence factors responsible for the pathogenesis of osteomyelitis have not been clearly identified.

The aim of this study was to detect *S. aureus* virulence factors that are associated with osteomyelitis and contribute to a chronic course of infection. To this purpose, we collected 41 *S. aureus* isolates, each 11 from acute osteomyelitis (infection period less than 2 months), 10 from chronic osteomyelitis (infection period more than 12 months), 10 from sepsis and 10 from nasal colonization. All isolates were analyzed for gene expression and in functional in-vitro systems. Adhesion assays to bone matrix revealed that all isolates equally bound to matrix structures, but invasion assays in human osteoblasts showed a high invasive capacity of chronic osteomyelitis isolates. The high invasion rate could not be explained by defined adhesins, as all infecting strains expressed a multitude of adhesins that act together and determine the level of adhesion. Following host cell invasion isolates from chronic osteomyelitis induced less cytotoxicity than all other isolates and a higher percentage of Small-colony-variant (SCV)-formation, which represents an adaptation mechanism during long-term persistence. Isolates from acute and chronic osteomyelitis strongly produced biofilm and highly expressed *agr* and *sarA* that regulate secreted virulence factors and induced an inflammatory response in osteoblasts. In conclusion, chronic osteomyelitis isolates were characterized by a high host cell invasion rate, 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 tissue destruction and bone deformation observed as typical complications of long-lasting bone infections.

Introduction

Staphylococcus aureus is by far the most common isolated microorganism in different types of osteomyelitis, including hematogenous osteomyelitis, postsurgical and implant-related infections (Lew and Waldvogel, 2004). Clinically, all types of osteomyelitis can develop from an acute and highly inflammatory stage into a chronic and persisting disease. Particularly in the chronic stage osteomyelitis is extremely difficult to treat and usually requires a combination of antibiotics, bone debridement and reconstructive surgery that often results in bone deformation, tissue loss and persistent disability, e.g. limb amputations (Rao et al., 2011). Consequently, osteomyelitis is a severe and

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costly clinical problem that has a high burden on the patient's quality of life.

The reasons for tissue destructive and persistent infections are incompletely understood, but most likely originate from the complex interaction of staphylococci with bone tissue and the multitude of virulence factors expressed by *S. aureus* (Lowy, 1998; Wright and Nair, 2010). To initiate an infection *S. aureus* expresses various surface proteins with adhesive functions (adhesins) (Clarke and Foster, 2006). The group of adhesins can be divided into proteins that are covalently bound to bacterial cell wall peptidoglycans (microbial surface components recognizing adhesive matrix molecules, MSCRAMMs) (Foster and Hook, 1998; Patti et al., 1994; Sinha and Herrmann, 2005) and proteins that are only secreted but rebind to the bacterial cell surface (secretable expanded repertoire adhesive molecules, SERAMs) (Chavakis et al., 2005). Fibronectin binding proteins (FnBPs), clumping factor (Clf) and collagen binding protein (Cna) belong to the group of MSCRAMMs and their role in bacterial adhesion to host cells, to host matrix and in invasion of host cells is well established (Hauck and Ohlsen, 2006; Patti et al., 1994; Sinha et al., 1999). FnBPs consist of FnBPA and B. Both FnBPs are widely expressed by the majority of clinical isolates and play a major role in the process of host cell invasion (Sinha et al., 1999, 2000). Two clumping factors (Clf), ClfA and ClfB, have been identified that mainly bind fibrinogen, but binding to other matrix components, such as cytokeratin, is also possible (McDevitt et al., 1995; Walsh et al., 2004). Cna preferentially binds to collagen I and has therefore been implicated as a major virulence factor of osteomyelitis and septic arthritis (Patti et al., 1994), whereas a clear association of Cna and osteomyelitis could not be demonstrated and many osteomyelitis strains were found negative for Cna (Ryding et al., 1997; Thomas et al., 1999). The extracellular adhesion protein (Eap) and the extracellular matrix binding protein (Emp) are anchorless proteins and belong to the group of SERAMs (Harraghy et al., 2003; Hussain et al., 2001; Palma et al., 1999), but their functions in bacterial adhesion and osteomyelitis development are less clear.

Additionally, *S. aureus* expresses toxins and exoenzymes that can defend staphylococci against immune cells and destroy host tissue, which enable the pathogens to enter deep tissue structures (Gordon and Lowy, 2008; Lowy, 1998). The expression of cytotoxic and proinflammatory secreted virulence factors is mainly controlled by a complex regulatory network containing the *agr*-, *sarA*- and *sae*-systems (Cheung et al., 2004; Reyes et al., 2011), as experiments in different infection models with single or double knock-out mutants revealed less virulence (Blevins et al., 2003; Wesson et al., 1998). Only recently the *agr*-regulated phenol-soluble modulins (PSMs) (Wang et al., 2007) have been identified as important *S. aureus* cytotoxic factors for cultured osteoblasts that mediate bacterial phagosomal escape and could contribute the establishment of deep bone infections (Grosz et al., 2014; Rasigade et al., 2013). After initial settling of the infection the bacteria need to adapt to the host tissue for persistence and to escape from the host immune system. Thereby, it is important to recognize *S. aureus* as a potential intracellular pathogen (Garzoni and Kelley, 2009; Sendi and Proctor, 2009). *S. aureus* can invade different types of host cells (Sinha and Fraunholz, 2010), including osteoblasts (Ellington et al., 1999; Hudson et al., 1995; Shi and Zhang, 2012). Within the intracellular location the bacteria dynamically change their phenotypes to small-colony-variants (SCVs). SCVs adapt their metabolism and their virulence factor expression, which enable the bacteria to persist within the intracellular location at low numbers for long-time periods (Proctor et al., 2006; Tuchscherr et al., 2011). The intracellular location most likely represents a shelter against many antimicrobial treatments and against the host immune defense.

Consequently, various infection strategies can contribute to establish an acute or chronic bone infection. To identify important and responsible bacterial virulence strategies for osteomyelitis we investigated clinical isolates from colonization, sepsis, acute and chronic osteomyelitis and characterized them for their virulence by gene expression and in-vitro assays. A better knowledge of the virulence patterns of acute and chronic osteomyelitis strains is the prerequisite to develop novel preventive (e.g., vaccination), diagnostic and therapeutic (e.g., novel antibiotics) strategies.

Material and methods

Bacterial isolates

S. aureus isolates were obtained from 41 patients from seven different hospitals in Argentina. The isolates were divided into four clinical categories: nasal colonization ($n = 10$), sepsis ($n = 10$), acute ($n = 11$) and chronic ($n = 10$) osteomyelitis. The acute and chronic osteomyelitis isolates utilized in this study were drawn from a previously characterized collection (Lattar et al., 2012). Those from acute osteomyelitis were obtained from patients with a less than 2-month infection course whereas those from chronic osteomyelitis were obtained from patients with an infection course exceeding 12 months. Additionally, we used the *S. aureus* laboratory strains Cowan I (ATCC 12598) and Newman as well as the clinical isolate 6850 (Balwit et al., 1994; Duthie and Lorenz, 1952). All staphylococci were analyzed for their growth in brain–heart-infusion (BHI) and in cell culture invasion medium showing faster growth in nutrition rich BHI, but there were always similar growth kinetics between the different isolates (Supp Fig. 1). Hemolysis was tested on sheep blood-agar plates, which was recorded semi-quantitatively. Furthermore, *spA*-typing (Mellmann et al., 2008) and susceptibility to methicillin was determined by using VITEK-2 automated systems (BioMérieux, Marcy l'Etoile, France) (Table 1).

