RESEARCH LETTER

MsrR contributes to cell surface characteristics and virulence in *Staphylococcus aureus*

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Abstract

MsrR, a factor contributing to methicillin resistance in *Staphylococcus aureus*, belongs to the LytR-CpsA-Psr family of cell envelope-associated proteins. Deletion of *msrR* increased cell size and aggregation, and altered envelope properties, leading to a temporary reduction in cell surface hydrophobicity, diminished colony-spreading ability, and an increased susceptibility to Congo red. The reduced phosphorus content of purified cell walls of the *msrR* mutant suggested a reduction in wall teichoic acids, which may explain some of the observed phenotypes. Microarray analysis of the *msrR* deletion mutant revealed only minor changes in the global transcriptome, suggesting that MsrR has structural rather than regulatory functions. Importantly, virulence of the *msrR* mutant was decreased in a nematode-killing assay as well as in rat experimental endocarditis. MsrR is therefore likely to play a role in cell envelope maintenance, cell separation, and pathogenicity of *S. aureus*.

Introduction

Staphylococcus aureus has the ability to adapt quickly to changing environmental conditions and possesses a respectable arsenal of virulence factors. A complex regulatory network controls the coordinated expression of surface-associated and exoproteins, which are important in host colonization and virulence (Bronner *et al.*, 2004; Cheung *et al.*, 2004). When exposed to inhibitory concentrations of cell wall-damaging antibiotics, *S. aureus* responds with the upregulation of the cell wall stress stimulon (Utaida *et al.*, 2003; McCallum *et al.*, 2006). The cell wall stress stimulon comprises genes assigned to diverse functional categories, including a number of genes involved in cell wall metabolism. Various members of the cell wall stress stimulon have been associated with methicillin resistance or vancomycin intermediate resistance in *S. aureus* (Utaida *et al.*, 2003; McAleese *et al.*, 2006). One of these genes is *msrR* (methionine sulfoxide reductase regulator), which contributes to the development of high-level resistance to β-lactam antibiotics (Rossi *et al.*, 2003). MsrR belongs to a group of membrane proteins sharing the predicted extracellular LytR-CpsA-Psr domain of unknown function. This protein family is mainly found in Gram-positive bacteria (Hübscher *et al.*, 2008). Few members of the LytR-CpsA-Psr family of proteins have been described so far: LytR of *Bacillus subtilis* is a transcriptional attenuator of itself and of the divergently transcribed *lytABC* operon, which encodes a lipoprotein (LytA), an *N*-acetylmuramoyl-L-alanine amidase (LytC), and LytB, a modifier protein of LytC (Lazarevic *et al.*, 1992). The streptococcal protein CpsA is a transcriptional activator of capsule gene expression (Cieslewicz *et al.*, 2001). BrpA (biofilm-regulatory protein A), a homologue of LytR in *Streptococcus mutans*, is involved in autolysis, biofilm formation, and virulence (Nakano *et al.*, 2005; Wen *et al.*, 2006). Psr was initially proposed to be a repressor of penicillin-binding protein 5 (PBP5) synthesis in *Enterococcus hirae*, but neither a role in repression of PBP5 synthesis nor in regulation of autolysis or resistance to β-lactam antibiotics could be confirmed (Sapunaric *et al.*, 2003). Experiments using the nematode *Caenorhabditis elegans* suggested that both Psr of *Enterococcus faecalis* and MsrR of *S. aureus* play roles in virulence (Bae *et al.*, 2004; Maadani *et al.*, 2007). The LytR-CpsA-Psr family proteins are

presumed to function in cell envelope maintenance, although their functional role has not been elucidated so far.

In this study, we demonstrate that the *S. aureus* LytR-CpsA-Psr family protein MsrR influences cell surface and envelope properties and promotes virulence in both a nematode and a rat endocarditis infection model.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. Cultures were grown in brain–heart infusion (BHI) (Difco) broth with shaking at 180 r.p.m. and 37 °C, unless stated otherwise. Ampicillin 100 μg mL^{-1}, tetracycline 10 μg mL^{-1}, erythromycin 10 μg mL^{-1}, or chloramphenicol 5–10 μg mL^{-1} was added when appropriate.

Allelic replacement of *msrR*

Fragments covering *c.* 0.9-kb regions downstream and upstream of *msrR* were amplified from strain BB270 with primers *msrR*_do_PstI (5′-CCGCTGCAGGATTAAAAATAAACAAGGCG-3′), *msrR*_do_HindIII (5′-CCGAAGCTTATATTAGAACCAATGCCCAC-3′) and *msrR*_up_EcoRI (5′-CCTGAATTCAGTAATGCGTGTAATACGTC-3′), *msrR*_up_BamHI (5′-CGTGGATCCTTTACCTACCTTATATCTTC-3′), respectively, and ligated at 3′ and 5′, respectively, of *ermB* in pEC1. The resulting insert was cloned into the *S. aureus* suicide plasmid pBT to yield pJH011. pJH011 was electroporated into strain RN4220 and erythromycin-resistant transformants were screened for loss of tetracycline resistance. The resulting Δ*msrR*::*ermB* deletion was transduced into *S. aureus* strain MSSA1112 by phage 80α (Berger-Bächi, 1983).

