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MsrR, a Putative Cell Envelope-Associated Element Involved in *Staphylococcus aureus sarA* Attenuation

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A novel membrane-associated protein, MsrR, was identified in *Staphylococcus aureus* which affects resistance to methicillin and teicoplanin, as well as the synthesis of virulence factors. MsrR belongs to the LytR-CpsA-Psr family of cell envelope-related transcriptional attenuators and was shown to be inducible by cell wall-active agents, such as β-lactams, glycopeptides, and lysostaphin. The expression of *msrR* peaked in the early exponential growth phase and decreased sharply thereafter. *msrR* mutants showed increased *sarA* transcription and an earlier and higher expression of RNAIII, resulting in altered expression of virulence factors such as alpha-toxin and protein A. These observations suggest that MsrR is a new component involved in *sarA* attenuation and the regulatory network controlling virulence gene expression in *S. aureus*.

Staphylococcus aureus, an inhabitant of the skin and mucous membranes in 10 to 30% of the healthy population, easily survives in the environment and is, moreover, the most common cause of community-acquired and nosocomial infections. The pathogenicity of *S. aureus* is based on an impressive repertoire of virulence factors. The diseases caused range from superficial infections of the skin to life-threatening infections such as septicemia, endocarditis, osteomyelitis, and toxic shock syndrome. Virulence factors comprise surface-associated and extracellular proteins such as toxins and enzymes. Cell surface-associated proteins, the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), function in adhesion and colonization of the host and evasion of host defenses. Cell surface-associated factors are needed primarily during the initial stages of infection and are expressed mainly in the early exponential growth phase. The exoenzymes, which damage the host tissue and promote dissemination of the pathogen and most soluble exoproteins, have a role in a later stage of the infection and are produced in the postexponential phase. The coordinate and timely expression of those virulence factors during the growth cycle is governed by a complex network comprising two-component sensor transducers, global regulatory systems, and transcription factors, which are important for the pathogen to adapt to the changing host environment during different stages of infection (2, 21, 23, 26). One of the two major global regulatory systems is the *agr* (accessory gene regulator) regulon. This locus consists of two divergent transcripts originating from the promoters P2 and P3, which produce RNAII and RNAIII, respectively. The RNAII transcript encodes four proteins, AgrA to AgrD. AgrA and AgrC form a two-component regulatory system whereby AgrC acts as the signal receptor and AgrA acts as sensor regulator. The signal is a small peptide processed from AgrD by AgrB. This self-encoded autoinducing peptide determines the specificity group of *S. aureus* (29). The autoinducer leads from the phosphorylation of AgrC to the phosphorylation of AgrA, which upregulates both P2 and P3 promoters of the *agr* regulon. The RNAIII molecule is the effector molecule, which controls the expression of cell wall-associated and virulence proteins in a growth-dependent manner (36). The second major regulatory system is the *sarA* (staphylococcal accessory regulator A) locus (14, 18). SarA is a transcriptional regulator that binds to a consensus motif in the promoter of its target genes and has positive and negative regulatory effects on extracellular protein synthesis (48). SarA interacts with the *agr* system by binding to the *agr* promoter region, to stimulate the transcription of RNAII and RNAIII from the P2 and P3 promoters, respectively (4). SarA forms part of a family of SarA-related proteins, which participate in the SarA-RNAIII regulatory cascade and have regulatory functions as well (16).

Global regulators and effector molecules are important in the life cycle of *S. aureus*. They not only govern virulence factors but also are involved in the modulation of antibiotic resistance levels. Compromised *agr* function was found to help clinical isolates of *S. aureus* develop vancomycin heteroresistance (39). The *sarA* and *agr* operons were found to affect methicillin resistance (37), and high activity of transcription factor σ^B was shown to correlate with increased teicoplanin resistance (9) and high-level methicillin resistance (46). In this work we describe a novel potential sensor of cell wall damage, which influences *sarA* and *agr* transcription.