Isolation and cultivation of human osteoblasts

Primary cultures of human osteoblasts (pHOB) were generated from normal trabecular bone specimens as described before (Robey and Termine, 1985) with some modifications. Briefly, the bone material was isolated using a sharp spoon. The resulting bone chips were washed carefully with phosphate-buffered saline (PBS) and treated with Trypsin/EDTA (PAA Laboratories GmbH) for 30 min at 37 °C. Subsequently, the chips were seeded in MEM Alpha Modification (PAA Laboratories GmbH) supplemented with 10% FCS, penicillin/streptomycin (PAA Laboratories GmbH), 0.2 mM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate disodium salt hydrate and 10 nM dexamethasone (Sigma-Aldrich Co. LLC). The expression of alkaline phosphatase, and osteocalcin were analysed by real time as specific marker for mature osteoblasts to detect the differentiation into osteoblasts (Disthabanchong et al., 2007).

Flow cytometric invasion assay

Osteoblasts were plated at 6×10^5 cells/well in 12-well plates two days before the assay. Cells were washed with PBS, and then 1 ml of 1% human serum albumin (HSA), 10 mmol/l HEPES (pH 7.4) in medium MEM Alpha modification (PAA), and 100 μl of a formalin (2%)-fixed fluorescein isothiocyanate-labeled bacteria suspension (optical density, 1) was prepared as described elsewhere (Sinha et al., 1999) and added to the cells. Culture dishes were preincubated for 15 min at room temperature to allow sedimentation of bacteria and were then shifted to 37 °C for 3 h. Cells were analyzed by flow cytometry as described elsewhere (Juuti et al., 2004). The invasiveness was expressed in relation to the laboratory strain Cowan I.

Table 1
Description of all the strains used in this study.

Colonization (nasal swabs); *N* = 10				Sepsis (blood culture); *N* = 10			
Isolate	**Spa type**	**Susceptibility**	**Hemolysis**	**Isolate**	**Spa type**	**Susceptibility**	**Hemolysis**
NA-1	t2164	MSSA	3	**HE-1**	t037	MRSA	2
NA-2	t5211	MSSA	0	**HE-6**	t149	MRSA	3
NA-5	t349	MSSA	3	**HE-10**	t149	MRSA	3
NA-10	t021	MSSA	2	**HE-16**	t149	MRSA	3
NA-11	t021	MSSA	1	**HE-20**	t4053	MSSA	1
NA-15	t645	MSSA	1	**HE-33**	t149	MRSA	0
NA-23	t2734	MSSA	3	**HE-48**	t084	MSSA	1
NA-26	t1654	MSSA	2	**HE-55**	t12362	MSSA	0
NA-52	t149	MRSA	0	**HE-60**	t189	MSSA	0
NA-57	t10307	MSSA	0	**HE-64**	t149	MRSA	0
Acute osteomyelitis (bone); *N* = 11				**Chronic osteomyelitis (bone); *N* = 10**			
Isolate	**Spa type**	**Susceptibility**	**Hemolysis**	**Isolate**	**Spa type**	**Susceptibility**	**Hemolysis**
aHU-10	t127	MSSA	1	**cHU-2**	t002	MRSA	2
aHU-36	t622	MSSA	3	**cHU-6**	t067	MRSA	3
aHU-64	t1987	MSSA	3	**cHU-20**	t359	MSSA	3
aHU-67	t122	MSSA	0	**cHU-44**	t024	MSSA	2
aHU-69	t267	MRSA	2	**cHU-51**	t311	MSSA	3
aHU-75	t002	MRSA	2	**cHU-52**	t267	MSSA	2
aHU-78	t024	MSSA	2	**cHU-70**	t267	MRSA	3
aHU-81	t359	MSSA	3	**cHU-71**	t166	MSSA	2
aHU-82	t359	MSSA	3	**cHU-74**	t359	MSSA	2
aHU-91	t149	MRSA	0	**cHU-85f**	t189	MSSA	0
aHU-109	t148	MRSA	3				

All strains were characterized for their Spa type, methicillin resistance and hemolysis. Hemolytic activity (mainly due to α-toxin expression) was determined on sheep blood agar plates after 24 h and is listed semi-quantitatively in four categories: 0, no hemolysis; 1, borderline; 2, effective; 3, very effective hemolysis.

Preparation of matrix from human bone tissue

Fragments of suprastructural aggregates from bone were extracted from human hips recovered after joint replacement. Briefly, the hips were carefully cleaned from adhering connective tissue and bone marrow followed by a demineralization step using sodium-EDTA (20 g/l, pH 7.4) for four weeks changing the demineralising buffer every 3 days. After demineralization, hips were rinsed in PBS and the remaining bone was mechanically disrupted into small pieces and homogenized using a Polytron. The obtained homogenates were clarified by centrifugation and used for adhesion assays.

Adhesion assay

Microtiter plates were coated with 5 μg/ml of ECM solution isolated from bone tissue. After washing, the plates were incubated with protein-free blocking buffer (Thermo Fisher Scientific Inc.) at 37 °C. Bacteria, grown for 2 h in BHI, were added and incubated for 3 h at 37 °C. Wells were washed and bound staphylococci were fixed with ice-cold methanol. Adherent bacteria were detected with primary antibodies to *S. aureus* (rabbit; 1:5000; Abcam, ab20920) and secondary antibodies (anti-rabbit IgG; 1:2000) conjugated to alkaline phosphatase applying chemiluminescence after adding p-nitrophenyl phosphate (0.1 M Tris, 1 mM $MgCl_2$, pH = 9.5). Finally, the absorbance was measured at 405 nm with an ELISA plate reader (Bio-Rad Inc.). The adherence capacity of *S. aureus* 6850 was set as 1.0.

Electron microscopy

For electron microscopy of infected extracellular matrix aliquots of bone extracts were absorbed to nickel grids coated with Formvar/carbon. The grids were subsequently washed with PBS and treated with 2% BSA in PBS. *S. aureus* was added and was incubated for 3 h at 37 °C in a humid chamber. After washing with PBS, samples were fixed with 2% formaldehyde for 30 min. Finally, the grids were washed with water and negatively stained with 2% uranyl acetate for 10 min. Electron micrographs were taken at 60 kV with a Philips EM 410 electron microscope.

For electron microscopy of cells primary isolated and cultivated bone cells were infected with live bacteria of *S. aureus* 6850 at an MOI of 50 as described for the lysostaphin protection assay. After 3 h of host cell invasion and lysostaphin treatment infected host cells were fixed in 2% (v/v) formaldehyde and 2.5% (v/v) glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, at 4 °C. After washing in PBS, cells were postfixed in 0.5% (v/v) osmiumtetroxide and 1% (w/v) potassium hexacyanoferrate (III) in 0.1 M cacodylate buffer for 2 h at 4 °C followed by washing with distilled water. After dehydration in an ascending ethanol series from 30% to 100% ethanol, cells were two times incubated in propylenoxide each for 15 min. During the propylenoxide treatment, cells were detached from the surface of the cell culture plates, and finally, embedded in Epon using beem capsules. Ultrathin sections were cut on an ultramicrotome, collected on copper grids and stained with 2% uranyl acetate. Electron micrographs were taken at 60 kV with a Phillips EM 410 electron microscope.

PCR assays

S. aureus isolates were grown on blood agar at 37 °C overnight. Subsequently, several colonies were harvested and resuspended in Tris-EDTA buffer containing 200 μg/ml Lysostaphin (Sigma-Aldrich Co. LLC). After cell lysis, genomic DNA was extracted using QIAamp DNA Minikit (Qiagen GmbH) according to manufacturer's instructions, and DNA concentration was determined by NanoPhotometer P330 (Implen GmbH). Primers and conditions used to amplify these genes are described in Table 2. PCR amplifications were performed in a thermal cycler (ICycler, Bio-Rad Laboratories). Genomic DNA was added to PCR mix containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphates, 2 pM of each, forward and reverse primers, and Taq polymerase (Segenetic). PCR products were analyzed by 2% agarose gel electrophoresis.