Construction of a complementation plasmid

A fragment containing *msrR*, including its own promoter and terminator regions, was amplified from strain BB270 using primers *msrR*_f_BamHI (5′-GGAGGATCCTTAATTAGTTACATCCTTTCATTAGACCTTAG-3′) and *msrR*_r_BamHI (5′-GGAGGATCCGCAATTCACGTTGTTATAGAAG-3′), and was cloned in the *Escherichia coli–S. aureus* shuttle vector pGC2. The resulting plasmid pGC2*msrR* was electroporated into RN4220, from where it was transduced into the strains of interest.

Northern blot analysis

Cells were harvested from cultures grown in Luria–Bertani (LB) broth and RNA extracted according to Cheung *et al.* (1992). Eight micrograms of total RNA were used for Northern hybridization and probed with digoxigenin-labelled DNA probes as described by Rossi *et al.* (2003).

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant genotype and phenotype	References or sources
Strains		
E. coli		
DH5α	F-ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k-, m_k+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1λ−	Invitrogen
S. aureus		
RN4220	NCTC8325-4, *rsbU*- r- m+; Mc^s	Kreiswirth *et al.* (1983)
BB270	NCTC8325 derivative, Type I SCC*mec* *rsbU*- Δ30 kb SmaI-F; Mc^r	Berger-Bächi (1983)
MSSA1112	Clinical isolate, *bla*; Mc^s	Entenza *et al.* (1997)
J156	MSSA1112, Δ*msrR*(171-984)::*ermB*; Mc^s Em^r	Rossi *et al.* (2003)
JH100	MSSA1112, Δ*msrR*::*ermB*; Mc^s Em^r	This study
JH100 pGC2*msrR*	MSSA1112, Δ*msrR*::*ermB*; pGC2*msrR*; Mc^s Em^r Cm^r	This study
COLn	COL, cured from pT181, Type I SCCmec, Mc^r	Katayama *et al.* (2004)
JH99	COLn, Δ*msrR*::*ermB*; Mc^r Em^r	This study
JH99 pGC2*msrR*	COLn, Δ*msrR*::*ermB*; pGC2*msrR*; Mc^r Em^r Cm^r	This study
Plasmids		
pEC1	*E. coli* plasmid, *ori* ColE1 *bla ermB*; Am^r, Em^r	Bruckner (1997)
pBT	*E. coli* plasmid, *ori* ColE1 *tetL*; Tc^r	Giachino *et al.* (2001)
pJH011	pBT with a 3.3-kb fragment comprising *ermB* flanked by the up- and downstream sequence of *msrR*; Tc^r	This study
pGC2	*E. coli–S. aureus* shuttle plasmid, *ori* ColE1-*ori* pC194 *bla cat*; *E. coli* Am^r *S. aureus* Cm^r	Skinner *et al.* (1988)
pGC2*msrR*	pGC2 containing a 1.3-kb fragment covering *msrR*; *E. coli* Am^r; *S. aureus* Cm^r	This study

Am, ampicillin; Cm, chloramphenicol; Em, erythromycin; Mc, methicillin; Tc, tetracycline; r, resistant; s, susceptible.

Microarray analysis

Overnight cultures were diluted 100-fold into fresh pre-warmed LB medium (Difco) and grown to an $OD_{600\,nm}$ of 0.6. Cells were then mixed (1 : 2) with RNAprotect Bacterial Reagent (Qiagen) as indicated by the manufacturer. RNA was isolated as described by Cheung *et al.* (1992) and purified using the RNeasy kit with on-column DNAseI digestion (Qiagen). RNA integrity was checked on an Agilent 2100 BioAnalyser. cDNA was synthesized using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen) and labelled with either Cy3- or Cy5-dCTP (GE Healthcare). The *S. aureus* PCR product

microarray and hybridization protocols are described in Witney *et al.* (2005). Slides were scanned using an Affymetrix 428 scanner and data were extracted using BLUEFUSE 3.0 (BlueGnome, Cambridge). Three biological replicates of both the wild type and the mutant were hybridized against each other in dye-swap experiments and data from all six arrays were normalized and analyzed using GENESPRING 7.2 (Silicon Genetics). The list of differentially expressed genes includes those with a twofold or greater change in the expression level and *t*-test values of $P \leq 0.05$ (Supporting Information, Table S1).

Oxacillin-susceptibility tests

Minimum inhibitory concentrations (MIC) of oxacillin were determined using Etest strips (AB Biodisk, Solna, Sweden). Qualitative differences in resistance levels were evaluated by swabbing 0.5 McFarland standard cell suspensions along an antibiotic gradient on rectangular LB agar plates. Gradient plates were incubated at 35 °C for 24 h.