MATERIALS AND METHODS

Strains and culture conditions. The strains and plasmids used are listed in Table 1. Strains were grown in Luria-Bertani broth (Becton Dickinson, Sparks, Md.) at 37°C unless specified otherwise. Transductions were performed with phage 85 (5). Erythromycin at 10 μg ml^{-1}, tetracycline at 10 μg ml^{-1}, or kanamycin at 20 μg ml^{-1} was added to the medium when needed. MICs of antibiotics were determined by Etest (AB-Biodisk, Solna, Sweden) on Mueller-Hinton agar plates with an inoculum of an 0.5 McFarland standard after 24 h of incubation at 35°C or by broth microdilution as recommended by the NCCLS (35). MICs of teicoplanin and vancomycin were determined on brain heart infusion agar after 48 h of incubation at 35°C. Antibiotic resistance levels were compared by swabbing an 0.5 McFarland standard suspension of overnight cultures along an antibiotic gradient in rectangular agar plates.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and phenotype[a]	Reference or source
Escherichia coli		
DH5α	F^- ϕ80d/acZΔM15 *recA1*	Invitrogen
Staphylococcus aureus		
RN4220	NCTC8325-4 r^- m^+; recipient for electroporations	25
J126	RN4220 *msrR*::*ermB*	This study
BB270	Essentially the same as NCTC8325; *rsbU mec*	6
BB1259	BB270 Ω2020*chr*::Tn*551*	44
J141	BB270 *msrR*::*ermB*	This study
J190	J141[pAW17(*msrAR*)]	This study
J191	J141(pAW17)	This study
BB938	Essentially the same as 8325; Tc^r	10
J161	BB938 *msrR*::*ermB*	This study
MSSA1112	Clinical isolate; *bla*	20
J156	MSSA1112 *msrR*::*ermB*	This study
J175	MSSA1112 *msrR*::pEC1($msrR_p$-*luc*+) $msrR^+$	This study
J198	MSSA112[pBUS($mecA_p$-*luc*+)]	This study
J192	J156[pBUS($mecA_p$-*luc*+)]	This study
J209	J156(pAW17)	This study
J210	J156[pAW17(*msrAR*)]	This study
Plasmids		
pAW17	*S. aureus-E. coli* shuttle vector *ori* pAMα1-*ori* ColE1 *aac-aph*; Gm^r Km^r	This study
pAW17 (*msrAR*)	1.5-kb fragment covering *msrAR* cloned in pAW17	This study
pBUS1	*S. aureus-E. coli* shuttle vector pAW8 (42) with multicloning site from pBluescript II SK (Stratgene) and the $rrnT1_4$ terminator sequence from pLL2443 (24)	S. Burger, unpublished data
pJR3	pBT with a 3.45-kb insert containing the *ermB* cassette from pEC1 substituting for *msrR*, flanked on both sides by 1-kb chromosomal sequences from BB270; Tet^r	This study
pEC1($msrR_p$-*luc*+)	2.3-kb *Kpn*I-*Eco*RI $msrR_p$-*luc*+ fragment cloned in pEC1; Ap^r Erm^r	This study
pBUS1($mecA_p$-*luc*+)	2.5-kb *Kpn*I-*Eco*RI $mecA_p$-*luc*+ fragment cloned in pBUS1; Tet^r	This study

[a] Abbreviations: Ap^r, ampicillin resistant; Erm^r, erythromycin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; Mc^r, methicillin resistant; Tc^r, teicoplanin resistant; Tet^r, tetracycline resistant.

Molecular biological methods. Standard molecular biology procedures were performed essentially according to the protocols of Sambrook et al. (40) and Ausubel et al. (3). Total RNA was isolated as described by Cheung et al. (12), with a FastRNA kit and a Fastprep reciprocating shaker (Bio 101, Vista, Calif.). Digoxigenin-labeled DNA was produced using the PCR DIG Probe synthesis kit (Roche, Rotkreuz, Switzerland). Primers used are listed in Table 2. Probes for *sarA* and RNAIII were those described earlier (9, 22). For Northern blots, 6 μg of total RNA was loaded per lane. Chromosomal sequencing was performed according to the protocol of A. Wada (45) on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). For sequencing the *msr* region a 1.8-kb fragment was amplified using primers JR1 and JR2. Homology searches were performed with the program Blast (http://www.ncbi.nm.nih.gov/BLAST) (1). Sequence data were analyzed with the GCG sequence analysis software package, version 9 (Genetics Computer Group, University of Wisconsin, Madison). Primer extension was performed using 40 μg of total RNA from early exponential growth and 3 pmol of the γ-^{32}P-labeled primer JR7 or JR8 for *msrR* or JR11 and JR12 for *msrA*, respectively, with Omniscript reverse transcriptase (Qiagen, Hilden, Germany), according to the instructions of the manufacturer. The sequencing reaction was performed with the Thermo Sequenase cycle sequencing kit (U.S. Biochemicals, Cleveland, Ohio).

Construction of mutants and plasmids. For allelic replacement of *msrR*, a 1-kb fragment covering the 5′ region of *msrR* was amplified from strain BB270 with primers JR3 and JR4 and cloned 5′ to the *ermB* resistance cassette of vector pEC1 (11). A 1-kb fragment covering the 3′ region of *msrR* amplified with primers JR5 and JR6 was subsequently cloned downstream of the *ermB* cassette. The insert was then subcloned into the suicide vector pBT (22), resulting in plasmid pJR3, which was electroporated into RN4220. Mutants with the *msrR*::*ermB* allelic replacement were selected for Erm^r and screened for loss of Tet^r. The *msrR*::*erm*B construct was then transduced into different genetic backgrounds. The plasmid pAW17(*msrAR*) used for complementation was constructed by cloning a 1.7-kb DNA fragment generated with primers JR9 and JR10, covering *msrAR*, into pAW17. Luciferase reporter gene fusions were constructed by amplifying the desired promoter regions and cloning them 5′ to the firefly luciferase gene *luc*+ of the pSP-*luc*+ vector (Promega, Madison, Wis.). For $msrR_p$-*luc*+, a DNA fragment covering 709 bp of the *msrR* promoter region of strain MSSA1112 was generated by PCR with upstream primer JR13, including a *Kpn*I linker, and downstream primer JR14, including an *Nco*I site. For $mecA_p$-*luc*+, a DNA fragment covering 895 bp of the *mecA* promoter region of the SCC*mec* element of *S. aureus* BB270 was generated by PCR with upstream primer JR15, including a *Kpn*I site, and downstream primer JR16, including an *Nco*I site. The PCR products obtained were digested with *Kpn*I and *Nco*I and

