

# SarZ Is a Key Regulator of Biofilm Formation and Virulence in *Staphylococcus epidermidis*

Li Wang,<sup>1,a</sup> Min Li,<sup>2,a</sup> Dandan Dong,<sup>1</sup> Thanh-Huy L. Bach,<sup>2</sup> Daniel E. Sturdevant,<sup>3</sup> Cuong Vuong,<sup>2,b</sup> Michael Otto,<sup>2</sup> and Qian Gao<sup>1</sup>

<sup>1</sup>Key Laboratory of Medical Molecular Virology, and Institutes of Medical Microbiology and Biomedical Sciences, Shanghai Medical College, Fudan University, Shanghai, People's Republic of China; <sup>2</sup>Laboratory of Human Bacterial Pathogenesis and <sup>3</sup>Research Technologies Section, Genomics Unit, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, Hamilton, Montana

**Biofilm-associated infection due to *Staphylococcus epidermidis*, the leading nosocomial pathogen, is a major problem for the public health system, but the regulation of this important phenotype is not completely understood. Using a highly discriminatory screening procedure for genes that influence biofilm formation, we identified the transcriptional regulator SarZ as a novel important determinant of biofilm formation and biofilm-associated infection, on the basis of the significant impact of *sarZ* on the transcription of the biosynthetic operon for biofilm exopolysaccharide. In addition, *sarZ* influenced the expression of a series of virulence genes, including genes that influence the expression of lipases and proteases, resistance to an important human antimicrobial peptide, and hemolysis. Our study indicates that the SarZ regulator has a key role in maintaining the typical *S. epidermidis* phenotype, which is characterized by pronounced biofilm formation and immune evasion, a likely reason for the success of *S. epidermidis* as a colonizing organism and pathogen in chronic, biofilm-associated infection.**

*Staphylococcus epidermidis* is the most important member of the coagulase-negative staphylococci, which are normal microflora on the human skin and mucous membranes that have attracted considerable attention more recently as dangerous nosocomial pathogens [1]. There are increasingly frequent reports about the devastating complications of indwelling medical device-related infections due to *S. epidermidis*, which prolong disease and result in higher morbidity and mortality, es-

pecially among immunocompromised patients [2]. In particular, *S. epidermidis* plays an important role in foreign body-related infections, such as catheter-related infections, prosthetic-valve-related endocarditis, prosthetic joint-related infections, and peritoneal dialysis-related infections [1, 3]. The repertoire of virulence factors in *S. epidermidis* is relatively limited, especially compared with the more virulent *S. aureus* [4]. In contrast, biofilm formation has long been recognized as a key virulence determinant for *S. epidermidis* [1, 5]. Bacteria in a biofilm are more resistant to attacks by innate host defense mechanisms and antibiotics; this increased resistance is based on the specific biofilm structure and metabolism [6, 7].

Several genes are directly or indirectly involved in the molecular mechanisms of biofilm formation in *S. epidermidis*, including *fbe*, *atlE*, *aap*, and the *ica* locus [8–11]. Undoubtedly the most important gene product involved in the accumulation phase of biofilm formation is the polysaccharide intercellular adhesin (PIA), the biosynthetic machinery of which is encoded by the *ica* locus [9]. PIA is a biofilm exopolysaccharide that surrounds the cells in a typical biofilm matrix and has an additional function in providing protection from antibacterial peptides and phagocytosis [12].

Received 21 August 2007; accepted 8 November 2007; electronically published 21 March 2008.

The authors declare that they have no conflicts of interest.

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, the Chinese National Natural Science Foundation (30670108), the National Program 863 (2006AA02Z328), and the Shanghai municipal science and technology commission (05PJ14025).

<sup>a</sup> L.W. and M.L. contributed equally to this article.

<sup>b</sup> Present affiliation: University of Massachusetts Medical School, Department of Anesthesiology, Worcester, Massachusetts.

Reprints or correspondence: Michael Otto, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, The National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840 (motto@niaid.nih.gov); or Qian Gao, Key Laboratory of Medical Molecular Virology, Shanghai Medical College, Fudan University, 138 Yi Xue Yuan Road, Shanghai 200032, People's Republic of China (qiangao@shmu.edu.cn).

The Journal of Infectious Diseases 2008; 197:1254–62

This article is in the public domain, and no copyright is claimed.

0022-1899/2008/19709-0007

DOI: 10.1086/586714

Biofilms represent the common mode of growth for many bacteria, including *S. epidermidis*, particularly under the environmental conditions found on the human skin [13]. Accordingly, there are numerous regulatory mechanisms by which *S. epidermidis* responds to environmental stimuli and coordinates the expression of biofilm structural factors and biofilm-specific metabolism [14, 15]. However, we are far from understanding the intricate details of this regulation. Several regulators have been found to impact biofilm formation by controlling the expression of biofilm factors, mostly PIA [16–23].

Many biofilm-related structural and regulatory genes have been discovered by transposon mutagenesis and subsequent in vitro screening for biofilm formation. A previous study that screened for biofilm determinants in *S. epidermidis* was particularly successful in that regard [24]. Since then, many researchers have tried to use similar methods to detect additional biofilm determinants, but due to the fact that biofilm formation is strongly dependent on the specific model system, many false-positive clones are usually found. To overcome this problem, we aimed to discover key biofilm determinants of *S. epidermidis* using a highly discriminatory screening procedure with 2 different, consecutive in vitro assays. By use of this procedure, we identified only 1 gene, *sarZ*, whose pivotal regulatory role in *S. epidermidis* biofilm formation and virulence we describe in the present article.

## METHODS

**Bacterial strains, plasmids, and growth conditions.** *Escherichia coli* was grown in Luria-Bertani medium, and *S. epidermidis* strain 1457 [25] was grown in tryptic soy broth (TSB) (Oxoid). When necessary, media were supplemented with ampicillin (100 µg/mL for *E. coli*), erythromycin (2.5 µg/mL for selection of Tn917-positive organisms) or chloramphenicol (20 µg/mL for pQG plasmids in staphylococci). For microarray experiments, cultures grown overnight were diluted 1:100 into 50 mL of TSB and incubated at 37°C with shaking at 180 rpm until grown to the late exponential growth phase.

**DNA manipulations.** Staphylococcal chromosomal DNA was extracted as described elsewhere [26]. Plasmids were prepared by use of standard protocols, except for modifications for staphylococci described elsewhere [21], and introduced by electroporation [27]. ExTaq Polymerase was obtained from Takara, restriction enzymes from New England Biolabs, and oligonucleotides from Invitrogen or Sigma.