Transmission electron microscopy (TEM)

Cells grown to an $OD_{600\,nm}$ of 0.7 or overnight, corresponding to an exponential or a stationary growth phase, respectively, were fixed with 2.5% glutaraldehyde in phosphate-buffered saline at pH 7.2 for 1 h. Electron microscopy was performed at the Center for Microscopy and Image Analysis, University of Zürich.

Adhesion to polystyrene

Adhesion to polystyrene plates, using BHI supplemented with 1% (w/v) glucose as a culture medium, was measured as described previously (Seidl *et al.*, 2008). To test whether biofilm formation depended on polysaccharide intercellular adhesin (PIA) production, proteins, or DNA, cultures were supplemented with 10 mM sodium metaperiodate ($NaIO_4$), 100 μg mL^{-1} proteinase K, or 100 μg mL^{-1} DNAseI, respectively.

Congo red tests

Congo red growth phenotypes were tested by spotting aliquots of 10^7 bacteria mL^{-1}, suspended in 0.9% NaCl, onto BHI agar plates supplemented with 0.08% (w/v) Congo red and 1% (w/v) glucose using a Microtiter AB80 Automatic Inoculator (Dynatech, Dübendorf, Switzerland). For MIC determination, twofold dilutions of Congo red (starting from 32 g L^{-1}) were incorporated into the agar. Congo red agar plates were incubated at 37 °C for 24 h and subsequently at room temperature for an additional 24 h. Congo red binding was assessed in 1 mL of bacteria grown for 4 h at 37 °C and concentrated to an $OD_{600\,nm}$ of 7. Cells were harvested, washed once with 10 mM sodium phosphate buffer, pH 6.8, 85 mM NaCl, and then resuspended in 1 mL of 0.2 mM Congo red diluted in the same buffer, and incubated for 30 min at room temperature with gentle agitation. Cells were harvested and the $OD_{480\,nm}$ of the supernatant was measured to determine the amount of unbound Congo red.

Colony spreading

Colony-spreading ability was assayed as described by Kaito & Sekimizu (2007) using BHI soft agar plates containing 0.24% (w/v) agar and 1% (w/v) glucose. Two microliters of overnight cultures were spotted onto the plates and surface spreading was monitored after 10 h of incubation.

Determination of relative cell surface hydrophobicity

Overnight cultures were diluted 200-fold in BHI broth and grown at 37 °C. Cells were collected by centrifugation at 5000 ***g*** for 5 min, washed twice with 0.15 M NaCl, adjusted to an $OD_{600\,nm}$ of 0.95–1.25 in 0.15 M NaCl, and subjected to a modified microbial adhesion to hydrocarbons (MATH) procedure (Rosenberg *et al.*, 1980). Briefly, 40 μL of hexadecane was mixed with 0.8 mL of cell suspension in a 2-mL polypropylene Eppendorf tube and vortexed twice for 30 s. The phases were allowed to separate for 15 min before the aqueous phase was removed to measure the $OD_{600\,nm}$. The percentage of affinity to hexadecane was calculated using the formula: % affinity = 100 × [1 − (OD after mixing/OD before mixing)]. Experiments were conducted in triplicate.

Preparation and quantification of wall teichoic acids (WTA)

Cell walls and WTA were prepared as described previously (Majcherczyk *et al.*, 2003). The amount of WTA was indirectly quantified by determination of the cell wall phosphorus content as described in Ames & Dubin (1960). Experiments were performed three times with three replicates per sample.

Nematode-killing assay

Infection of *C. elegans* strain Bristol N2 was carried out as described previously (Sifri *et al.*, 2003). Survival was calculated using the Kaplan–Meier method, and survival differences were tested for significance using the log rank test (GRAPHPAD PRISM, version 4.0).

Rat endocarditis model

Catheter-induced aortic vegetations were produced in rats as described previously (Heraief *et al.*, 1982). Groups of rats were inoculated with 10^4 CFUs from cultures in the

exponential growth phase. This is the minimal inoculum producing endocarditis in $>90\%$ of the animals [90% infective dose (ID_{90})] challenged with the wild-type strain, and thus allowed differentiation with putative less-virulent strains. Rats were sacrificed 16 h postinoculation, and bacterial titers, expressed as the mean of $\log_{10}$ CFUs g^{-1} of tissue, were determined in vegetations, spleens, and blood cultures. Statistical differences comparing infection rates were evaluated using the χ^2-test with the Yates correction.

Results

Allelic replacement of *msrR*

The original *msrR* mutant J156 still contained the N-terminal and transmembrane segments (Rossi *et al.*, 2003). To rule out the possibility that previous observations were confounded by the MsrR remains, the complete *msrR* ORF was deleted by allelic replacement in the clinical endocarditis isolate, MSSA1112, yielding strain JH100. As in the truncated mutant J156, β-lactam susceptibility increased slightly in the new mutant JH100. Resistance was restored to the original values by introduction of the wild-type *msrR* in *trans* on plasmid pGC2*msrR* (Fig. 1). Complete deletion of MsrR in the methicillin-resistant strain COLn had the same effect on oxacillin resistance as the truncation of MsrR reported by Rossi *et al.* (2003) (Fig. 1). No effects on the growth rate or spontaneous and Triton X-100-induced autolysis were observed as in the truncated mutant (data not shown), suggesting that both mutants were identical in this respect.