TABLE 2. Primers

Primer	Sequence[a] (5′-3′)
JR1	GGGATCCTGAGCTAAAGTTAAGTCGCC
JR2	TGAATTCAAACCATTATGGTGTGGCTGG
JR3	CGGATCCTGGCATATTCTACACCGC
JR4	GGAATTCAGTTATGCCTGATGCGCTTGG
JR5	CATTGACTGCAGCAATCAAGAACTCATACGAAG
JR6	GACTAAGCTTCGCTTCAACATCCATAGC
JR7	CGTTGTCATTAGTTTCTTTATC
JR8	TCAGAAGAATGATAGGTAATTTC
JR9	ATTTCAGATCTGTACCGTATTTTAATATTG
JR10	ATTTCAGATCTGAGTATGATAGAAATCG
JR11	GCAAAATAAGCTGTATTAATATTC
JR12	CAAATGGTTTCGTCATACAC
JR13	GGGTACCTGAGCTAAAGTTAAGTCGCC
JR14	TATCCATGGTTACCTACCTTATATCTTC
msrR	AATGGACCAGTAAAAAATGATG
msrR for rev	CCTCGGATACCAAAACTCAAAC
msrA	GGGATCCTGAGCTAAAGTTAAGTCGCC
msrA for rev	GAGGTACCTTTTGGTGTATGACGAAACC
sarR	CTTCTAATTCTGAAATCAG
sarR for rev	GACATTAATGATTTAGTCAAC
hla	GTGATGGAAAATAGTTGATGAG
hla for rev	GTGAATTTGTTCACTGTGTCG
spa	TGAATTCGTAAACTAGGTGTAGG
spa for rev	CGGTACCAGGCTTGTTATTGTCTTCC

[a] Restriction enzyme sites used in this work are underlined.

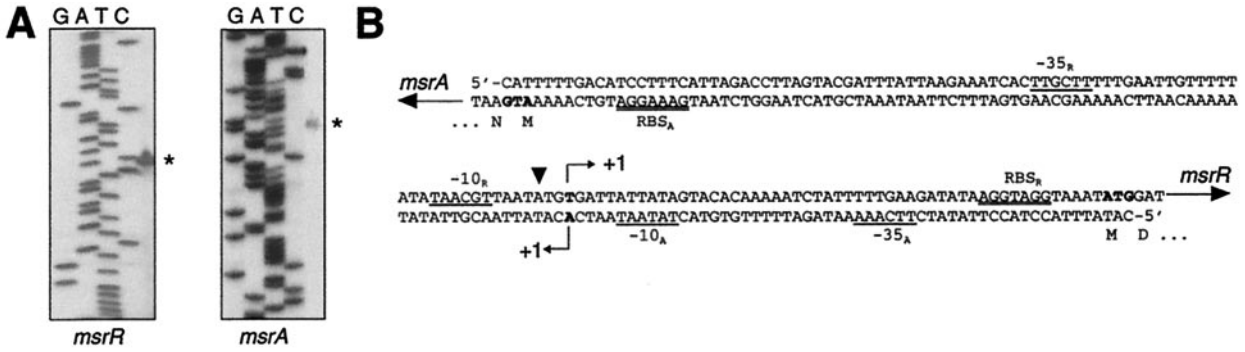

FIG. 1. Genetic organization of the *msrAR* region. (A) Transcriptional start points (*) for *msrR* and *msrA* were determined by primer extension as outlined in Materials and Methods. (B) *msrA-msrR* intercistronic region. Transposon Tn*551* integration site (inverted triangle), transcriptional starts of *msrA* and *msrR* (+1), ribosomal binding sites (RBS), and −10 and −35 boxes are indicated.

cloned in frame with the 5′ end of the luciferase gene of plasmid pSP-*luc*+. Sequence analysis confirmed the identity of the constructs. The 2.3-kb *Kpn*I-*Eco*RI fragment, including the *msrR* promoter region fused to the luciferase coding region, was subsequently cloned into suicide plasmid pEC1 to obtain plasmid pEC1(*msrR*$_p$-*luc*+), which was electroporated into RN4220 and transduced into MSSA1112, resulting in strain J175. Plasmid pBUS1(*mecA*$_p$-*luc*+) was generated by cloning the 2.5-kb *Kpn*I-*Eco*RI fragment, including the *mecA* promoter region fused to the luciferase coding region, into the *Escherichia coli-S. aureus* shuttle vector pBUS1. All constructs were verified by restriction analysis, Southern blotting, and pulsed-field gel electrophoresis.

Enzymatic assays. Luciferase activity was determined as described earlier (9) using the luciferase assay substrate and a Turner Designs TD-20/20 luminometer (Promega). Spontaneous autolysis was measured in cells that were grown at 37°C, harvested at mid-exponential phase, washed twice with cold phosphate-buffered saline, and resuspended in 0.05 M Tris-HCl (pH 7.5), by determining turbidity loss as optical density at 600 nm (OD_{600}) when incubated with shaking (200 rpm) at 37°C. Penicillin-binding proteins (PBPs) from membrane preparations of exponentially growing cells were labeled with [^{3}H]penicillin and separated on sodium dodecyl sulfate-polyacrylamide gels as described earlier (8).

Nucleotide sequence accession number. The nucleotide sequence was deposited in the EMBL and GenBank nucleotide sequence databases under accession number AF311784.