Table 2
PCR primers and conditions used in PCR/RT-PCR assays.

Gene name[a]	Primer description	Primer sequence	Product length	Type of PCR/ positive control	Reference
bbp[b]	Forward	5′-AACTACATCTAGTACTCAACAACAG-3′	575 nt	PCR/Cowan I	Tristan et al. (2003)
	Reverse	5′-ATGTGCTTGAATAACACCATCATCT-3′			
clfA[b]	Forward	5′-ATTGGCGTGGCTTCAGTGCT-3′	292 nt	PCR/Newman	Tristan et al. (2003)
	Reverse	5′-CGTTTCTTCCGTAGTTGCATTTG-3′			
clfB	Forward	5′-GCTGCAAAAATGCAAGATCA-3′	343 nt	PCR/Newman	This paper
	Reverse	5′-TTGCCGCCATAAATGTGTTA-3′			
cna[b]	Forward	5′-AAAGCGTTGCCTAGTGGAGA-3′	192 nt	PCR/6850	This paper
	Reverse	5′-AGTGCCTTCCCAAACCTTTT-3′			
eap [c]	Forward	5′-AGTCATTGATTACAACAA-3′	206 nt	PCR/Newman	Joost et al. (2009)
	Reverse	5′-CTTATTAAATGTTAAGCTTG-3′			
efb	Forward	5′-CGAAGGATACGGTCCAAGAG-3′	217 nt	PCR/Newman	This paper
	Reverse	5′-ATCAGTTTTCGCTGCTGGTT-3′			
emp	Forward	5′-GCATCAGTGACAGAGAGTGTTGACAAA-3′	948 nt	PCR/Newman	This paper
	Reverse	5′-TTATACTCGTGGTGCTGGTAAGCTACC-3′			
fnbA	Forward	5′-CTCCATTGCTTTGTACAGTTTTAG-3′	1151 nt	PCR/Newman	This paper
	Reverse	5′-GTGTGACAAGGGTTTATGATGAC-3′			
fnbB[b]	Forward	5′-TAAATCAGAGCCGCCAGTGGAG-3′	416 nt	PCR/Newman	This paper
	Reverse	5′-GTCCTTGCGCTTGACCATGTTC-3′			
isdA	Forward	5′-CTGCGTCAGCTAATGTAGGA-3′	332 nt	PCR/Newman	Verkaik et al. (2010)
	Reverse	5′-TGGCTCTTCAGAGAAGTCAC-3′			
sdrC	Forward	5′-CGCATGGCAGTGAATACTGTTGCAGC-3′	725 nt	PCR/Newman	Campbell et al. (2008)
	Reverse	5′-GAAGTATCAGGGGTGAAACTATCCACAAATTG-3′			
sdrD[c]	Forward	5′-CCACTGGAAATAAAGTTGAAGTTTCAACTGCC-3′	514 nt	PCR/Newman	Campbell et al. (2008)
	Reverse	5′-CCTGATTTAACTTTGTCATCAACTGTAATTTGTG-3′			
sdrE	Forward	5′-CAGTAAATGTGTCAAAAGA-3′	767 nt	PCR/Newman	Peacock et al. (2000)
	Reverse	5′-TTGACTACCAGCTATATC-3′			
agrA	Forward	5′-AACTGCACATACACGCTTACA-3′	145 nt	RT-PCR/Newman	Tuchscherr et al. (2011)
	Reverse	5′-GGCAATGAGTCTGTGAGATTT-3′			
aroE	Forward	5′-CTATCCACTTGCCATCTTTTAT-3′	123 nt	RT-PCR/Newman	Tuchscherr et al. (2011)
	Reverse	5′-ATGGCTTTAATATCACAATTCC-3′			
aur	Forward	5′-ACCGTGTGTTAATTCGTGTGCTA-3′	65 nt	RT-PCR/Newman	Gustafson and Oscarsson (2008)
	Reverse	5′-ATGGTCGCACATTCACAAGTTT-3′			
clfA	Forward	5′-GAATCAGCTCCACAGAGTACAG-3′	81 nt	RT-PCR/Newman	This paper
	Reverse	5′-TCTCATTCTAGGCGCACTTG-3′			
clfB	Forward	5′-ACAAGCAAACGGTATTTGTT-3′	102 nt	RT-PCR/Newman	This paper
	Reverse	5′-CTTTACCGCTACTTTCTTCG-3′			
eap	Forward	5′-AGTCATTGATTACAACAA-3′	206 nt	RT-PCR/Newman	Joost et al. (2009)
	Reverse	5′-CTTATTAAATGTTAAGCTTG-3′			
emp	Forward	5′-CAGAATCGCCTAGATATACACATCCA-3′	103 nt	RT-PCR/Newman	This paper
	Reverse	5′-GCATGCCCTGGTGTAACAAAATT-3′			
fnbA	Forward	5′-ACAAGTTGAAGTGGCACAGCC-3′	74 nt	RT-PCR/Newman	Tuchscherr et al. (2011)
	Reverse	5′-CCGCTACATCTGCTGATCTTGTC-3′			
fnbB	Forward	5′-CACCGAAAACTGTGCAAGCA-3′	118 nt	RT-PCR/Newman	Vaudaux et al. (2002)
	Reverse	5′-TTCCTGTAGTTTCCTTATCAGCAACTT-3′			
gyrB	Forward	5′-AATTGAAGCAGGCTATGTGT-3′	138 nt	RT-PCR/Newman	Tuchscherr et al. (2011)
	Reverse	5′-ATAGACCATTTTGGTGTTGG-3′			
psmα	Forward	5′-TATCAAAAGCTTAATCGAACAATTC-3′	176 nt	RT-PCR/Newman	Li et al. (2010)
	Reverse	5′-CCCCTTCAAATAAGATGTTCATATC-3′			
saeR	Forward	5′-ATTTACGCCTTAACTTTAGGTG-3′	120 nt	RT-PCR/Newman	This paper
	Reverse	5′-AACTGGTTGATGATGGTATTT-3′			
sarA	Forward	5′-ACATGGCAATTACAAAAATCAATGAT-3′	151 nt	RT-PCR/Newman	Vaudaux et al. (2002)
	Reverse	5′-TCTTTCTCTTTGTTTTCGCTGATG-3′			
sdrE	Forward	5′-CTTTGCAGTTGCACAACCAG-3′	87 nt	RT-PCR/Newman	This paper
	Reverse	5′-TCGCCAACTTTGATTGTTTG-3′			
sigB	Forward	5′-ATGTACGTTTATTGAAGGATTG-3′	103 nt	RT-PCR/Newman	Renzoni et al. (2004)
	Reverse	5′-TAATTTCTTAATTGCCGTTCTC-3′′			
spA	Forward	5′-CAGATAACAAATTAGCTGATAAAAACAT-3′	174 nt	RT-PCR/Newman	Roberts et al. (2006)
	Reverse	5′-CTAAGGCTAATGATAATCCACCAAATAC-3′			

[a] Except noted otherwise, conditions for PCR run were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; 72 °C for 10 min; final hold at 12 °C.
[b] Conditions for PCR run were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; 72 °C for 10 min; final hold at 12 °C.
[c] Conditions for PCR run were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; 72 °C for 10 min; final hold at 12 °C.

Real time PCR

The bacterial RNA was extracted using the RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's instructions and to the suggestions of the protocol described by Garzoni et al. (2007). Subsequently, the cDNA was obtained using the QuantiTect reverse transcription kit (Qiagen GmbH). For the RT-PCR we used the iQ™SYBR®Green Supermix (Bio-Rad Inc.). The reaction mixtures were incubated for 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C using the C1000 Thermal Cycler (Bio-Rad Inc.). PCR efficiencies, melting-curve analysis and expression rates were calculated with the CFX Manager Software (Bio-Rad Inc.). In order to analyze the expression of the virulence factors, genes for the shikimate 5 dehydrogenase (*aroE*) and gyrase B (*gyrB*)

were taken as housekeeping genes. All data were normalized to these two genes. Utilized primers are listed in Table 2.