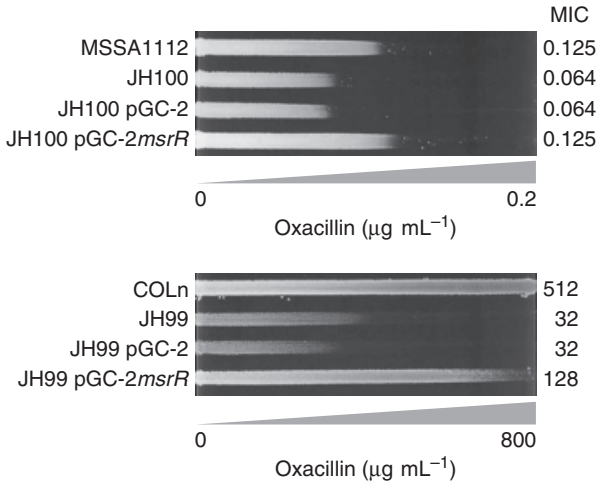

Fig. 1. Effect of the deletion of the entire *msrR* ORF on oxacillin susceptibility in a methicillin-susceptible (MSSA1112) and a methicillin-resistant (COLn) background. The wild-type strains, their respective *msrR* mutants JH100 and JH99, and the mutants harboring either an empty plasmid (pGC2) or an *msrR* complementation plasmid (pGC2*msrR*) were swabbed along antibiotic gradients as indicated. The corresponding MIC values (μg mL^{-1}) are shown.

Microarray analysis

Inactivation of *msrR* was previously reported to result in premature *sarA* transcription (Rossi *et al.*, 2003). If MsrR were to act as a regulator of *sarA*, which controls the expression of exoproteins and virulence factors (Cheung *et al.*, 1992; Chien *et al.*, 1999), changes in the whole-genome transcriptome would be expected. However, differential microarrays from exponentially growing cells, at the time point of the highest *msrR* expression (Rossi *et al.*, 2003), surprisingly identified very few and only very small transcriptional changes. Thirteen genes were upregulated and 18 genes were downregulated in JH100 compared with MSSA1112 (Table S1). Among these genes were representatives of the purine and pyrimidine biosynthesis pathways that appear to be regulated under diverse conditions (Dunman *et al.*, 2001; Resch *et al.*, 2005; Fox *et al.*, 2007; Sobral *et al.*, 2007), and therefore probably do not reflect an *msrR*-specific effect. Moreover, differences in expression levels were very low (median of 2.3). The only gene with a more than fourfold increase was SA1196 (hypothetical protein, similar to DNA damage repair protein), mapping downstream of *msrR*. This upregulation was very likely due to a polar effect from the allelic replacement. Under the conditions chosen, microarray analysis did not reveal any transcriptional changes in known virulence factors or global regulators upon deletion of *msrR*. The influence of MsrR on *sarA* transcription originally reported by Rossi *et al.* proved to be strain and culture dependent. Repetitive comparisons between wild type and both *msr*R mutants mostly showed no differences in *sarA* transcription as shown in Fig. 2.

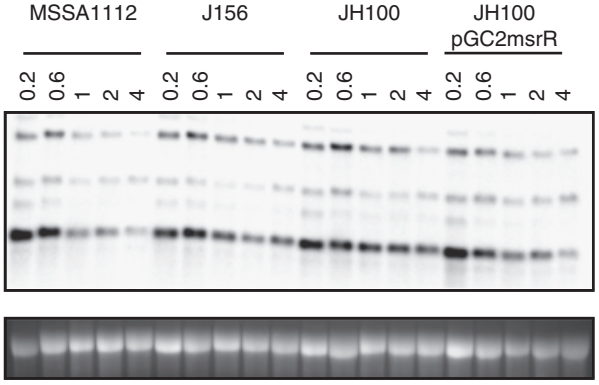

Fig. 2. Northern blots of *sarA* transcripts of wild-type MSSA1112, truncated *msrR* mutant J156, complete *msrR* deletion mutant JH100, and complemented *msrR* mutant JH100 pGC2*msrR*. $OD_{600\,nm}$ time points of cell harvesting are given above the graph.

Cell morphology

The deletion of *msrR* had a profound effect on cell morphology as illustrated by TEM (Fig. 3). The mutant JH100 contained a subpopulation of strikingly enlarged cells. In the exponential growth phase, the average longitudinal diameter of JH100 was 1.30 ± 0.16 μm compared with 1.11 ± 0.12 μm of MSSA1112, corresponding to an average increase of 16.9% ($P < 0.001$, *t*-test). Differences in septal diameters were less pronounced, resulting in a higher ratio of longitudinal to septal diameter in JH100 of 1.19 ± 0.09 compared with 1.13 ± 0.08 in MSSA1112 ($P < 0.001$). Cells of both the wild type and the mutant were slightly smaller in the stationary growth phase, but JH100 retained on average a 5.2% longer longitudinal diameter than MSSA1112. There was no apparent difference in the appearance of the cell wall. Interestingly, a few cells of strain JH100 showed an aberrant septum positioning that was parallel to the already initiated or completed septum (Fig. 3g and h), while none of the wild-type cells displayed parallel septa.