RESULTS

Insertion site characterization. The insertion Ω2020::Tn*551* was reported to increase susceptibility to β-lactams in *S. aureus*, but its nature remained unsolved (44). Sequencing of the insertion site revealed that the transposon had integrated into the intercistronic region of two divergently transcribed genes, tentatively termed *msrA* and *msrR*, which are separated by 136 nucleotides (Fig. 1). The *msrA* gene was shown to code for a peptide methionine sulfoxide reductase, MsrA2, specific for the S isomer of methionine sulfoxide (34). The *msrR* gene has not yet been characterized; the deduced amino acid sequence includes a hypothetical protein of 327 amino acids with 44% identity and 66% similarity to *Enterococcus faecalis* Psr (U42211) and 42% identity and 66% similarity to *Bacillus subtilis* LytR, which belong to the LytR-CpsA-Psr family of cell envelope-related transcriptional attenuators. A predicted transmembrane domain in the N-terminal part of MsrR divides MsrR into a shorter, presumably intracellular domain and a larger, extracellular domain. Analysis of the MsrR amino acid sequences obtained by the public genome sequencing projects revealed that the protein is strictly conserved among *S. aureus* strains.

The open reading frames of *msrA* and *msrR* are each terminated by a structure resembling a putative rho-independent terminator, suggesting that they lie on monocistronic transcripts. Interestingly, primer extension showed that *msrA* and *msrR* share the same nucleotide for the transcriptional start (Fig. 1). The Tn*551* insertion mapped 2 nucleotides upstream of the *msrA* transcriptional start. Northern blots showed that Ω2020::Tn*551* abolished the transcription of both *msrA* and *msrR* (data not shown).

Phenotype of *msrR* inactivation. To determine if only one of the two inactivated genes was responsible for the increased β-lactam susceptibility, we replaced *msrR* with an *ermB* resistance cassette in strain RN4220 and transduced the inactivated *msrR*::*ermB* gene into the methicillin-resistant strain BB270. The resulting strain, J141, showed a fourfold-lower resistance to oxacillin than did the parent (Fig. 2), suggesting that *msrR* inactivation was responsible for the reduced resistance. Oxacillin resistance could be restored by complementing strain J141 with plasmid pAW17(*msrAR*), supporting the MsrR effect on oxacillin resistance levels (Fig. 2). Transduction of *msrR*::*ermB* into the teicoplanin-intermediate-resistant strain BB938 reduced the teicoplanin MIC for the strain twofold, from 16 to 8 μg ml^{-1}, showing that MsrR had an effect on teicoplanin resistance as well.

Expression of *msrR* during growth. The transcription profile of *msrR* during growth in liquid culture was determined by two different methods. Both the Northern blots with the wild-type strain MSSA1112 and the *msrR*$_p$-luciferase reporter activity in

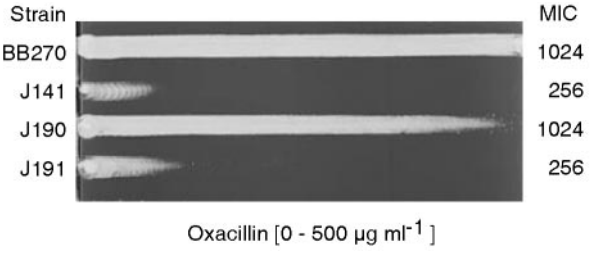

FIG. 2. Effects of *msrR* inactivation and complementation of oxacillin resistance. The methicillin-resistant strain BB270, its *msrR* mutant J141, strain J190 [J141 complemented by pAW17(*msrAR*)], and control strain J191 [J141(pAW17)] were swabbed along a plate containing an oxacillin gradient. The corresponding MICs of oxacillin (in micrograms per milliliter) for the strains are indicated.

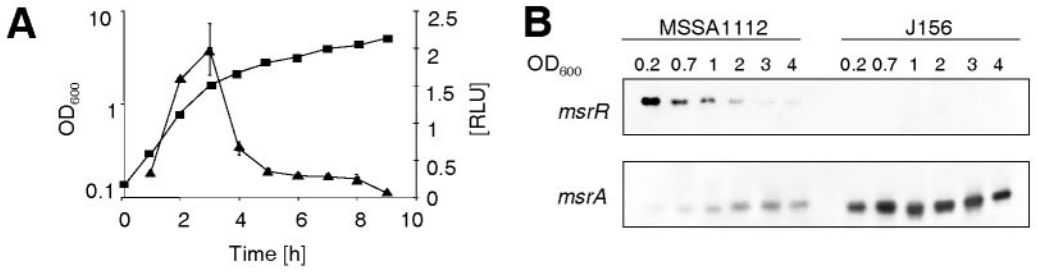

FIG. 3. Expression profiles of *msrA* and *msrR*. (A) Expression of $msrR_p$-*luc*+ during growth of *S. aureus* strain J175 was determined by quantifying the luciferase activity (triangles). Bacterial growth was monitored by measuring the OD_{600} (squares). RLU, relative light units. (B) Northern blot of *msrA* and *msrR*. Total RNA of strain MSSA1112 and its *msrR* mutant J156 was harvested at different growth stages indicated as OD_{600} values above the lanes.

the isogenic strain J175 showed that *msrR* transcription peaked in the early exponential growth phase and decreased towards the late exponential and stationary phases (Fig. 3). The transcription profile of *msrA* was the reverse, lower in the exponential phase and increasing towards the stationary phase. Interestingly, in the *msrR* mutant J156, *msrA* transcription was strongly enhanced over the entire growth cycle compared to that in the wild type (Fig. 3B). The sizes of the *msrA* and *msrR* mRNAs, 0.5 and 1 kb, respectively, indicated that both genes are located on monocistronic transcripts, as predicted from the nucleotide sequence.