**Transposon mutagenesis and identification of insertion loci.** Transposon mutagenesis was performed using plasmid pTV1ts, which contains transposon Tn917, as described elsewhere [28]. The presence of the erythromycin cassette was used in Southern blot analysis to confirm single transposon insertion. The probe used for Southern hybridization was a 500-bp polymerase chain reaction (PCR) product of the erythromycin cassette (ob-

tained with primers *ermB5* [5'-CGAAATTGGAACAGGTAAAAG-3'] and *ermB3* [5'-GCGTGTTCATTGCTTGATG-3']). Adaptor PCR [29] was used to identify the insertion locus. Adaptors carrying *Hind*III and *Acc*I restriction sites were ligated to *Hind*III-cleaved fragments of *S. epidermidis* genomic DNA. Fragments were amplified for subsequent DNA sequencing by use of the purified ligation product as the template for the first PCR and the diluted product of the first PCR as the template of a sequential PCR (primers: adaptor1 [5'-AGCTTGTAGCAGATGATGCTGT-3'], adaptor2 [5'-MKACAGCATCATCTGCTACA-3'], Tn917 5-1 [5'-CCATGTTAAACC-CATAGATAAG-3'], and Tn917 5-2 [5'-CATAACTTTAGGG-TTAACCATAC-3']).

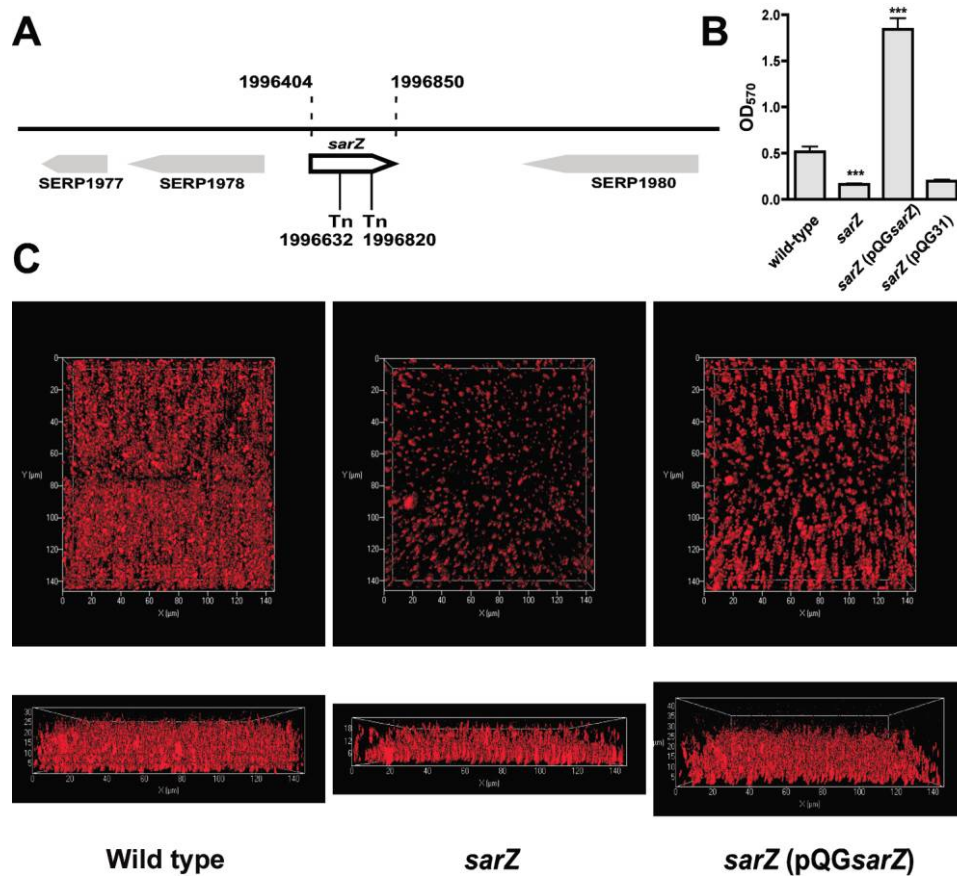
**Complementation of the *sarZ* mutant.** The chloramphenicol resistance cassette (*cat*) from plasmid pBT1 [30] was amplified by PCR with primers Cat5 (5'-CGGAAGCTTAAGCTTCAAA-CGAAATTGGATAAAGTGG-3') and Cat3 (5'-CGGGGATCCTTATAAAAGCCAGTCATTAGGCC-3'), cut with *Hind*III and *Bam*HI, and ligated into the *Hind*III and *Bam*HI cleaved vector pYJ90 (containing origins of replication from both pUC19 and pE194 [31]). The resulting plasmid pQG31 was transformed into *S. epidermidis* as negative control. The *sarZ* gene including a 500-bp sequence upstream of *sarZ* was amplified by PCR from the genomic DNA of *S. epidermidis* strain 1457 with primers SarZ5 (5'-CGCGATCCTCCATGAGCTTTCGCTGTAA-3') and SarZ3 (5'-CCGGAATTCTCGCTATTGCTAAATTGAAAGT-3'), introducing *Bam*HI/*Eco*RI restriction sites. The 1-kb *sarZ* fragment was ligated into *Bam*HI/*Eco*RI digested pQG31, which resulted in plasmid pQGsarZ.

**Semiquantitative biofilm assay.** Semiquantitative biofilm assays were performed as described elsewhere [21, 23]. Subsequently, cells were either directly stained with safranin or fixed by Bouin's fixative. The fixative was removed after 1 h and wells were washed with PBS. Organisms in the wells were then stained with crystal violet, and the floating stain was washed off with slowly running water. After drying, the stained biofilm was read with a MicroELISA autoreader (BioRad) at 570 nm (crystal violet stain) or 490 nm (safranin stain).

**Primary attachment assay.** A method described elsewhere [21] was used, with slight modifications. *S. epidermidis* strains were grown to the early stationary phase, and cell suspensions were adjusted with TSB to a 600-nm optical density (OD<sub>600</sub>) of 1.0. The diluted cultures were pipetted into 96-well tissue culture plates and incubated at 37°C for 1 h. The subsequent procedures are the same as those used for the semiquantitative biofilm assay.

**Immunoblot analysis of PIA production.** PIA was isolated and detected by immuno-dot blots as described [21]. The degree of immunoreactivity was measured and quantitated with a photodetection system (Quantity One 4.6; BioRad).