Cell aggregation and biofilm formation

When JH100 was grown with constant agitation in BHI broth, enhanced sedimentation was observed (Fig. 4a) and a ring of adherent bacteria formed at the liquid–air interface. Complementation of JH100 with pGC2*msrR*, containing the *msrR* wild-type allele, reversed the effect. On polystyrene surfaces, JH100 formed a thicker biofilm than MSSA1112, and the amount of biofilm could be reduced again to wild-type levels by complementation with the plasmid pGC2*msrR* (Fig. 4b), showing that biofilm formation was indeed influenced by *msrR* inactivation. Dispersal of the biofilm by proteinase K and DNAseI, but not by $NaIO_4$ (Fig. 4c), demonstrated that it was composed mostly of protein and extracellular DNA and not PIA.

Congo red sensitivity

On Congo red agar, MSSA1112 and JH100 formed crusty colonies that slowly turned black during incubation, an appearance that has been linked to PIA production. However, the correlation of colony appearance on Congo red agar and production of PIA in *S. aureus* is debatable (Knobloch *et al.*, 2002), and also not supported here by the lack of susceptibility of the JH100 biofilm to metaperiodate. A remarkable observation was the reduced colony size of the mutant JH100 compared with the wild type on Congo red plates (Fig. 4d), although the colonies did not differ in size on plates without Congo red (data not shown). Reduced growth of JH100 could be attributed to a strongly increased susceptibility of JH100 to Congo red, with the MIC decreasing from 16 to $< 0.5\ g\ L^{-1}$. Congo red resistance was restored by introducing plasmid pGC2*msrR*. The Congo red-binding capacity of JH100 was identical to that of MSSA1112 in an *in vitro* binding assay (data not shown).

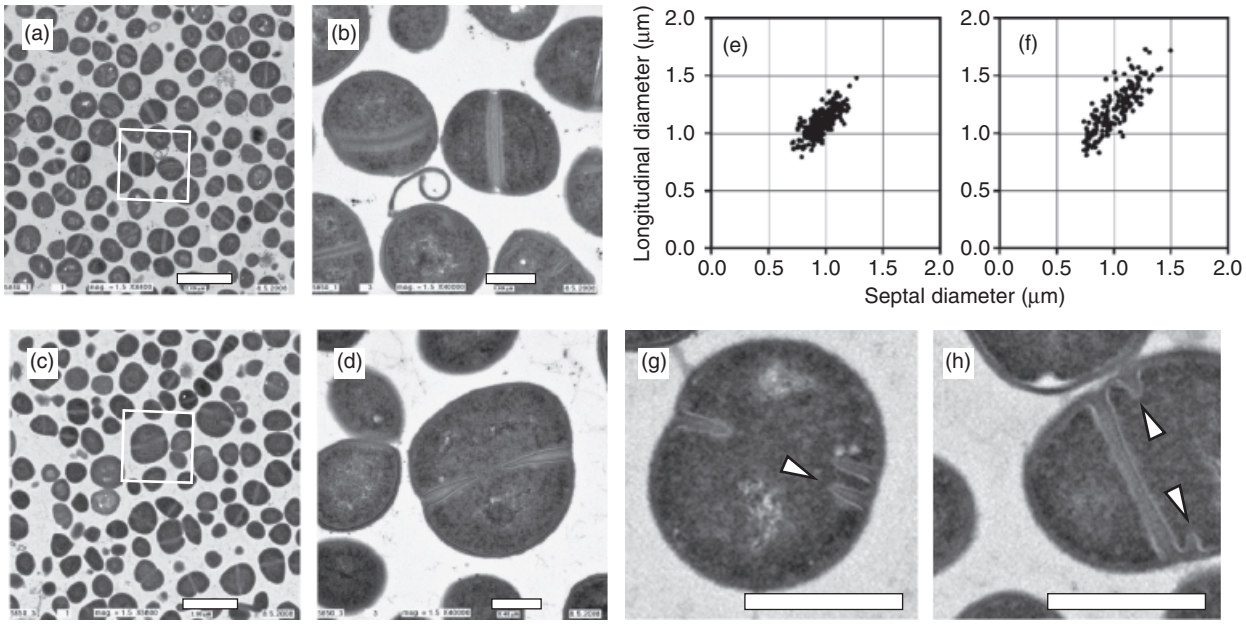

Fig. 3. Cell morphology. TEM pictures of the wild-type strain MSSA1112 (a and b) and of the *msrR* mutant JH100 (c and d). The white box in (a) and (c) indicates the region shown at a higher magnification in (b) and (d). The white bars indicate 2 μm in (a) and (c) and 0.4 μm in (b) and (d). (e and f) Ratios between the longitudinal and the septal diameters of MSSA1112 (e) and of JH100 (f) in exponential and stationary growth phases. Diameters of all cells within five microscopic fields (80–120 cells per strain and growth phase) with visible septa (either complete or incomplete) were measured using IMAGEJ (National Institutes of Health NIH). (g and h) Initiation of parallel septa (white arrows) observed in cells of the *msrR* mutant JH100. The white bar indicates 1 μm.