Induction of *msrR* by antibiotic stress. Knowing that *msrR* inactivation resulted in lower oxacillin resistance, we analyzed the response of *msrR* to different cell wall-active antibiotics by measuring the $msrR_p$-luciferase reporter activity in strain J175. The antibiotics were added at concentrations corresponding to their MICs to exponentially growing cultures. Since staphylococci are known to show a strong inoculum effect with glycopeptides, vancomycin and teicoplanin were added at 5× their MICs. Transcription of *msrR* was found to be induced approximately two- to fivefold by (i) β-lactam derivatives such as oxacillin, cefoxitin, and imipenem; (ii) vancomycin, and to a lesser extent teicoplanin; (iii) antibiotics interfering with peptidoglycan precursor formation, such as fosfomycin and bacitracin; and (iv) the glycyl-glycine endopeptidase lysostaphin, which specifically cleaves the characteristic staphylococcal peptidoglycan pentaglycine interpeptide (Table 3). Northern blots showed that induction started immediately after addition of the antibiotic. Induction of *msrR* reached its maximum after 30 min and decreased thereafter, presumably due to the antibiotic's effect on cell growth. The extent of the induction was found to be concentration dependent and was higher when higher oxacillin concentrations were added (data not shown).

Non-cell wall-active antibiotics such as trimethoprim, which acts on folate synthesis, or ciprofloxacin, which inhibits DNA gyrase, had no effect on *msrR* expression (Table 3). Similar results were obtained by Northern blot analyses when protein synthesis inhibitors such as tetracycline or erythromycin were tested for their effects on *msrR* expression (data not shown).

Effects of *msrR* on *sarA* and *agr*. SarA is a global regulator which controls the synthesis of virulence factors and stimulates the transcription of the *agr* operon from the P2 and P3 promoters (17; see review by Arvidson and Tegmark [2]). The *sar* locus is composed of three overlapping transcripts, designated *sarA* (0.56 kb), *sarC* (0.8 kb), and *sarB* (1.2 kb), originating from the P1, P3, and P2 promoters, respectively, all encoding the SarA protein (30). An impressive upregulation of the three *sarA* transcripts was observed in *msrR* mutants, particularly during the early exponential growth phase, up to an OD_{600} of 1 to 2, as shown in Fig. 4. The increase of *sarA* transcripts in the mutant coincided precisely with the time of the otherwise highest *msrR* transcription in the wild type (Fig. 3A).

The SarA protein acts as an activator of the *agr* operon. We therefore measured transcription of RNAIII, the effector molecule of the *agr* operon. RNAIII expression, which usually begins in the stationary phase, started to increase in the *msrR* mutant already during the exponential growth phase and appeared also slightly stronger than in the wild type, as shown in Fig. 4.

Effects of *msrR* on virulence factors. The global regulators *sarA* and *agr* control a large number of cell wall-associated and excreted proteins. We therefore analyzed the influence of *msrR* inactivation on *spa* (coding for protein A) and *hla* (coding for alpha-toxin) transcription, respectively. The *spa* transcription appeared to be reduced in the mutant compared to its parent, especially in the stationary phase. In the very early exponential phase, at an OD_{600} of 0.2, though, there appeared to be more *spa* transcripts in the mutant than in the wild type. The transcription of *hla*, in contrast, was enhanced and started

TABLE 3. Induction of *msrR* in strain J175 by antibiotics

Antibiotic	MIC (μg ml^{-1})	Fold induction[a]
Oxacillin	0.125	3.2 ± 0.50
Cefoxitin	3	3 ± 0.59
Imipenem	0.032	3 ± 0.97
Vancomycin[b]	1.5	4.5 ± 1.30
Teicoplanin[b]	1.5	1.8 ± 0.28
Bacitracin	48	5 ± 1.48
Fosfomycin	1.5	2.5 ± 1.27
Lysostaphin	0.008	4.7 ± 1.84
Trimethoprim	0.75	0.96 ± 0.09
Ciprofloxacin	0.25	0.98 ± 0.09

[a] Fold increase in *msrR* transcription in strain J175 after 30 min of exposure to antibiotics, determined by measuring the luciferase activity of $msrR_p$-luc^+. The values shown represent the means of three independent assays.

[b] Glycopeptides were added at 5× MICs due to scavenging of glycopeptides by the cell wall, resulting in an inoculum effect.

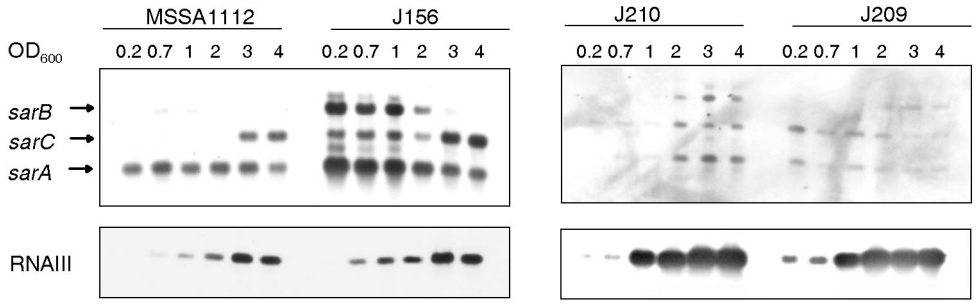

FIG. 4. Effect of *msrR* on the expression of the global regulators *sarA* and RNAIII. Shown are Northern blots of *sarA* and RNAIII transcripts of the wild-type strain MSSA1112 and its derivatives J156 (*msrR*), J210 [J156 pAW17(*msrAR*)], and J209 (J156 pAW17) harvested during growth at the OD_{600} values indicated.

earlier during growth in the mutant than in the wild type (Fig. 5).