Measurement of protein expression and cell death induction

For measurement of cytokine release and cell death, primary osteoblasts were seeded in 12-well plates and were stimulated with live staphylococci. After the lysostaphin step, cells were incubated with new culture medium, and after 24 h culture supernatants were collected and the lysostaphin step was repeated before adding new culture medium. Conditioned media were centrifuged to remove cells and cellular debris, and samples were frozen at −20 °C until measurement of chemokine levels. The levels of the chemokines RANTES in the cell culture supernatants were measured by ELISA following the manufacturer's instructions (Biosource). Remaining cells were used to measure cell death by propidium iodide (PI) staining and flow cytometry, as described elsewhere (Löffler et al., 2010).

Lysostaphin protection assay to analyse intracellular bacteria

Internalization and intracellular replication of live bacteria was determined as described elsewhere (Tuchscherr et al., 2011). Briefly, cells were plated at a concentration of 1×10^5 cells in 25 cm^2 cell bottles and were stimulated with live bacteria with an MOI (multiplicity of infection) of 50. After a 3 h invasion period, the cells were washed and lysostaphin (20 μg/ml) was added for 30 min in order to lyse all extracellular or adherent staphylococci. Then, the cells were supplemented with fresh culture medium. The washing, the lysostaphin step, and medium exchange were repeated daily to remove all extracellular staphylococci which might have been released from the infected cells. The quality of the lysostaphin-step to eliminate all extracellular and adherent bacteria was controlled in additional experiments (Supp Fig. 2). To detect live intracellular bacteria host cells were lysed in 3 ml H_2O (for 25 cm^2 bottles) after different time periods (0, 1, 2, 4, and 7 days post infection). To determine the number of colony forming units (CFU), serial dilutions of the cell lysates were plated on Blood agar plates and were incubated overnight at 37 °C.

Biofilm assay

For quantitation of the biofilm-forming capacity, a test similar to those described previously was used (O'Toole and Kolter, 1998) with some modifications. Briefly, *S. aureus* strains were cultivated overnight in TSB supplemented with 0.25% glucose. The culture was diluted 1:200 in TSB-glucose with 0.25% glucose, and 200 μl of this cell suspension was used to inoculate sterile 96 well polystyrene microtiterplates (Greiner, Germany). After cultivation for 7 days at 37 °C, the wells were gently washed with 200 μl of sterile PBS. The plates were air-dried, and the remaining surface-adsorbed cells of the individual wells were stained with 1% crystal violet (100 μl/well) for 15 min. Then, 100 μl EtOH/Aceton (80:20) was added. Absorbance was measured with a Biorad iMark Microplate reader (Biorad) at 655 nm. As controls, we used *S. carnosus* TM300 (negative control) and *S. epidermidis* RP62A (positive control).

Statistics

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA, www.graphpad.com). Categorical distributions were compared using *t* test analysis and Fisher's exact test. Furthermore, we used Mann–Whitney *U* test to compare variables between two groups and two-way-ANOVA for complex studies.

Ethical statements

The isolation of human cells and preparation of matrix from human bone tissue were approved by the local ethics committee (Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Medizinischen Wilhelms-Universität Münster). For our study, written informed consent was obtained (Az. 2010-155-f-S).

Results

Isolates from chronic osteomyelitis are highly invasive in osteoblasts

Bacterial adherence to host structures is an initial step in infection development. To analyze the adhesive capacity of the clinical isolates we performed an adhesion assay to extracellular matrix prepared from human bone tissue which is preserved in its authentic three-dimensional composition and thereby provides many binding epitopes for adhesins. In this assay the adherence of strain 6850 was set as 1. All isolates from colonization, sepsis, acute and chronic osteomyelitis showed similar adhesion to matrix structures (Fig. 1A). These results were confirmed by electron microscopy. Representative isolates from acute and chronic osteomyelitis bound to collagen fibrils (Fig. 1B and F) as well as to other matrix components (Fig. 1D) in bacterial clusters (Fig. 1F) and as single bacteria (Fig. 1C and E). *S. aureus* does not only adhere to matrix structures, but also to host cells, which is followed by bacterial uptake. To investigate the host cell invasion of the different isolates, we performed a flow cytometric invasion assay using formalin-fixed FITC-labeled staphylococci and determined the relative invasiveness of all isolates in relation to the invasiveness of the laboratory strain Cowan I (Fig. 2). A comparison between all groups revealed that isolates from chronic osteomyelitis were significantly more invasive than isolates from colonization or sepsis, whereas the invasiveness of isolates from acute osteomyelitis was not significantly enhanced. Furthermore, the group of chronic osteomyelitis did not include isolates with an invasion rate lower than 0.7. By contrast, all other groups contained isolates with very low invasiveness (0.03, 0.013 and 0.13 respectively, Fig. 2A). The bacterial location within osteoblasts was demonstrated by electron microscopy (Fig. 2B).

Isolates from osteomyelitis are associated with fnbB and highly express fnbA and emp

Adherence to host cells and host cell invasion is determined by the expression of a multitude of adhesins that have redundant functions. The presence of adhesins can largely vary among clinical isolates (Dreisbach et al., 2010; Sibbald et al., 2006). To reveal an association between osteomyelitis isolates and defined adhesins, we determined the presence of different genes coding for adhesins (*bbp*, *clfA*, *clfB*, *cna*, *eap*, *efb*, *emp*, *fnbA*, *fnbB*, *isdA*, *sdrC*, *sdrD*, *sdrE*) by PCR. We found several adhesins in all isolates from colonization, sepsis, acute and chronic osteomyelitis (*clfA*, *clfB*, *eap*, *efb*, *emp*, *fnbA*, *isdA*, *sdrC*), whereas the presence of some adhesins varied between the isolates (*bbp*, *cna*, *fnbB*, *sdrC*, *and sdrE*) listed in Table 3. To evaluate whether the presence of certain genes correlates with osteomyelitis development, we compared all osteomyelitis isolates with isolates derived from colonization and sepsis. We found a significantly higher prevalence of *fnbB* in the osteomyelitis isolates, suggesting a role for this adhesin in the interaction with bone tissue (Table 3).

As the relevance of genes is largely determined by their levels of expression, we additionally performed real-time PCR experiments.