**Quantitative reverse-transcription (RT) PCR.** Oligonucleotide primers and probes were designed using Primer Express. Quantitative RT-PCR was performed as described [26]. The experiments were performed in triplicate, with 16S ribosomal RNA as a control. The primers



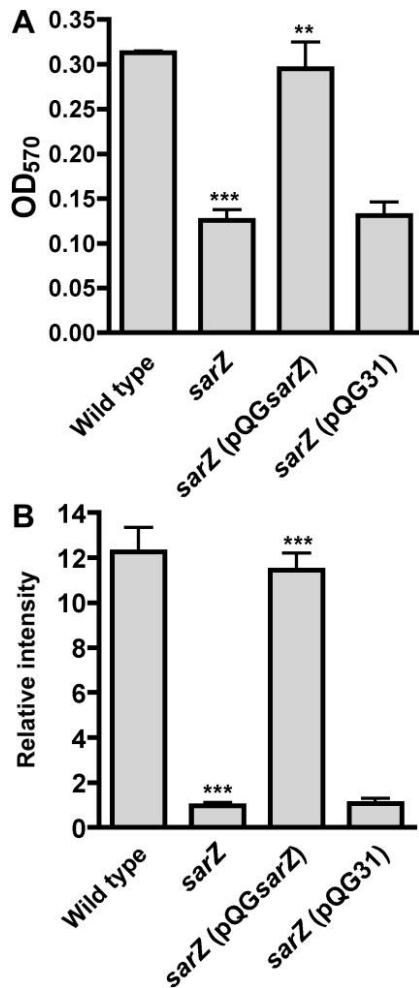
**Figure 1.** SarZ influence on in vitro biofilm formation. *A*, Location of the *sarZ* gene in the genome of *Staphylococcus epidermidis* (strain RP62A genome sequence [32]) and insertion loci of the 2 transposon mutants. The mutant with the insertion locus at 1996632 bp was selected for further investigation. *B*, Semiquantitative biofilm assay in microtiter plates performed with fixation and crystal violet staining. \*\*\* $P < .001$ ; *sarZ* vs. wild-type strain; *sarZ* (pQG*sarZ*), vs. *sarZ* (pQG31). Data are means  $\pm$  standard error of the mean of 4 independent experiments. *C*, Confocal laser scanning microscopy of static biofilm formation. Biofilms were stained with propidium iodide. OD<sub>570</sub>, optical density 570 nm.

and probes used were Lipase-F [5'-TGTC AAGGAGGACG-TGTTGAT-3'], Lipase-R [5'-CGCATGATGCCCTTGTATGT-3'], and Lipase-probe [5'-ATATTTAGCTGCATGCGCTGCACCGT-3']; SspB-F [5'-AATCGATATAATGCAGAATCAGTAATGAG-3'], SspB-R [5'-CTCGTTAGATGTTAGTCCTGTAAATTGG-3'], and SspB-probe [5'-ATTTACATCCTAATTTAAGAGGTCAC-GACT-3']; IcaB-F [5'-GACAATGCCTTTCCTGTTTTGAA-3'], IcaB-R [5'-AGAACCAATGTGGTTCGTAATAAGAA-3'], and IcaB-probe [5'-AATATCATATTCCAGCAACAGG]; RNA III-F [5'-ACTAAATCACCGATTGTAGAAATGATATCT-3'], RNA III-R [5'-ATTTGCTTAATCTAGTCGAGTGAATGTTA], and RNA III-probe [5'-TGCCATTATAACTTCACTCCTTTCGA-3']; Ndqr-F [5'-GTGAATATAGATAGTATTTAATGGCAATGC-3'], Ndqr-R [5'-TTATCGAAGCCGCCAATAGG-3'], and Ndqr-probe [5'-ATCGIATCCATGAGCTTTCGCTGTAAGCATT-3'].

**Microarray experiments.** RNA isolation, DNA removal, reaction cleanup, cDNA synthesis and hybridization, and estimation of labeling efficiency were performed as described elsewhere [26]. Biotinylated *S. epidermidis* cDNA was hybridized to cus-

tom Affymetrix GeneChips (RMLChip 3) with 98.8% coverage of genes from RP62A [32] (2465 probe sets of 2494 open reading frames) and scanned in accordance with standard GeneChip protocols (Affymetrix). Each experiment was replicated at least 3 times. Affymetrix GeneChip Operating Software (GCOS, version 1.4) was used to perform the preliminary analysis of the custom chips at the probe-set level. Subsequent data analysis was performed as described elsewhere [26]. To be included in the final gene list, gene expression had to have been changed at least 2-fold for 1 of the treatments. The complete set of microarray data was deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, available at <http://www.ncbi.nlm.nih.gov/geo/>) and is accessible through GEO Series accession number GSE8523.

**Confocal laser scanning microscopy.** *S. epidermidis* was inoculated from precultures grown overnight at a dilution of 1:100 with TSB that contained 0.5% glucose in 8-well polystyrene chambers (Lab-Tek II; Nunc). After 24 h of incubation at 37°C without shaking, supernatants were gently removed, and biofilm



**Figure 2.** SarZ influence on primary attachment and intercellular accumulation. *A*, Primary attachment assays performed with the Bouin fixation method and crystal violet staining. Data are means  $\pm$  standard error of the mean (SEM) of 4 independent experiments. *B*, Immuno-dot blot detection of polysaccharide intercellular adhesin (PIA) with  $\alpha$ PIA antiserum and photodigital evaluation. Data are means  $\pm$  SEM of 3 independent experiments. \*\* $P < .01$ ; \*\*\* $P < .001$ ; *sarZ* vs. wild-type strain; *sarZ* (pQG:*sarZ*) vs. *sarZ* (pQG31). OD<sub>570</sub>, optical density 570 nm.

layers were washed and resuspended in 400  $\mu$ L of saline. Biofilm formation was made visible by staining cells with 4  $\mu$ mol/L propidium iodide (Sigma) for 10 min. Images were acquired on a Zeiss LSM 5 Pascal laser-scanning confocal unit with an Axiovert 100 microscope with a 63  $\times$  1.4—NA oil-immersion objective. Zeiss 3D software (Image VisArt) was used for the 3-dimensional visualization of biofilm structures.