Effects of MsrR on *mecA* transcription and autolytic activities. Since *msrR* inactivation reduced oxacillin resistance, the transcription profile of *mecA*, the gene encoding the low-affinity PBP2′, which is responsible for methicillin resistance, was analyzed in a wild-type strain and in the corresponding *msrR* mutant. For this purpose, a plasmid containing a *mecA* promoter-luciferase reporter gene fusion was introduced in parent strain MSSA1112, yielding strain J198, and into the corresponding *msrR* mutant J156, yielding strain J192. The *mecA* transcription, though, was not affected in either strain by *msrR* inactivation (data not shown). The effect of *msrR* inactivation was further analyzed for the expression pattern of the endogenous PBPs in two genetically distinct methicillin-resistant *S. aureus* strains. However, the PBP patterns were identical for the methicillin-resistant strains and their corresponding *msrR* mutants. There was also no change in the rate of spontaneous autolysis in parent and mutant strains (data not shown).

DISCUSSION

MsrR shares high sequence identity and similarity with Psr, the PBP5 synthesis repressor of *E. faecalis* and *Enterococcus hirae*, and with LytR of *B. subtilis*. All belong to the LytR-CpsA-Psr family of cell envelope-related transcriptional attenuators, whose exact function has yet to be defined. Psr of *E. hirae*, postulated to be a global regulator of cell wall synthesis genes, was shown to alter the autolytic response by reducing the cell wall's content of rhamnose, a sugar occurring in nonpeptidoglycan polysaccharides and teichoic acids, and to repress PBP5 synthesis (31). Interestingly, the enterococcal PBP5, which is associated with increased levels of ampicillin resistance, has sequence similarity with the staphylococcal methicillin resistance-determining PBP2′, encoded by *mecA*. The *B. subtilis* LytR was shown to act as an attenuator of the expression of its own gene and of the divergently transcribed *lytABC* operon, which codes for a lipoprotein, a modifier of an *N*-acetylmuramoyl-L-alanine amidase, and the amidase itself (28). It is worth noting that MsrR seemed to influence its own expression (M. Bischoff and J. Rossi, unpublished results) and that of the divergently transcribed *msrA* as well, with the latter being strongly upregulated in *msrR* mutants.

The most important finding was that MsrR appeared to act as an attenuator of *sarA* transcription. This is strongly supported by the remarkable *sarA* upregulation in *msrR* mutants occurring during exponential phase, precisely the period of otherwise peak *msrR* activity in the wild type. It is further supported by the downregulation of *sarA* transcription in the mutants to wild-type levels through *msrAR trans*-complementation (data not shown). The effect, though, is presumably not mediated directly by MsrR, since no DNA-binding motifs were found in the short predicted internal N-terminal fragment of MsrR. Additional factors have therefore to be postulated as intermediates leading from MsrR to *sarA* attenuation. The same may be true for Psr, which was postulated to be a global regulator of cell wall synthesis in *E. hirae* but seemed to act as neither a transcriptional repressor nor an activator in *Enterococcus faecium* (38), suggesting that Psr, like MsrR, may be a mediator for the regulatory effects.

Since SarA was known to be an activator of the *agr* operon (18), the drastic changes in *sarA* transcription levels in the *msrR* mutants were expected to influence *agr* expression. The premature initiation and increased RNAIII transcription observed in the *msrR* mutant may thus be explained by an increased and earlier SarA production.

The altered activity profile of the global regulators *sarA* and *agr* upon *msrR* inactivation was predicted to have consequences for the synthesis of a variety of virulence factors (19). We therefore investigated whether the *msrR* mutation influenced the transcription of *spa*, known to be repressed by SarA and RNAIII (13, 19), and of *hla*, which, on the other hand, is upregulated by these two regulators (14). Consistently with the previously demonstrated effects of *msrR* inactivation on *sarA*

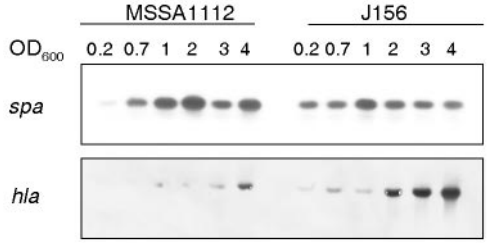

FIG. 5. Northern blots of *spa* and *hla* transcripts in the wild-type strain MSSA1112 and its *msrR* mutant J156, harvested during growth at the OD_{600} values indicated and probed with *hla* or *spa*, respectively.

and RNAIII expression, we could observe a clear increase in *hla* expression in the *msrR* mutant, which was again not only stronger but appeared also earlier in growth. Expression of *spa* seemed to be downregulated in the mutant, especially during later growth stages, again consistent with the already-demonstrated effect of the two global regulators. However, the reduction of *spa* transcripts in the mutant was less pronounced than expected, pointing out the complex regulation of this cell wall-associated protein. It has been shown that further regulatory elements, such as SarS (SarH1) (15, 43), Rot (32), and ArlSR (21), are involved in regulation of *spa* transcription.