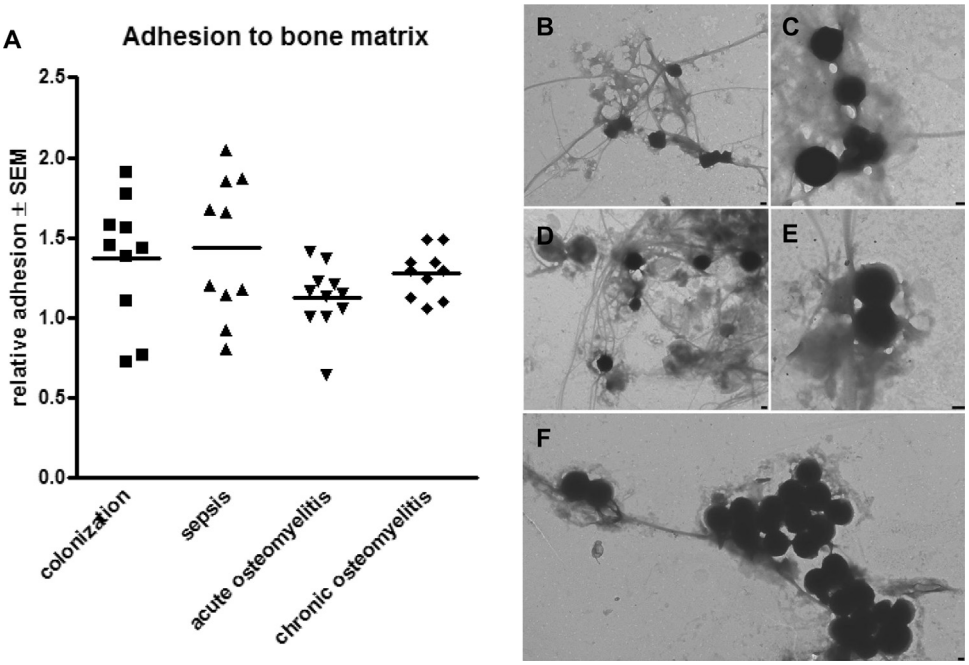

Fig. 1. Adherence of *S. aureus* isolates to extracellular bone matrix. (A) The adhesive capacity of the 41 *S. aureus* isolates was quantified by ELISA. All results were determined relative to *S. aureus* 6850 (adherence was set to 1.0) and data shown are the mean ± SEM of three independent experiments in six fold, showing no significant differences between the groups (Mann–Whitney *U* test). (B–F) Transmission electron microscopy was performed with two representative strains from acute and chronic osteomyelitis (HU-91 for B–D and HU 71 for E and F), respectively. Both strains bound to collagen fibrils (A, E) as well as to other matrix components (C) in bacterial clusters (E) and as single bacteria (B, D). Bars: 200 nm.

Here we found that all infecting strains (derived from sepsis and osteomyelitis) showed higher expression of *fnbA* and *emp* than the colonizing isolates (Fig. 3). The expression of all other adhesins (*clfA*, *clfB*, *fnbB*, *sdrE* and *spA*) did not reveal any significant elevations in the groups of osteomyelitis isolates compared to isolates from sepsis or colonization (data not shown).

Isolates from chronic osteomyelitis are less cytotoxic after infection of osteoblasts, but nevertheless induce an inflammatory response

Host cell invasion can be followed by different courses of infection. The intracellular release of bacterial cytotoxic factors can

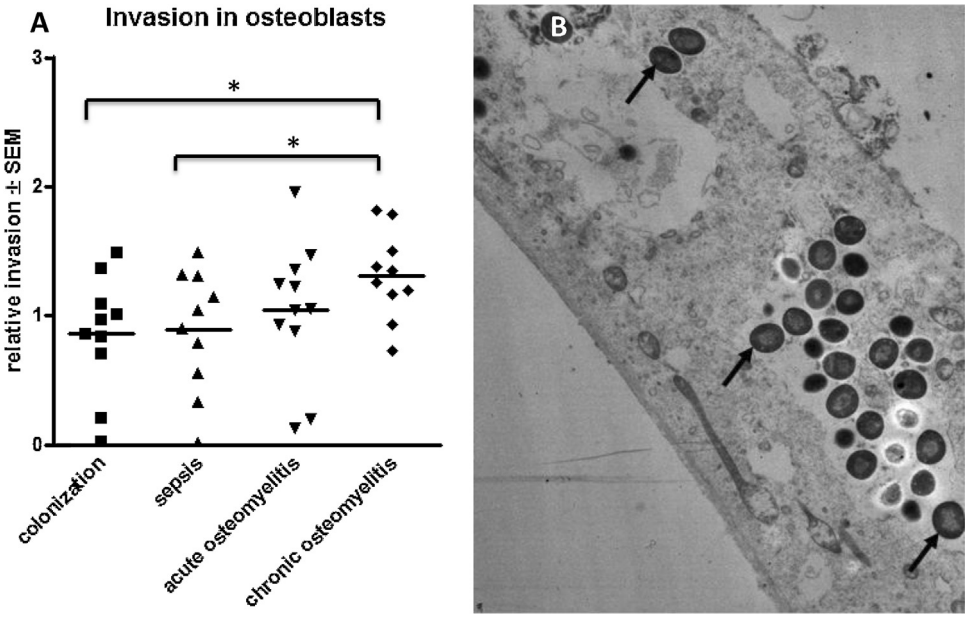

Fig. 2. Invasion of *S. aureus* isolates in human osteoblasts. (A) The capacity of *S. aureus* strains to invade human osteoblasts was analyzed by flow cytometry. Cowan I was used as reference strain (invasion rate was set to 1.0). The results represent means ± SEM of three independent experiments in duplicate and demonstrate that isolates from chronic osteomyelitis showed a significant increased invasion rate in comparison with isolates of the others groups. * $p \leq 0.05$ (Mann–Whitney *U* test). (B) Additionally, the bacterial localization of *S. aureus* 6850 within human osteoblasts was determined by electron microscopy. *S. aureus* was internalized by human osteoblasts and morphologically intact bacteria were detected intracellularly (arrows).

Table 3
Prevalence of adhesins among the clinical *S. aureus* isolates.

Number (percentage in %) of positive isolates					Statistic analysis
Gene	**Colonization ($n=10$)**	**Sepsis ($n=10$)**	**Acute osteomyelitis ($n=11$)**	**Chronic osteomyelitis ($n=10$)**	**Fisher's exact test**
bbp	4(40.0)	1(10.0)	1(9.09)	0(0.00)	0.0931
cna	6(60.0)	4(40.0)	3(27.3)	1(10.0)	0.0516
fnbB	4(40.0)	4(40.0)	9(81.8)	10(100)	0.0009
sdrD	9(90.0)	10(100)	11(100)	10(100)	0.4878
sdrE	5(50.0)	9(90.0)	9(81.8)	10(100)	0.13

The Fisher's exact test was used to compare non osteomyelitis isolates (colonization and sepsis) versus osteomyelitis isolates (acute and chronic).

severely damage the infected host cells, whereas downregulation of cytotoxic components allows for silent persistence in morphologically intact host cells (Grundmeier et al., 2010). To evaluate post-invasion events, we measured cytotoxicity after infection with the different groups of clinical isolates in osteoblasts. Twenty four hours post infection we determined the rate of dead host cells using a PI flow cytometric assay. We found that clinical isolates derived from chronic osteomyelitis were less cytotoxic than isolates from acute osteomyelitis or colonizing strains (Fig. 4A), although chronic osteomyelitis isolates invaded osteoblasts in higher numbers (Fig. 2A). Consequently, isolates from chronic osteomyelitis better preserved their host cells after invasion. These findings are reflected by the integrity of the cultured cell layers. After infection with isolates from acute osteomyelitis, many cells were detached (Fig. 4C), whereas infection with isolates from chronic osteomyelitis usually resulted in less cell damage leaving an almost intact cell layer, as in control cells (Fig. 4B and D). The infection of osteoblasts with *S. aureus* led to an inflammatory reaction of the host cells that was measured by the chemokine RANTES release after 24 h post infection (Fig. 4E). The osteoblast infected with isolates from osteomyelitis had an increased release compared to the osteoblasts infected with isolates from colonization.