**Protease assay.** Protease activity was determined by an agar plate assay. The test agar contained 1.0% skim milk and 1.0% agar. Cultures were grown at 37°C for 10 h and 15 mL of culture filtrate were then lyophilized. The lyophilysates were dissolved in 3 mL of 20 mmol/L Tris-HCl containing 1 mmol/L CaCl<sub>2</sub> (pH, 7.8) and passed through 0.22-mm pore filters. Next, 50  $\mu$ L were loaded into holes in the plates, which were incubated at 37°C for 8 h.

**Killing assays.** *S. epidermidis* cells were grown to exponential growth phase (OD<sub>600</sub>, 2.5–3.0), harvested, washed twice with 10 mmol/L sodium phosphate buffer (pH, 6.5) that included 100 mmol/L NaCl, and resuspended in the same buffer. The cells were diluted to a final concentration of 10<sup>6</sup> cells in each sample, exposed to a range of concentrations of the human antimicrobial peptide human  $\beta$ -defensin 3 (hBD3), and incubated at 37°C for 3 h. Appropriate dilution series of the samples were spread on TSB agar plates and incubated at 37°C for 24 h for colony counting. hBD3 was synthesized by the Peptide Synthesis Unit, Research Technology Branch, National Institute of Allergy and Infectious Diseases.

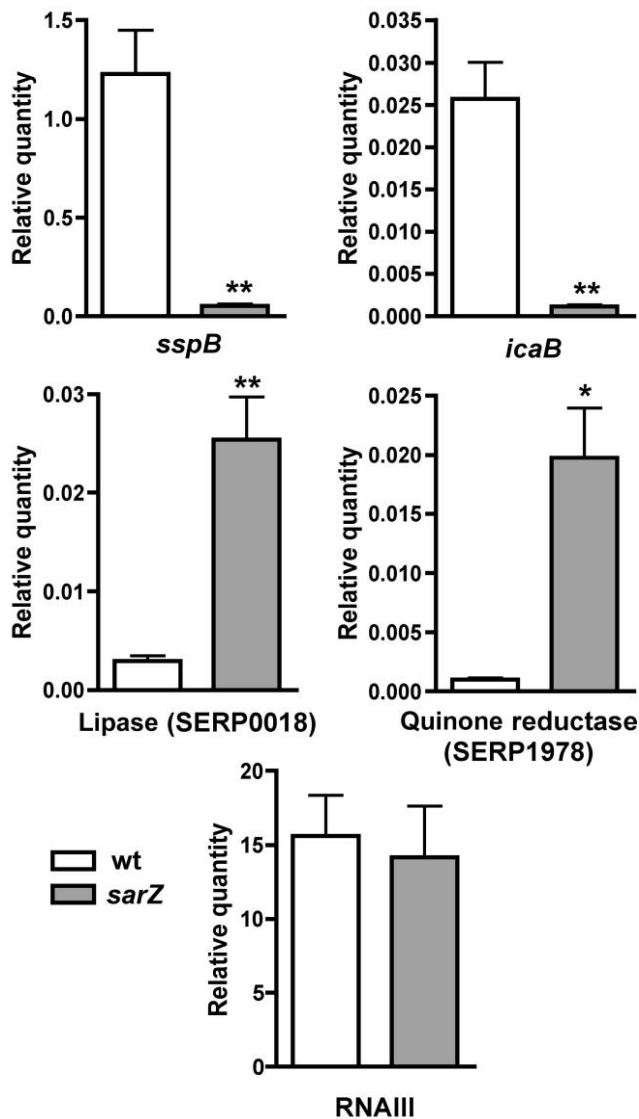
**Rat model of central venous catheter-related infection.** The rat model of central venous catheter (CVC)-related infection was as described elsewhere [23]. In brief, healthy male Sprague-Dawley rats (weight, 500 g) (Academia Sinica) were surgically dissected, and silastic lumen-within-lumen catheters (inside diameter, 0.12 mm) were inserted into their right external jugular veins. Twenty-four hours after CVC placement, 1  $\times$  10<sup>6</sup> cfu of *S. epidermidis* were inoculated into the catheters. The catheters were flushed with heparin every day. The rats were sacrificed on day 8 and the bacteria recovered from hearts, livers, kidneys, blood, and catheters were counted. All animal work was approved by the ethics committee of Fudan University.

**Mouse model of subcutaneous foreign body infection.** A murine model of subcutaneous foreign body infection like that

**Table 1. Regulatory gene changes in the *sarZ* mutant strain of *Staphylococcus epidermidis*, compared with wild-type *S. epidermidis* strain 1457.**

Gene number	Gene or gene product	Factor of regulation
<b>Down-regulated genes</b>		
SERP1397	<i>sspA</i>	11.11
SERP2388	Lipase	3.70
SERP2390	<i>sspB</i>	2.63
SERP2391	<i>sspC</i>	5.56
SERP2292	<i>icaR</i>	2.17
SERP2293	<i>icaA</i>	6.67
SERP2294	<i>icaD</i>	7.69
SERP2295	<i>icaB</i>	6.67
SERP2296	<i>icaC</i>	2.94
<b>Up-regulated genes</b>		
SERP0018	Lipase	7.68
SERP1970	Hypothetical protein	12.99
SERP1971	Hypothetical protein	12.59
SERP1978	NAD(P)H-dependent quinone reductase	36.84
SERP2258	putative hemolysin	2.33
SERP2297	Lipase	3.15

**NOTE.** The entire list of differentially expressed genes can be found on the National Center for Biotechnology Information Gene Expression Omnibus (GEO, available at <http://www.ncbi.nlm.nih.gov/geo/>) and is accessible through GEO Series accession number GSE8523.



**Figure 3.** *SarZ* gene regulatory effects by quantitative reverse transcription polymerase chain reaction. Data are means  $\pm$  standard error of the mean of 3 independent experiments. \* $P < .05$ ; \*\* $P < .01$ ; vs. wild-type (wt) strain.