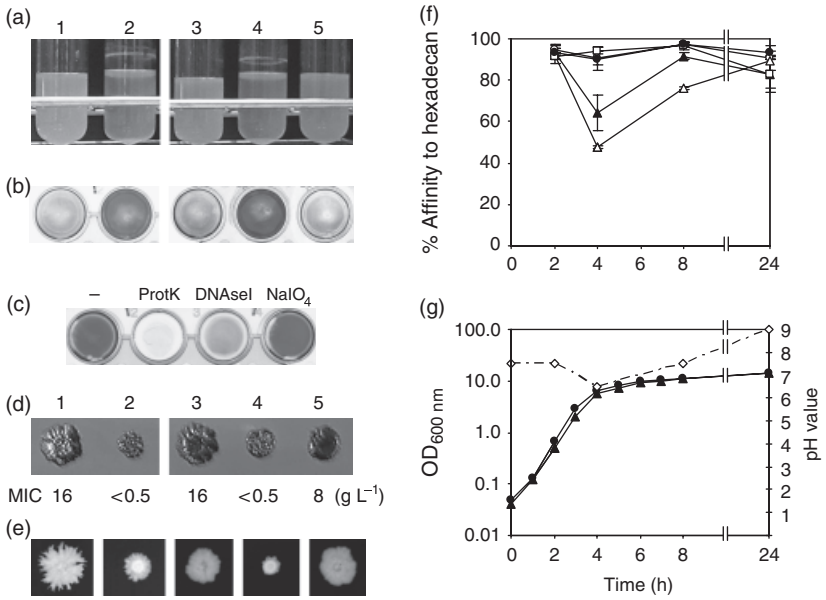

Fig. 4. Cell surface-associated characteristics of (1) MSSA1112, (2) JH100, (3) MSSA1112 pGC2, (4) JH100 pGC2, and (5) JH100 pGC2*msrR*. (a) Growth in glass tubes. (b) Saffranin-stained biofilms formed in polystyrene 12-well plates after growth in BHI broth with 1% (w/v) glucose. (c) Effect of proteinase K (Prot K), DNAseI, and $NaIO_4$ on biofilm formation of the *msrR* mutant JH100. (−), untreated control. (d) Growth on BHI agar supplemented with 0.08% (w/v) Congo red and 1% (w/v) glucose. The respective Congo red MICs as obtained by LB agar dilution are given. (e) Colony-spreading ability on BHI 0.24% (w/v) agar plates supplemented with 1% (w/v) glucose. (f) Relative cell surface hydrophobicity illustrated by the affinity to hexadecane after growth in BHI broth for 2, 4, 8, and 24 h. The data represent the means and SDs from three replicates of a representative experiment. Closed circles, MSSA1112; closed triangles, JH100; open circles, MSSA1112 pGC2; open triangles, JH100 pGC2; and open squares, JH100 pGC2*msrR*. (g) Representative growth curves of MSSA1112 and the *msrR* mutant JH100 in BHI broth and changes in the pH value of the culture media during growth. Closed circles, MSSA1112; closed triangles, JH100; and open diamonds, pH values (which were identical for both strains).

Surface-spreading ability

MSSA1112 spread rapidly on soft agar, forming giant colonies with dendritic borders. This spreading ability was clearly diminished by the deletion of *msrR* (Fig. 4e). On plates supplemented with chloramphenicol, to maintain the pGC2-control or -complementing plasmids, all surface areas appeared smoother (Figs 4e, 3–5). Complementation of the *msrR* mutant with pGC2*msrR* restored spreading.

Relative cell surface hydrophobicity

Relative cell surface hydrophobicity was assessed over the growth cycle by MATH (Fig. 4f and g). MSSA1112 showed a constant high affinity to hexadecane (> 90%) over the entire growth curve. In the mutant JH100, the relative cell surface hydrophobicity decreased after 4 h at the transition to the stationary growth phase, with an affinity to hexadecane around 50%; and increased again after 8 h, reaching wild-type values after 24 h. Strikingly, the decrease in the hydrophobicity of JH100 correlated with a decline in the pH value in the medium. The mutant carrying plasmid pGC2*msrR* behaved like the wild type. Samples of the 4-h cultures of MSSA1112 and JH100 were examined microscopically after incubation with hexadecane. While hexadecane droplets were sparsely populated by the *msrR* mutant, they were completely covered by the wild-type cells (data not shown).