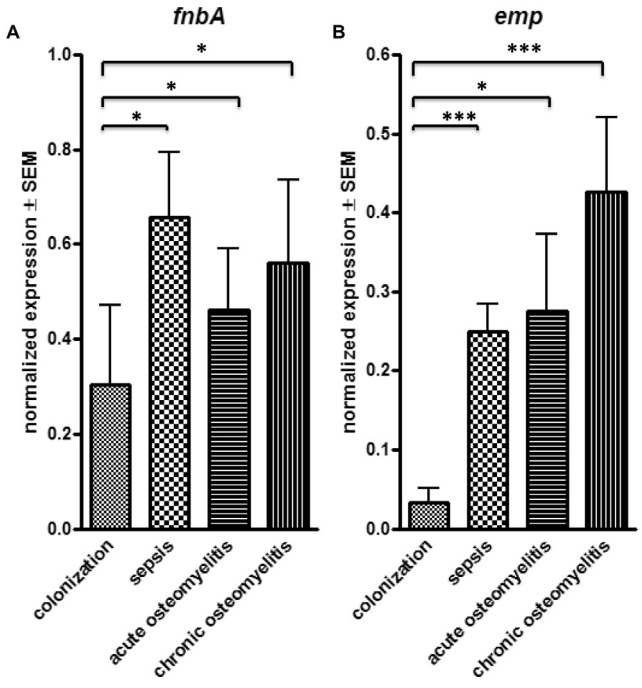

Fig. 3. Expression rate of *S. aureus* isolates for the adhesins *fnbA* and *emp*. All isolates were cultivated in BHI for 2 h (for *fnbA*, A) and 6 h (for *emp*, B), respectively. The isolated RNA was transcribed into cDNA to perform RT-PCR. *aroE* and *gyrB* were used as reference genes. The results represent means ± SEM of three independent experiments in triplicate and demonstrated an increased expression of both adhesins in the groups of all infecting isolates in comparison with colonizing strains. * $p \leq 0.05$, *** $p \leq 0.001$ (Mann–Whitney *U* test).

It is well known that *S. aureus* virulence factors that induce an inflammatory and cytotoxic reaction in the host are largely regulated by a network, involving *agr*, *sarA* and *sae* (Cheung et al., 2004). Consequently, we measured the expression of these regulatory factors in our collection of strains. We found elevated expression of *agr* and *sarA* in isolates not only from acute but also from chronic osteomyelitis (Fig. 5A and B). The upregulation of these regulatory factors was in parallel to an increased inflammatory response that could be measured in osteoblasts after infection (Fig. 4E). The expression of *sae* was not elevated in the isolates from acute and chronic osteomyelitis compared to isolates from colonization (Fig. 5C). The expression of PSMalpha was not found different in the groups of isolates (data not shown). Further on, we found elevated expression of the protease aureolysin (*aur*) only in isolates from chronic osteomyelitis (Fig. 5D). Taken together, isolates from acute and chronic osteomyelitis both induced a high inflammatory response in osteoblasts, although isolates from chronic osteomyelitis are less cytotoxic and release more proteases than isolates from acute osteomyelitis.

Isolates from acute and chronic osteomyelitis strongly produce biofilm and persist within osteoblasts, but isolates from chronic osteomyelitis form a higher percentage of SCVs

Recent research associated biofilm production and intracellular persistence with chronic infections, such as osteomyelitis (Brady et al., 2008; Sendi and Proctor, 2009). Hence, we determined the capacity of all isolates to produce biofilm in an in-vitro assay. We found that isolates from acute and chronic osteomyelitis formed higher levels of biofilm than isolates from colonization and sepsis (Fig. 6A), suggesting that biofilm might play a role in osteomyelitis development.

Bacterial host cell invasion can be followed by intracellular long-term persistence. During persistence the bacteria need to adapt their metabolism and their virulence factor expression to the intracellular location that is associated with SCV formation (Tuchscherr et al., 2010, 2011). To investigate whether isolates from chronic osteomyelitis can better adapt to the intracellular location and can better persist than isolates from acute osteomyelitis, we performed long-term infection experiments. We infected cultured osteoblasts with the clinical isolates from acute or chronic osteomyelitis, respectively, and analyzed the infected host cells for up to 7 days. During the infection course the amounts of intracellular bacteria were decreased in all cases, but all isolates were able to persist at low bacterial numbers inside osteoblasts (Fig. 6B). Although we observed a tendency of the chronic isolates to persist in higher bacterial numbers than the acute isolates, the differences were not significant. We further evaluated the intracellular development of SCVs, which is a sign for adaptation (Fig. 6C). Here, we observed a significant higher percentage of SCVs with the chronic isolates after 7 days of intracellular persistence.

Taken together, all osteomyelitis isolates strongly formed biofilm and were able to persist in osteoblasts for several days, but

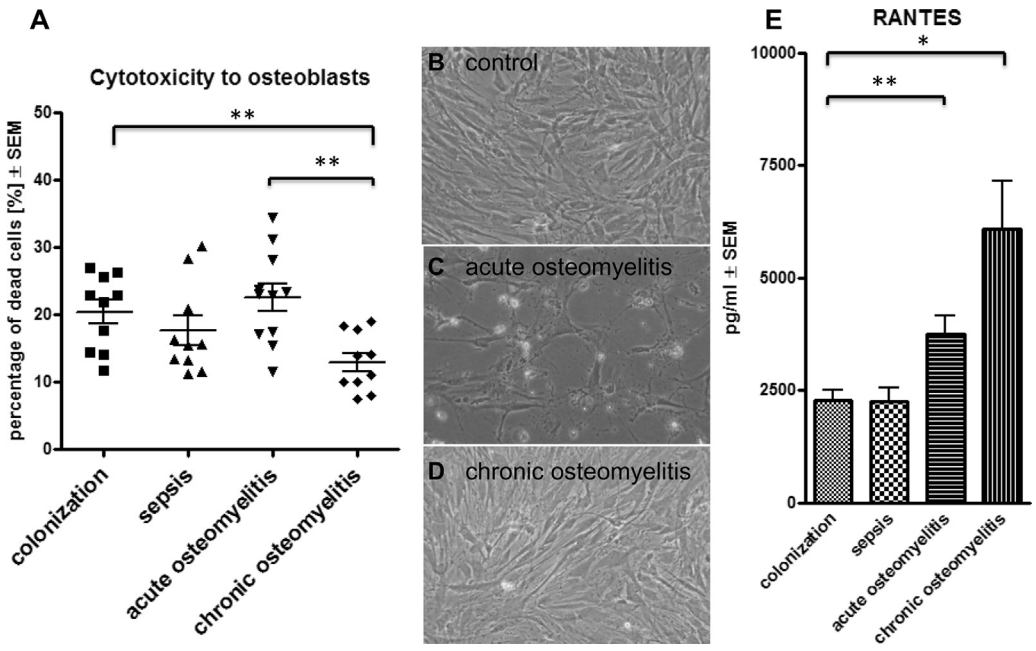

Fig. 4. Cytotoxic and proinflammatory effects of *S. aureus* isolates in human osteoblasts. (A) The cytotoxicity of *S. aureus* isolates on human osteoblasts was tested by a PI cytotoxicity assay 24 h post infection. The results represent means ± SEM of three independent experiments in duplicate and show that the group of isolates from chronic osteomyelitis is less cytotoxic than the groups of isolates from acute osteomyelitis and from colonization. ** $p \leq 0.01$ (Mann–Whitney *U* test). Microscopic photographs were taken from (B) uninfected osteoblasts, (C) osteoblasts infected with the strain (HU-69) from acute osteomyelitis and (D) osteoblasts infected with the strain (HU-20) from chronic osteomyelitis. (E) 24 h post infection the chemokine RANTES was measured in the supernatants of infected osteoblasts by ELISA. The results represent means ± SEM of three independent experiments in duplicate and demonstrate an increased chemokine release by osteoblasts infected with isolates from osteomyelitis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann–Whitney *U* test).

the isolates from chronic osteomyelitis showed a higher tendency to adapt by developing SCV phenotypes.