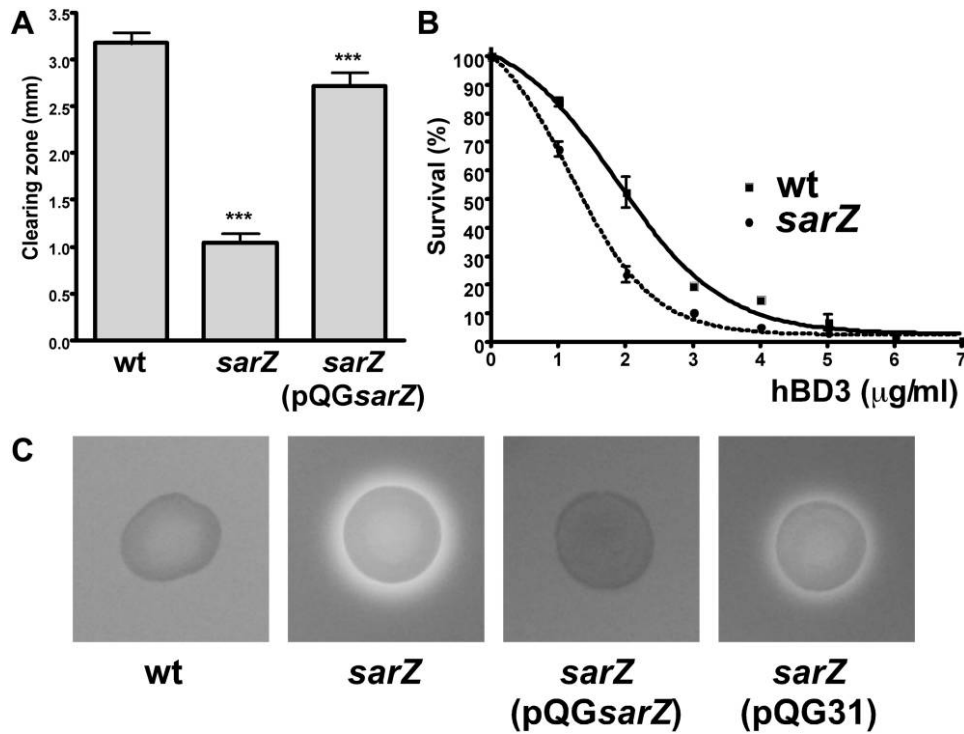
described by Kadurugamuwa et al. [33] was used, with slight modifications. In brief, eighteen male BALB/c mice (weight 20 g) (Academia Sinica); were randomly and evenly divided into 2 groups and treated with *S. epidermidis* strain 1457 and *S. epidermidis sarZ* mutant strain, respectively. One-centimeter long silicon catheters (14 gauge) were implanted subcutaneously on each side at the flank of each mouse before injection of  $10^7$  cfu to the catheter bed. After 10 days, the mice were sacrificed. Catheters were aseptically removed and manipulated by use of the method described elsewhere [33], except that we used a sonication time of 30 min. Then serial dilutions of the wash fluid were plated on TSB plates and the recovered *S. epidermidis* colonies were counted.

## RESULTS

**Transposon mutagenesis identified *sarZ* as key biofilm determinant.** To detect novel genes that influenced biofilm formation and biofilm-associated infection due to *S. epidermidis*, Tn917 transposon mutagenesis was performed in the clinical isolate *S. epidermidis* 1457. In contrast to previous, similar approaches, a large number of clones ( $\sim 20,000$ ) were tested to account for the insertion bias of Tn917 [34]. Furthermore, clones were screened by use of 2 consecutive, different in vitro tests to discover genes that showed a consistent influence on biofilm formation. The 20,000 clones were first assayed by biofilm formation in round-bottom Greiner microtiter plates and subsequent staining with safranin without fixation, as described elsewhere [21]. About 100 clones were found that were then screened using Costar (Corning) plates with fixation and crystal violet staining. Surprisingly, this consecutive, exclusive method yielded only 2 clones that were deficient in biofilm formation, both of which had a single Tn917 insertion at 2 different locations in the *sarZ* (SERP1979) gene (figure 1A). This gene shares 79% identity and 91% similarity in amino acid sequence with the virulence regulator gene *sarZ* of *S. aureus* [35]. Both have a helix-turn-helix multiple antibiotic resistance protein motif characteristic of the SarA protein family in staphylococci [36]. To confirm that *sarZ* was involved in biofilm formation, a single transposon mutant was selected for further studies and complemented with a plasmid expressing *sarZ*, which resulted in restored biofilm formation (figure 1B). Of note, growth of the mutant strain was indistinguishable from that of the wild-type strain (data not shown). Finally, 3-dimensional biofilm formation was monitored by use of confocal laser-scanning microscopy, which confirmed that *sarZ* had a strong impact on biofilm development (figure 1C). These findings indicated that *sarZ* is a key determinant of biofilm formation in *S. epidermidis*.

***SarZ* influences primary attachment and exopolysaccharide-dependent biofilm accumulation.** Biofilm formation is a 2-step process that involves initial attachment to a surface and subsequent accumulation due to proliferation and cell-cell adhesion [5]. Primary attachment assays demonstrated that the first step of biofilm formation is controlled by *sarZ* (figure 2A). Further, immunoblot analysis of polysaccharide intercellular adhesion (PIA), the main factor influencing biofilm accumulation in *S. epidermidis* strain 1457, showed that *sarZ* also has an impact on the second step of biofilm formation (figure 2B). Thus, *sarZ* has a profound influence on both the initial and accumulative phases of biofilm development, which is due at least in part to the regulation of PIA production.

**The *sarZ* regulon comprises a specific set of *S. epidermidis* virulence genes.** The Sar family of DNA-binding proteins are transcriptional regulators with a frequently global influence on gene expression, notably including the expression of virulence genes [36]. To investigate whether *sarZ* plays a similar role in *S. epidermidis*, we determined the *sarZ* regulon by genome-wide



**Figure 4.** SarZ influences proteolytic activity, resistance to human  $\beta$ -defensin 3 (hBD3), and hemolysis. *A*, Proteolytic activity of 5 $\times$  concentrated stationary-phase supernatants detected in skim milk agar plates. \*\*\* $P < .001$ ; *sarZ* vs. wild-type (wt) strain; *sarZ* (pQG*sarZ*) vs. *sarZ*. Data are means  $\pm$  standard error of the mean (SEM) of 3 independent experiments. *B*, Killing assay with different concentrations of the human antimicrobial peptide hBD3. Data are means  $\pm$  SEM for 3 independent experiments. *C*, Hemolysis. Corresponding volumes of stationary-phase cell suspensions with the same optical density were inoculated on sheep blood agar plates and incubated at 37°C for 24 h.

analysis of gene expression using *S. epidermidis* Affymetrix gene chips (RMLchip3). Interestingly, whereas the *sarZ* regulon comprised  $\sim$ 80 genes, the most dramatic regulatory effects occurred in genes that were implicated in virulence (table 1). Of note, all genes of the *ica* operon for PIA biosynthesis and 3 different *S. epidermidis* protease genes—*sspA*, *sspB*, and *sspC*—showed strong up-regulation by *sarZ*. Further, 3 lipase genes were regulated by *sarZ*, but in an inconsistent manner. Finally, 3 genes in close proximity to the *sarZ* gene, 2 that coded for hypothetical