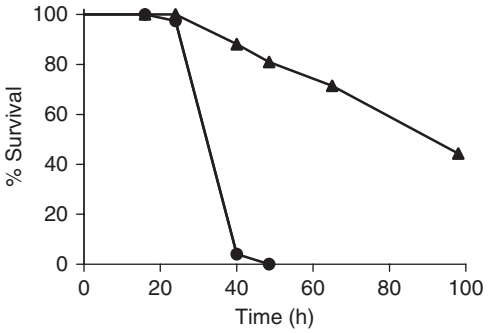

Fig. 5. Survival of nematodes that were fed wild-type strain MSSA1112 (circles, $n = 90$) and *msrR* mutant JH100 (triangles; $n = 90$). Data are representative of one of three independent experiments.

Teichoic acid content

Differences in cell surface characteristics, such as altered spreading capacity and changes in hydrophobicity, have been reported to depend on teichoic acids and their

composition (Fedtke *et al.*, 2007; Kaito & Sekimizu, 2007). Measurements of the phosphorus content of wild type ($0.85 \pm 0.07\ \mu mol\ mg^{-1}$ of purified cell walls) and mutant cell walls (0.63 ± 0.07) showed a distinct and statistically significant 25% decrease in the amount of WTA in the mutant ($P < 0.05$, Student's *t*-test). In the cell wall of the complemented mutant JH100 pGC2*msrR* (0.86 ± 0.01), wild-type levels of phosphorus could be measured. The muropeptide fingerprint patterns remained identical in the wild type and the *msrR* mutant (data not shown).

Virulence

Caenorhabditis elegans killing was highly attenuated in the MSSA1112 *msrR* null mutant JH100 (Fig. 5) ($P < 0.01$, log rank test), confirming the similar findings of Bae *et al.* (2004) in strain Newman. Moreover, reduced virulence of JH100 was confirmed in a rat model of experimental endocarditis. While all animals ($n = 9$) challenged with the ID_{90} of the wild-type strain MSSA1112 (10^4 CFU) could be infected, only seven of 16 (43%) rats inoculated with JH100 developed infected vegetations ($P < 0.05$ by a χ^2-test). The bacterial load within infected vegetations, though, was identical in the wild type (mean $7.8 \pm 2\ \log_{10}\ CFUs\ g^{-1}$) and the *msrR* mutant ($7.53 \pm 2\ \log_{10}\ CFUs\ g^{-1}$). Eight of nine animals infected with the wild type and seven of 16 animals infected with the *msrR* mutant showed positive blood cultures ($P < 0.05$, χ^2-test). Infection was also assessed in spleens. All animals had infected spleens irrespective of the challenging strain, demonstrating appropriate inoculation of the animals.

Discussion

Members of the LytR-CpsA-Psr family are associated with cell wall maintenance and envelope properties, such as autolysis, capsule biosynthesis, and biofilm formation, and have been reported to influence virulence and to have a regulatory function (Lazarevic *et al.*, 1992; Cieslewicz *et al.*, 2001; Chatfield *et al.*, 2005; Wen *et al.*, 2006).

Microarrays showed that *msrR* deletion had a negligible impact on the global transcriptome during early growth, at the time point of the highest *msrR* transcription. The MsrR-dependent reduction of *sarA* expression observed by Rossi *et al.* (2003) could neither be confirmed by microarray data nor by successive Northern blots, suggesting that it was likely a temporal effect and/or dependent on the growth conditions.

Inactivation of *msrR* had a marked impact on cell surface properties, resulting in a prominent reduction of the cell surface hydrophobicity. The effect was transient, and restored in the early stationary phase. The consistently high surface hydrophobicity of wild-type *S. aureus* may therefore depend on MsrR or a factor influenced by MsrR in a growth phase-dependent manner. In contrast, Nakano *et al.* (2005) observed an increased cell surface hydrophobicity upon *brpA* deletion in *S. mutans*. Because MsrR is more closely related to Psr of *Enterococcus* than to BrpA (Hübscher *et al.*, 2008), it may have a different biological role, even though it belongs to the same protein family and shares functional similarities.

MsrR seems to prevent biofilm formation, because its deletion increased cell-to-cell aggregation. The biofilm of the *msrR* mutant did not depend on PIA, but consisted of protein and DNA. PIA-independent biofilms have been described (Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005), and components associated with biofilm formation include proteins (Kajimura *et al.*, 2005; Biswas *et al.*, 2006), teichoic acids, and DNA (Sadovskaya *et al.*, 2006; Rice *et al.*, 2007). Rice *et al.* (2007) demonstrated that reduced DNA release, due to reduced cell lysis, accounted for the defect in biofilm formation of *cidA* mutants. The *msrR* mutant, however, did not exhibit altered autolytic activity (data not shown) and results of the microarrays did not indicate altered gene expression of known key components of biofilm formation. Like the inactivation of *msrR* in *S. aureus*, inactivation of *brpA* in *S. mutans* also caused increased cell sedimentation (Wen & Burne, 2002), but in contrast to the *msrR* mutants, *brpA* mutants showed limited biofilm formation and enhanced autolytic activities (Wen & Burne, 2002; Nakano *et al.*, 2005; Wen *et al.*, 2006).