Discussion

S. aureus osteomyelitis is a severe inflammatory disease of bone tissue associated with bone destruction. Often bone infections develop into a chronic course with a high risk of relapse that is very difficult and longsome to treat (Lew and Waldvogel, 2004; Rao et al., 2011; Wright and Nair, 2010). Up to now, bacterial virulence factors or infection strategies that promote the development of osteomyelitis and are associated with a chronic course of disease are only partly understood. In this study, we analyzed a subset of clinical *S. aureus* strains with focus on isolates from patients with

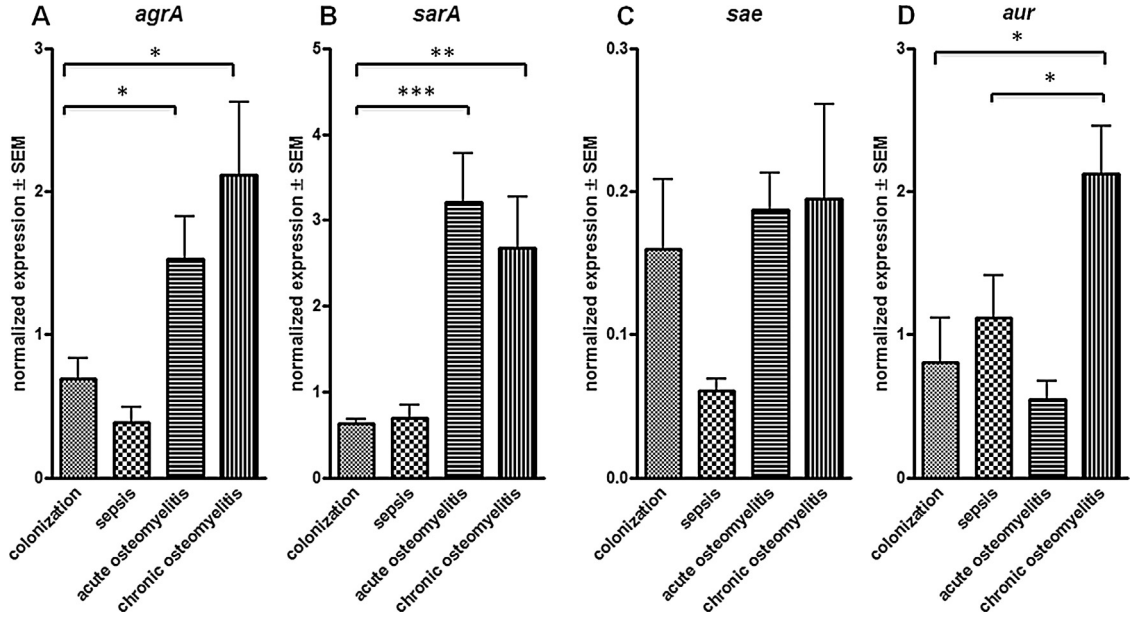

Fig. 5. *agr*, *sarA*, *sae* and *aur* expression in *S. aureus* isolates. (A–D) All *S. aureus* isolates were cultivated in BHI for 6 h to determine the expression of *agr*, *sarA*, *sae* and *aur* via RT-PCR. The results represent means ± SEM of three independent experiments in triplicate, revealing that the regulator genes *agr* and *sarA* were upregulated in isolates of osteomyelitis but not *sae*. The expression of the protease aureolysin (*aur*) was increased only in isolates from chronic infection.

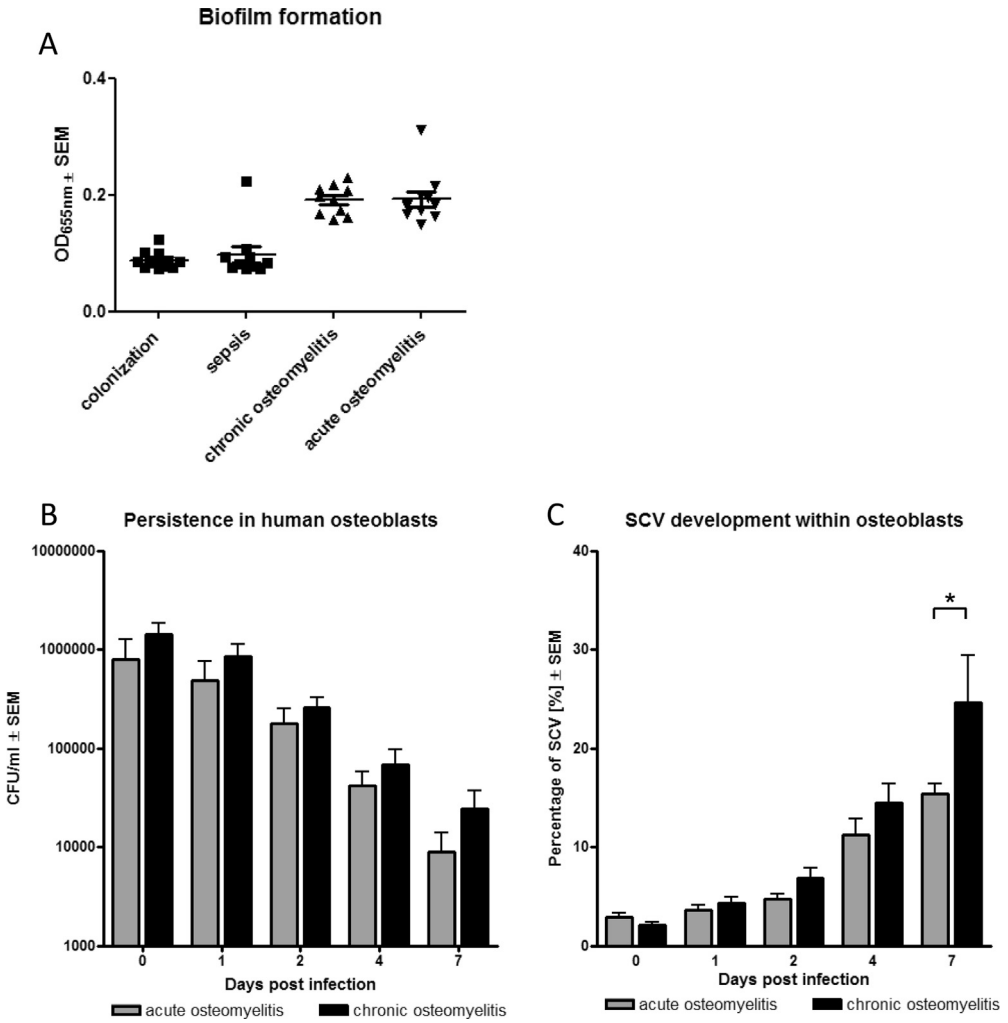

Fig. 6. Biofilm production and intracellular persistence of *S. aureus* isolates from acute and chronic osteomyelitis. Biofilm production was measured by the crystal violet method in all staphylococcal strains after incubation of 7 days at 37 °C. OD values were measured at 655 nm and are presented as means ± SEM of six independent experiments. The values show enhanced biofilm production in strains from acute and chronic osteomyelitis compared with strains from colonization and sepsis. *** $p \leq 0.001$ (unpaired *t*-test). (B, C) Human osteoblasts were infected with isolates from acute and chronic osteomyelitis. After the indicated time points (1–7 days) host cells were lysed and the numbers (B) and phenotypes (C) of the intracellular persisting bacteria were determined by serial dilutions and plating. The results represent means ± SEM of three independent experiments and reveal a higher percentage of SCV development in the group of isolates from chronic osteomyelitis. * $p \leq 0.05$ (two-way-ANOVA).

acute and chronic osteomyelitis. To find out pathogenic properties related with osteomyelitis, we compared strains from osteomyelitis to isolates of nasal colonization and sepsis.

In our osteoblast cell culture systems, we were able to demonstrate that chronic osteomyelitis isolates were characterized by a high invasion rate within host cells compared to colonizing strains. The high invasion rate could not be explained by an increased adherence to bone matrix, as isolates from the different groups showed similar capacities to adhere to extracellular matrix. Our results are in line with previous work that failed to demonstrate differences in adherence of isolates from colonization and defined pathologies, e.g. endocarditis, sepsis, osteomyelitis and arthritis, to matrix or matrix components, e.g., fibronectin (Peacock et al., 2000; Ythier et al., 2010). These findings indicate that adherence to matrix is a general phenomenon of colonizing and infecting *S. aureus* strains that is not specific for osteomyelitis development.