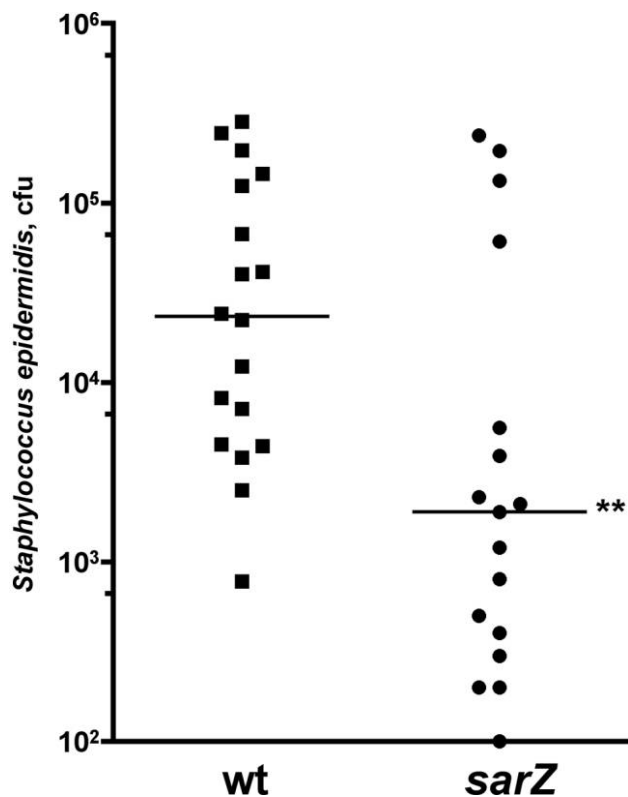
genes and 1 that coded for a quinone reductase, were strongly down-regulated by *sarZ*. Quantitative RT-PCR of the *icaB* gene in the *ica* operon, 1 of the down-regulated lipase genes (SERP0018), the *sspB* protease gene, and the strongly repressed quinone reductase gene, confirmed the data obtained with the microarray and demonstrated that the observed increased expression of PIA was caused by up-regulation of the transcription of PIA biosynthetic genes (figure 3). In addition, we used quantitative RT-PCR to determine expression of RNAlII, the regula-

**Table 2. Metastatic disease caused by *Staphylococcus epidermidis* strain 1457 and *sarZ* mutant strain in a rat model of central venous catheter-associated infection.**

Site	<i>S. epidermidis</i> 1457		<i>S. epidermidis sarZ</i>		<i>P</i> <sup>b</sup>
	Animals, no. <sup>a</sup>	CFU/g of tissue, median, (range)	Animals, no. <sup>a</sup>	CFU/g of tissue, median, (range)	
Heart	4/7	33 (0–6666)	1/6	0 (0–4000)	NS
Liver	6/7	161 (0–10,000)	4/6	83 (0–3333)	NS
Kidney	4/7	33 (0–20,000)	1/6	0 (0–33)	NS
Blood	4/7	20 (0–40)	1/6	0 (0–20)	NS
Catheter	7/7	39,500 (6500–192,000)	6/6	25,750 (0–24,000)	NS
Overall infection rate, %	71.4 $\pm$ 20.2		38.3 $\pm$ 29.8		.014

<sup>a</sup> Data are the number of animals from which bacteria were recovered/total number of animals.

<sup>b</sup> The no. of bacteria found in each organ or site tested (heart, liver, kidney, blood, and catheter) and the total infection rate (in all organs tested) were analyzed by the Fisher exact test. NS, not significant ( $P > .05$ ).



**Figure 5.** Murine model of subcutaneous catheter infection. Catheter tubing was inserted under the skin of mice and infected with  $1 \times 10^7$  cfu of the wild-type (wt) *Staphylococcus epidermidis* strain 1457 or *sarZ* mutant strains. After 10 days, catheters were removed and colony-forming units on the catheters were counted. Data were analyzed using the Mann-Whitney test. Horizontal line, median.  $**P = .007$ .

tory molecule of the staphylococcal *agr* system [37], because the SarA regulator and some other Sar paralogs are known to have a strong regulatory influence on *agr* expression [38]. However, we did not detect significant differences in RNIII expression between the wild-type and *sarZ* mutant strains, which demonstrated that *sarZ* does not influence *agr* expression in *S. epidermidis*.

**SarZ controls proteolytic and hemolytic activity.** The virulence of *S. epidermidis* is largely defined by its ability to evade host defenses [4, 39]. One mechanism it uses to subvert a key part of innate host defense is the proteolytic degradation of antimicrobial peptides, which play a crucial role in controlling bacterial colonization on epithelia and contribute to the killing of bacteria in the neutrophil phagosome after infection. Proteolytic activity was significantly decreased in the *sarZ* mutant strain and restored in the complemented strain (figure 4A), indicating that *sarZ* has an impact on protease secretion, an established virulence factor for *S. epidermidis*. To test whether the *sarZ*-dependent up-regulation of *S. epidermidis* proteases resulted in decreased degradation of antimicrobial peptides, we determined susceptibility to the important epithelial human antimicrobial peptide hBD3 [40]. We found that there was a slight, yet signif-

icant, increase in susceptibility to hBD3 for the *sarZ* mutant strain (figure 4B), indicating that *sarZ*-dependent gene regulation had an impact on the immune evasion properties of *S. epidermidis*.

The secretion of hemolytic toxins is an important determinant of staphylococcal virulence. Among the *sarZ* down-regulated genes, we detected 1 gene whose expression was increased ~2–3-fold in the *sarZ* mutant. This gene had similarities to hemolysins, such as the *S. lugdunensis* slush peptides [41] and the gonococcal growth inhibitor peptide AGS of *S. haemolyticus* [42]. Interestingly, the *sarZ* mutant exhibited pronounced  $\beta$  hemolysis on sheep agar plates, a phenotype known from the synergistic activities of  $\delta$ -toxin and either  $\alpha$ - or  $\beta$ -toxin in *S. aureus* [43], but not commonly seen in *S. epidermidis* (figure 4C), which indicates that *sarZ* regulates hemolysis, possibly by an effect on the gene SERP2258.