Since *msrR* was expressed mainly during early exponential growth in wild-type strains, effects of inactivation are expected to manifest mainly during this growth phase and to a lesser extent over the remaining growth cycle. The early growth phase is thought to mimic some of the conditions needed in the first steps in the infection cycle of *S. aureus*, namely, the adhesion to and colonization of the host. MsrR may therefore play a more important role in vivo than in vitro, since except for reduced resistance to certain antibiotics, the doubling time and growth yield in vitro seemed not to be affected by *msrR* inactivation.

How the MsrR function correlates with glycopeptide and methicillin resistance remains unclear. Many factors are known to influence methicillin resistance levels. Resistance can be lowered by preventing the production of PBP2′ and/or by reducing the expression of the endogenous PBPs. Peptidoglycan precursor formation or changes in the peptidoglycan composition, as well as control of autolytic activities, can affect resistance levels (reviewed in reference 7). The global regulators *sarA* and *agr* were also shown to be involved in some way in methicillin resistance (37). Glycopeptide resistance depends, in most instances, on an abundant production of cell wall precursors (27, 33) and genes involved in cell wall synthesis (41). Since neither peptidoglycan composition and cross-linking (K. Ehlert, personal communication) nor changes in the PBP patterns or autolytic activities were apparent upon *msrR* inactivation, other reasons for the reduced resistance have to be sought. The effect of MsrR on resistance levels may be attributed to its effect on the regulation of the global regulators, which, besides virulence factors, may affect cell wall metabolism. Alternatively, changes in cell wall constituents other than peptidoglycan, such as teichoic acids, polysaccharides, or capsule, are conceivable, since *Streptococcus mutans* LytR, similarly to the *B. subtilis* LytR, was shown to be involved in biofilm formation (47). An analogous function for MsrR may be postulated. Predicated upon the upregulation of *msrR* by inhibition of cell wall synthesis or degradation of the peptidoglycan, the primary function of MsrR may be in the response to signals following cell wall damage or restructuring. It is conceivable that MsrR acts as a sensor that relays an external signal, possibly generated by cell wall metabolism during growth or upon damage, to the cytoplasm. MsrR may be an intrinsic low-level defense mechanism against cell wall damage that may occur during infection and may affect the ability of *S. aureus* to survive in different environments. Further experiments will have to show if MsrR is of importance in adhesion to and invasion and infection of the host, since its inactivation disturbs the timing of virulence factor production.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Arvidson, S., and K. Tegmark.** 2001. Regulation of virulence determinants in *Staphylococcus aureus*. Int. J. Med. Microbiol. **291:**159–170.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley and Sons, Inc., New York, N.Y.
4. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular architecture of the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **178:**4563–4570.
5. **Berger-Bächi, B.** 1983. Increase in transduction efficiency of Tn*551* mediated by the methicillin resistance marker. J. Bacteriol. **154:**533–535.
6. **Berger-Bächi, B.** 1983. Insertional inactivation of staphylococcal methicillin resistance by Tn*551*. J. Bacteriol. **154:**479–487.
7. **Berger-Bächi, B., and S. Rohrer.** 2002. Factors influencing methicillin resistance in staphylococci. Arch. Microbiol. **178:**165–171.
8. **Berger-Bächi, B., A. Strässle, and F. H. Kayser.** 1989. Natural methicillin resistance in comparison with that selected by *in vitro* drug exposure in *Staphylococcus aureus*. J. Antimicrob. Chemother. **23:**179–188.
9. **Bischoff, M., J. M. Entenza, and P. Giachino.** 2001. Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus*. J. Bacteriol. **183:**5171–5179.
10. **Bischoff, M., M. Roos, J. Putnik, A. Wada, P. Glanzmann, P. Giachino, P. Vaudaux, and B. Berger-Bächi.** 2000. Involvement of multiple genetic loci in *Staphylococcus aureus* teicoplanin resistance. FEMS Microbiol. Lett. **194:**77–82.
11. **Brückner, R.** 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. FEMS Microbiol. Lett. **151:**1–8.
12. **Cheung, A. L., K. Eberhardt, and V. A. Fischetti.** 1994. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem. **222:** 511–514.
13. **Cheung, A. L., K. Eberhardt, and J. H. Heinrichs.** 1997. Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. Infect. Immun. **65:**2243–2249.
14. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc. Natl. Acad. Sci. USA **89:**6462–6466.
15. **Cheung, A. L., K. Schmidt, B. Bateman, and A. C. Manna.** 2001. SarS, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus*. Infect. Immun. **69:**2448–2455.
16. **Cheung, A. L., and G. Zhang.** 2002. Global regulation of virulence determinants in *Staphylococcus aureu*s by the SarA protein family. Front. Biosci. **7:**1825–d1842.
17. **Chien, Y., A. C. Manna, and A. L. Cheung.** 1998. SarA level is a determinant of *agr* activation in *Staphylococcus aureus*. Mol. Microbiol. **30:**991–1001.
18. **Chien, Y., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. J. Biol. Chem. **274:**37169–37176.
19. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. **183:**7341–7353.
20. **Entenza, J. M., J. Vouillamoz, M. P. Glauser, and P. Moreillon.** 1977. Levofloxacin versus ciprofloxacin, flucloxacillin, or vancomycin for treatment of experimental endocarditis due to methicillin-susceptible or -resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41:**1662–1667.
21. **Fournier, B., A. Klier, and G. Rapoport.** 2001. The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **41:**247–261.
22. **Giachino, P., S. Engelmann, and M. Bischoff.** 2001. σ^B activity depends on RsbU in *Staphylococcus aureus*. J. Bacteriol. **183:**1843–1852.
23. **Giraudo, A. T., A. L. Cheung, and R. Nagel.** 1997. The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch. Microbiol. **168:**53–58.
24. **Jana, M., T. T. Luong, H. Komatsuzawa, M. Shigeta, and C. Y. Lee.** 2000. A method for demonstrating gene essentiality in *Staphylococcus aureus*. Plasmid **44:**100–104.
25. **Kreiswirth, B., J. Kornblum, R. D. Arbeit, W. Eisner, J. N. Maslow, A.**