Adherence to host cells is mainly mediated by *S. aureus* FnBPs that bind to host cell α5β1-integrin via fibronectin as a bridging molecule (Sinha et al., 1999, 2000). This binding process is a signal for the host cells to take up the bacteria which requires an intact cytoskeleton (Jevon et al., 1999; Sinha et al., 1999). In our experiments we demonstrated that the gene for *fnbB* is highly prevalent among isolates from osteomyelitis and that *fnbA* is strongly transcribed in all infecting strains. The high prevalence and expression of *fnbs* most likely largely determine the host cell invasion rate of osteomyelitis isolates, but it cannot explain the differences between isolates from acute and chronic osteomyelitis. Yet, other adhesins besides FnBPs can contribute to adherence and invasion of host cells, such as the SERAMs Eap and Emp. Eap has been described to bind to several matrix components, to exert immunomodulatory functions and to enhance the internalization process into host cells (Haggar et al., 2003, 2004; Palma et al., 1999; Athanasopoulos et al., 2006), whereas the binding functions of Emp to host cells are largely unknown (Hussain et al., 2001). Analysis of the gene expression in our strain collection revealed a high expression of *emp* in infecting isolates, particularly in strains from chronic osteomyelitis. To investigate the role of Emp in osteomyelitis development in more detail, extensive studies on the binding functions and virulent capacity of this protein will be required.

In previously published work further adhesins have been proposed to contribute to osteomyelitis development, such as Cna and bone sialoprotein-binding protein (Bbp) (Elasri et al., 2002; Tung et al., 2000; Campoccia et al., 2009; Kornblum et al., 1990). In-vivo osteomyelitis models indicate that Cna plays a crucial role in

hematogenous osteomyelitis (Elasri et al., 2002), but is not important for the development of local osteomyelitis (Johansson et al., 2001). Hematogenous osteomyelitis is a rare form of osteomyelitis that is most frequent in children and elderly patients (Jones et al., 2011), whereas other local forms of osteomyelitis, e.g. post-traumatic osteomyelitis or osteomyelitis due to vascular insufficiency, prevail in clinical practice (Lew and Waldvogel, 2004). Accordingly, we did not find a high prevalence or expression of *cna* in our osteomyelitis isolates, supporting the hypothesis that in many forms of osteomyelitis it does not play a crucial role in pathogenesis. Another adhesin that has been associated with hematogenous osteomyelitis is Bbp (Tristan et al., 2003). *bbp* and *sdrE* represent allelic variants of the same gene (Tung et al., 2000) and we found it highly prevalent in all isolates from colonization and infection, but an association with osteomyelitis could not be made. As all colonizing and infecting strains express a multitude of adhesins with redundant functions, the adhesive capacity of each strain is determined by the coaction of all expressed adhesins rather than by a single adhesin. Furthermore, the expression of virulence factors in culture medium does not always reflect virulence factor expression during the infection. Yet, our study helps to narrow down the huge number of possible involved adhesins, but to finally proof the impact of defined factors additional in-vivo infection experiments in adequate animal models with bacterial knock-out mutants will be required.

In recent studies *S. aureus* chronic infections have been associated with biofilm formation and intracellular persistence (Brady et al., 2008; Sendi and Proctor, 2009). Biofilm is a tight adherent matrix mainly composed of adhesive proteins, e.g., FnBPs, ClfA/B and protein A, biofilm associated protein (BAP), and polysaccharides that protect bacteria against the immune systems and antibiotics. Usually biofilm is associated with foreign material-related infections, such as joint replacements that require a removal of the prosthetic implant to clear the infection and to avoid septic dissemination of pathogens (Archer et al., 2011; Otto, 2010). In our strain collection we found high production of biofilm in isolates from acute and chronic osteomyelitis, suggesting that biofilm formation might also play a role in other forms of osteomyelitis. Biofilm could form on non-vital tissue, such as dead bone fragments, as well, and contribute to bone deformation processes, protection of bacteria and therapy failure.

Furthermore, *S. aureus* is more and more recognized as an intracellular pathogen that can invade different types of host cells, including osteoblasts (Ellington et al., 1999), and persist within the intracellular location for long time periods (Garzoni and Kelley, 2009). During persistence the bacteria adapt to the intracellular milieu by dynamically changing their phenotypes to SCVs, which have a reduced and changed metabolism and low expression of *agr* and secreted virulence factors (Tuchscherr et al., 2011). Thereby, SCVs preserve the integrity of their host cells and largely avoid activation of the host immune system (Tuchscherr et al., 2010, 2011). To analyze whether isolates from chronic osteomyelitis feature more characteristics of persisting phenotypes than isolates from acute osteomyelitis, we measured cytotoxicity and host cell inflammation. Indeed, isolates from chronic osteomyelitis induced less cell damage than isolates from the other groups, indicating that they better sustain their host cells. In line with low cytotoxicity we found that isolates from chronic osteomyelitis developed a higher percentage of SCVs during persistence, suggesting that they can better adapt to their host cells.

On the other hand, we detected that acute as well as chronic osteomyelitis isolates expressed high levels of *agr* and *sarA* that induce the expression of toxins and exoproteins and cause inflammation in the host cells (e.g., RANTES expression). In many infection models *agr* is described as important regulator for virulence (Kornblum et al., 1990) and also clinical research indicates that *agr*-negative strains are compromised to settle an infection, but can develop during the infection course (Traber et al., 2008). The impact of *agr* and *sarA* in osteomyelitis development is further supported by a murine model showing that mutation of *agr* and/or *sarA* result in a reduced ability to cause bone infections (Blevins et al., 2003). The ability of chronic isolates to strongly upregulate *agr* and *sarA* might account for the fact that chronic osteomyelitis can be accompanied by severe bone thickening and deformations resulting from enhanced local inflammation (Horst et al., 2012; Jones et al., 2011; Lew and Waldvogel, 2004; Rasigade et al., 2013). In our osteomyelitis-isolates we did not find the expression of PSMalpha increased, but interestingly, we found the expression of the protease aureolysin significantly elevated in isolates from chronic osteomyelitis, suggesting that persisting bacteria need to adapt their metabolism to survive in the glucose-poor, but protein-rich environment of bone tissue. The proteolytic activity of bacteria could also account for the destruction of bone tissue.

Although our study may appear limited by the small sample sizes of only 10 (or 11) isolates in each group, we obtained significant differences in functional assays. In this study we measured not only gene expression, but we also performed extensive and time-consuming functional tests (e.g., analysis of adhesion, invasion, cytotoxicity and persistence in primary cell culture systems) with all isolates, which cannot be performed automatically at a large scale. The strength of this study is that we were able to discriminate between acute and chronic osteomyelitis isolates, as different pathogenic mechanisms might be involved. In summary we found that isolates from chronic osteomyelitis are characterized by a higher rate of host cell invasion and better adaptation to the intracellular environment (less cytotoxic effects, high expression of proteases and high rate of SCV-formation) than isolates from acute osteomyelitis, colonization and sepsis. The high invasion rate could not be attributed to the presence or expression of defined adhesins, as all isolates disposed of a multitude of adhesins with redundant functions. Acute as well as chronic osteomyelitis isolates showed high production of biofilm and expression of *agr* and *sarA*. Both regulators are known to be important for infection development and inflammation that can explain tissue destruction and deformation processes in infected bone tissue. The high capacity of chronic osteomyelitis isolates to invade osteoblasts and to adapt to the intracellular environment should be considered for the development of novel preventive (e.g., effective vaccination against intracellular pathogens) and therapeutic (e.g., antibiotics acting on intracellular persisting and adapted bacteria) strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2014.07.013.

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