**SarZ is an important determinant of *S. epidermidis* biofilm-associated infection.** Biofilm-associated infection on implants is the main type of infection caused by *S. epidermidis* [1]. To assess whether *sarZ* impacts the virulence of *S. epidermidis* during this type of infection, we used 2 different animal models: a CVC-related infection model in rats and a subcutaneous foreign material infection-related model in mice. In the CVC model, the rats were inoculated with  $1 \times 10^6$  cfu of wild-type or mutant strains through the catheters 24 h after surgery. The bacteria recovered from the organs, blood, and catheters were counted after sacrificing rats on the day 8 of infection. Although the specific infection rates of the single organs and catheters did not show statistically significant differences, the overall infection rate was significantly different between the rats infected with the *S. epidermidis* strain 1457 wild-type and *sarZ* mutant strains ( $P = .014$ , Fisher exact test) (table 2). In the subcutaneous murine catheter infection model (which involved infection with  $1 \times 10^7$  cfu), we detected a significant difference in the amount of bacteria recovered from the infected catheters of mice inoculated with the wild-type strain and mice inoculated with the *sarZ* mutant strains ( $P = .006$ , Mann-Whitney test) (figure 5). Thus, both animal models indicated that *sarZ* contributes significantly to the development of biofilm-associated infection due to *S. epidermidis*.

## DISCUSSION

*S. epidermidis* is the most frequent cause of foreign body-related infections, in which the formation of biofilms plays a preeminent role. However, the development of an *S. epidermidis* biofilm and the regulation of biofilm expression in this important opportunistic pathogen are not fully understood. Using a transposon mutagenesis approach with a 2-step screening procedure, we identified the SarA paralog SarZ of *S. epidermidis* as a key factor that influences biofilm formation.

The Sar family of transcriptional regulators comprises at least 12 paralogs in *S. aureus*, including SarA, SarR, SarS, SarU, SarX, SarV, Rot, and MgrA, which interact in a complicated regulatory network [36]. Many of these paralogs affect the expression of *S. aureus* virulence genes, such as the regulation of protease and surface protein expression. In addition, the Rot protein has a pivotal function as a mediator of quorum-sensing regulation in *S. aureus* [44, 45]. Finally, several Sar paralogs have been implicated in the control of biofilm formation. SarA regulates the expression of genes in the *ica* locus directly, by binding to the *ica* promoter [20, 46]. In addition, SarA and some other Sar paralogs may influence biofilm formation indirectly, by regulating the quorum-sensing system *agr*, which has been implicated in regulating biofilm formation via the control of surfactant-like peptides [7, 47]. In *S. epidermidis*, there are several Sar paralogs, but only the SarA protein has been characterized in more detail [48]. Similar to its role in *S. aureus*, SarA in *S. epidermidis* controls biofilm formation [20].

Recently, one role of *S. aureus* SarZ in controlling virulence has been described [35]. This Sar paralog has 53% similarity in amino acid sequence to SarA of *S. aureus*. Interestingly, SarZ of *S. epidermidis* is very similar to its homolog in *S. aureus*, although with no significant similarity with *S. epidermidis* SarA has been detected. In silkworm and mouse infection models, an *S. aureus sarZ* mutant strain exhibited less virulence than the wild-type strain [35]. In our study, *sarZ* deficiency resulted in decreased virulence in infections typical of *S. epidermidis*, indicating that this regulator plays a key role in staphylococcal virulence.

While *S. aureus* SarZ has been reported to influence hemolytic activity in a positive way by up-regulation of  $\alpha$ -hemolysin production [35], results from our study indicate a contrasting influence of *S. epidermidis* SarZ on hemolytic activity. As *S. epidermidis* is not commonly hemolytic, the finding that *S. epidermidis* has the means to cause hemolysis, which may be mediated in part by the not yet further characterized *sarZ*-regulated SERP2258 gene, is of great interest. One may only speculate as to why hemolytic factors are not normally produced in *S. epidermidis*, although they might have considerable potential as virulence factors. As a possible explanation, it has been suggested that the expression of virulence genes to the degree found in virulent *S. aureus* is counterproductive for *S. epidermidis* colonization and transmission [49]. Therefore, *S. epidermidis* as a species has evolved to suppress the acquisition and expression of determinants that cause significant harm to the human host.

There are indications that staphylococci produce colonization factors and acute virulence factors, such as toxins and exoenzymes, at different times during infection, or during different modes of existence; they produce different factors during the initial infection period, compared with the later infection period, and during infection, compared with colonization [22, 50]. Accordingly, there are global regulators that control the switch

between these modes, most notably the quorum-sensing regulator *agr*, which suppresses the production of colonization factors and promotes secretion of acute virulence factors [37]. Similarly, the *sarZ* regulator up-regulates genes involved in biofilm formation and colonization, and it appears to have an additional role in suppressing hemolytic factors. It will be an important part of future research to identify conditions under which *sarZ* is repressed, possibly to allow for the development of a more acute phase during *S. epidermidis* pathogenesis.

## Acknowledgments

We thank Jenna Carter for help in the transposon mutant screening procedure.

## References

1. Vuong C, Otto M. *Staphylococcus epidermidis* infections. *Microbes Infect* **2002**; 4:481–9.
2. Raad I, Alrahwan A, Rolston K. *Staphylococcus epidermidis*: emerging resistance and need for alternative agents. *Clin Infect Dis* **1998**; 26:1182–7.
3. von Eiff C, Peters G, Heilmann C. Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infect Dis* **2002**; 2:677–85.
4. Otto M. Virulence factors of the coagulase-negative staphylococci. *Front Biosci* **2004**; 9:841–863.
5. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* **1999**; 284:1318–22.
6. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* **2001**; 358:135–8.
7. Yao Y, Sturdevant DE, Otto M. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. *J Infect Dis* **2005**; 191:289–98.
8. Heilmann C, Hussain M, Peters G, Gotz F. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol Microbiol* **1997**; 24:1013–24.
9. Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D, Gotz F. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* **1996**; 20:1083–91.
10. Hussain M, Herrmann M, von Eiff C, Perdreau-Remington F, Peters G. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infect Immun* **1997**; 65:519–24.
11. Nilsson M, Frykberg L, Flock JI, Pei L, Lindberg M, Guss B. A fibrinogen-binding protein of *Staphylococcus epidermidis*. *Infect Immun* **1998**; 66:2666–73.
12. Vuong C, Voyich JM, Fischer ER, et al. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol* **2004**; 6:269–75.
13. Watnick P, Kolter R. Biofilm, city of microbes. *J Bacteriol* **2000**; 182:2675–9.
14. Kong KF, Vuong C, Otto M. *Staphylococcus* quorum sensing in biofilm formation and infection. *Int J Med Microbiol* **2006**; 296:133–9.
15. Mack D, Davies AP, Harris LG, Rohde H, Horstkotte MA, Knobloch JK. Microbial interactions in *Staphylococcus epidermidis* biofilms. *Anal Bioanal Chem* **2007**; 387:399–408.
16. Conlon KM, Humphreys H, O’Gara JP. Regulation of *icaR* gene expression in *Staphylococcus epidermidis*. *FEMS Microbiol Lett* **2002**; 216:171–7.
17. Conlon KM, Humphreys H, O’Gara JP. *icaR* encodes a transcriptional repressor involved in environmental regulation of *ica* operon expression and biofilm formation in *Staphylococcus epidermidis*. *J Bacteriol* **2002**; 184:4400–8.