Interestingly, *brpA* mutants produced longer chains than the wild type, which might indicate a defect in cell separation (Wen & Burne, 2002; Chatfield *et al.*, 2005; Nakano *et al.*, 2005; Wen *et al.*, 2006). Although the autolysis rate of the *msrR* mutant did not differ from the wild type, the increased cell size and the occasional occurrence of parallel septa suggested impaired cell separation and aberrant septum initiation and positioning. Whereas a proportion of mutant cells were larger than wild-type cells, cell wall thickness, and the appearance was not affected nor was there an alteration in peptidoglycan composition. These results suggest that peptidoglycan synthesis was not influenced by MsrR. Enlarged cells and the formation of parallel septa have been described before in *S. aureus*, in a conditional mutant of *pbpA* (Pereira *et al.*, 2007). As in the *msrR* mutant, only some of the cells were affected.

Interestingly, the *msrR* mutant was highly susceptible to Congo red dye. Both the wild type and the mutant possessed a single protein, which specifically bound Congo red with similar binding affinity (data not shown), arguing against a direct interaction between Congo red and MsrR. There are various possible Congo red interaction mechanisms that include the formation of hydrogen bonds via the H-atoms of the amino groups, an electrostatic interaction via the negatively charged sulfonate groups, a hydrophobic interactions via the aromatic rings, and a steric intercalation between β-sheets (Nilsson, 2004). Hence, the presence of congo red is also likely to unspecifically influence cell envelope compounds, for example by introducing a negative

charge or by inducing conformational changes. Therefore, altered cell surface properties such as increased aggregation and changes in hydrophobicity could render the *msrR* mutant more susceptible to Congo red.

Kaito and Sekimizu observed that *S. aureus* is able to spread on wet surfaces such as semi-solid agar plates (Kaito & Sekimizu, 2007). Bacterial surface motility depends on surface-active compounds of the cell envelope, which improve wettability (Harshey, 2003), and on rapid growth (Kinsinger *et al.*, 2005). For *S. aureus*, it has been shown that mutations in *tagO*, *dlt*, or *ypfP*, which are involved in WTA or lipoteichoic acid biosynthesis, diminish spreading ability, and it was proposed that teichoic acids might function as surfactants (Kaito & Sekimizu, 2007). The products of the *dltABCD* operon are important for alanylation of teichoic acids and YpfP is involved in glycolipid synthesis, and the lack of this protein significantly reduces the amount of lipoteichoic acids (Peschel *et al.*, 1999; Fedtke *et al.*, 2007). The growth rate was not affected by *msrR* deletion, but a diminished WTA content could be detected, which could be complemented by pGC2*msrR*. Teichoic acids are known to affect the regulation of autolysins (Bierbaum & Sahl, 1987) and both *dlt* and *ypfP* mutants have reduced autolytic activities and defects in biofilm formation (Peschel *et al.*, 1999; Fedtke *et al.*, 2007). However, autolysis was not affected in *msrR* mutants and biofilm formation was enhanced, which would imply changes different from those induced by *dlt* or *ypfP*. Alternatively, it is conceivable that the increased cell aggregation of the *msrR* mutant prevented spreading on semi-solid agar plates, and that physico-chemical changes in cell surface properties enhanced the phenotype.

A pathogen's surface characteristics can contribute significantly to its infectivity (Jordan *et al.*, 2008). Therefore, the effects of MsrR on the cell envelope of *S. aureus* may be the reason for the attenuated nematode-killing capacity of the *S. aureus msrR* deletion mutant as well as its decreased virulence in the rat endocarditis model. The attenuated virulence could also be attributed to the reduction in WTA because this surface structure has been implicated previously in staphylococcal pathogenicity (Weidenmaier *et al.*, 2005a, b). It might appear intriguing that although the *msrR* mutant produces more biofilm, it is less virulent *in vivo*. However, a lack of correlation between biofilm formation and virulence has been observed previously in several experimental models, including the model of endocarditis (Kristian *et al.*, 2004; Bizzini *et al.*, 2006).

Members of the LytR-CpsA-Psr protein family described so far have generally been shown to affect cell envelope properties. This study adds MsrR to this group, showing that MsrR inactivation influences cell size, aggregation, biofilm formation, colony spreading, Congo red susceptibility, and WTA content. MsrR seems to be particularly important during exponential growth, when the demand for nutrients is high and the cells are undergoing cell separation. This corresponds well to the high expression level of *msrR* during early growth stages (Rossi *et al.*, 2003). Moreover, this study confirmed and extended previous results indicating that MsrR is important in staphylococcal virulence. The mode of action of MsrR remains unclear, but the decreased levels of WTA in the *msrR* mutant suggest an influence on this major component of the staphylococcal cell envelope, which will be further investigated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Differentially regulated genes in the *msrR* deletion mutant JH100 in exponential growth phase.

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