18. Handke LD, Slater SR, Conlon KM, et al. SigmaB and SarA independently regulate polysaccharide intercellular adhesin production in *Staphylococcus epidermidis*. *Can J Microbiol* **2007**; 53:82–91.
19. Knobloch JK, Jager S, Horstkotte MA, Rohde H, Mack D. RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor sigmaB by repression of the negative regulator gene *icaR*. *Infect Immun* **2004**; 72:3838–48.
20. Tormo MA, Marti M, Valle J, et al. SarA is an essential positive regulator of *Staphylococcus epidermidis* biofilm development. *J Bacteriol* **2005**; 187:2348–56.
21. Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M. Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J Infect Dis* **2003**; 188:706–18.
22. Vuong C, Kocianova S, Yao Y, Carmody AB, Otto M. Increased colonization of indwelling medical devices by quorum-sensing mutants of *Staphylococcus epidermidis* in vivo. *J Infect Dis* **2004**; 190:1498–505.
23. Xu L, Li H, Vuong C, et al. Role of the *luxS* quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. *Infect Immun* **2006**; 74:488–96.
24. Heilmann C, Gerke C, Perdreau-Remington F, Gotz F. Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect Immun* **1996**; 64:277–82.
25. Mack D, Nedelmann M, Krokotsch A, Schwarzkopf A, Heesemann J, Laufs R. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. *Infect Immun* **1994**; 62:3244–53.
26. Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, Otto M. Gram-positive three-component antimicrobial peptide-sensing system. *Proc Natl Acad Sci U S A* **2007**; 104:9469–74.
27. Augustin J, Gotz F. Transformation of *Staphylococcus epidermidis* and other staphylococcal species with plasmid DNA by electroporation. *FEMS Microbiol Lett* **1990**; 54:203–7.
28. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Gotz F. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem* **1999**; 274:8405–10.
29. Wang S, He J, Cui Z, Li S. Self-formed adaptor PCR: a simple and efficient method for chromosome walking. *Appl Environ Microbiol* **2007**; 73:5048–51.
30. Bruckner R. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xyloso*. *FEMS Microbiol Lett* **1997**; 151:1–8.
31. Ji Y, Marra A, Rosenberg M, Woodnutt G. Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection. *J Bacteriol* **1999**; 181:6585–90.
32. Gill SR, Fouts DE, Archer GL, et al. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* **2005**; 187:2426–38.
33. Kadurugamuwa JL, Sin L, Albert E, et al. Direct continuous method for monitoring biofilm infection in a mouse model. *Infect Immun* **2003**; 71:882–90.
34. Bae T, Banger AK, Wallace A, et al. *Staphylococcus aureus* virulence genes identified by bursa aurealis mutagenesis and nematode killing. *Proc Natl Acad Sci U S A* **2004**; 101:12312–7.
35. Kaito C, Morishita D, Matsumoto Y, Kurokawa K, Sekimizu K. Novel DNA binding protein SarZ contributes to virulence in *Staphylococcus aureus*. *Mol Microbiol* **2006**; 62:1601–17.
36. Cheung AL, Zhang G. Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. *Front Biosci* **2002**; 7:d1825–42.
37. Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *Embo J* **1993**; 12:3967–75.
38. Chien Y, Manna AC, Cheung AL. SarA level is a determinant of *agr* activation in *Staphylococcus aureus*. *Mol Microbiol* **1998**; 30:991–1001.
39. Otto M. Bacterial evasion of antimicrobial peptides by biofilm formation. *Curr Top Microbiol Immunol* **2006**; 306:251–8.
40. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* **2001**; 276:5707–13.
41. Donvito B, Etienne J, Denoroy L, Greenland T, Benito Y, Vandenesch F. Synergistic hemolytic activity of *Staphylococcus lugdunensis* is mediated by three peptides encoded by a non-*agr* genetic locus. *Infect Immun* **1997**; 65:95–100.
42. Frenette M, Beaudet R, Bisailon JG, Sylvestre M, Portelance V. Chemical and biological characterization of a gonococcal growth inhibitor produced by *Staphylococcus haemolyticus* isolated from urogenital flora. *Infect Immun* **1984**; 46:340–5.
43. Hebert GA, Hancock GA. Synergistic hemolysis exhibited by species of staphylococci. *J Clin Microbiol* **1985**; 22:409–15.
44. Boisset S, Geissmann T, Huntzinger E, et al. *Staphylococcus aureus* RNAlII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev* **2007**; 21:1353–66.
45. Said-Salim B, Dunman PM, McAleese FM, et al. Global regulation of *Staphylococcus aureus* genes by Rot. *J Bacteriol* **2003**; 185:610–9.
46. Valle J, Toledo-Arana A, Berasain C, et al. SarA and not sigmaB is essential for biofilm development by *Staphylococcus aureus*. *Mol Microbiol* **2003**; 48:1075–87.
47. Vuong C, Saenz HL, Gotz F, Otto M. Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis* **2000**; 182:1688–93.
48. Fluckiger U, Wolz C, Cheung AL. Characterization of a *sar* homolog of *Staphylococcus epidermidis*. *Infect Immun* **1998**; 66:2871–8.
49. Massey RC, Horsburgh MJ, Lina G, Hook M, Recker M. The evolution and maintenance of virulence in *Staphylococcus aureus*: a role for host-to-host transmission? *Nat Rev Microbiol* **2006**; 4:953–8.
50. Wright JS 3rd, Jin R, Novick RP. Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc Natl Acad Sci U S A* **2005**; 102:1691–6.