McGeer, D. E. Low, and R. P. Novick. 1993. Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. Science **259:**227–230.
26. **Kullik, I., P. Giachino, and T. Fuchs.** 1998. Deletion of the alternative sigma factor σ^B in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J. Bacteriol. **180:**4814–4820.
27. **Kuroda, M., K. Kuwahara-Arai, and K. Hiramatsu.** 2000. Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. Biochem. Biophys. Res. Commun. **269:**485–490.
28. **Lazarevic, V., P. Margot, B. Soldo, and D. Karamata.** 1992. Sequencing and analysis of the *Bacillus subtilis lytRABC* divergon—a regulatory unit encompassing the structural genes of the *N*-acetylmuramoyl-L-alanine amidase and its modifier. J. Gen. Microbiol. **138:**1949–1961.
29. **Lyon, G. J., J. S. Wright, T. W. Muir, and R. P. Novick.** 2002. Key determinants of receptor activation in the *agr* autoinducing peptides of *Staphylococcus aureus*. Biochemistry **41:**10095–10104.
30. **Manna, A. C., M. G. Bayer, and A. L. Cheung.** 1998. Transcriptional analysis of different promoters in the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. **180:**3828–3836.
31. **Massidda, O., R. Kariyama, L. Daneo-Moore, and G. D. Shockman.** 1996. Evidence that the PBP 5 synthesis repressor (*psr*) of *Enterococcus hirae* is also involved in the regulation of cell wall composition and other cell wall-related properties. J. Bacteriol. **178:**5272–5278.
32. **McNamara, P. J., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J. Bacteriol. **182:**3197–3203.
33. **Moreira, B., S. Boyle-Vavra, B. L. M. De Jonge, and R. S. Daum.** 1997. Increased production of penicillin-binding protein 2, increased detection of other penicillin-binding proteins, and decreased coagulase activity associated with glycopeptide resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41:**1788–1793.
34. **Moskovitz, J., V. K. Singh, J. Requena, B. J. Wilkinson, R. K. Jayaswal, and E. R. Stadtman.** 2002. Purification and characterization of methionine sulfoxide reductases from mouse and *Staphylococcus aureus* and their substrate stereospecificity. Biochem. Biophys. Res. Commun. **290:**62–65.
35. **NCCLS.** 2000. Performance standards for antimicrobial susceptibility testing; 10th informational supplement M100-SID(M7). NCCLS, Wayne, Pa.
36. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:**3967–3975.
37. **Piriz Duran, S., F. H. Kayser, and B. Berger-Bächi.** 1996. Impact of *sar* and *agr* on methicillin resistance in *Staphylococcus aureus*. FEMS Microbiol. Lett. **141:**255–260.
38. **Rice, L. B., L. L. Carias, R. Hutton-Thomas, F. Sifaoui, L. Gutman, and S. D. Rudin.** 2001. Penicillin-binding protein 5 and expression of ampicillin resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. **45:**1480–1486.
39. **Sakoulas, G., G. M. Eliopoulos, R. C. Moellering, Jr., C. Wennersten, L. Venkataraman, R. P. Novick, and H. S. Gold.** 2002. Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. Antimicrob. Agents Chemother. **46:** 1492–1502.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Sieradzki, K., and A. Tomasz.** 1998. Suppression of glycopeptide resistance in a highly teicoplanin-resistant mutant of *Staphylococcus aureus* by transposon inactivation of genes involved in cell wall synthesis. Microb. Drug Resist. **4:**159–168.
42. **Strandén, A. M., M. Roos, and B. Berger-Bächi.** 1996. Glutamine synthetase and heteroresistance in methicillin-resistant *Staphylococcus aureus*. Microb. Drug Resist. **2:**201–207.
43. **Tegmark, K., A. Karlsson, and S. Arvidson.** 2000. Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **37:**398–409.
44. **Vasilieva, E., H. Labischinski, and B. Berger-Bächi.** 1997. Identification and mapping of new chromosomal sites affecting response to β-lactams in *Staphylococcus aureus*. Int. J. Antimicrob. Agents **8:**13–21.
45. **Wada, A.** 2001. An improved method for purifying bacterial genomic DNAs for direct sequencing by capillary automated sequencer. Tech. Tips Online. http://research.bmn.com/tto.
46. **Wu, S. W., H. de Lencastre, and A. Tomasz.** 1996. Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J. Bacteriol. **178:**6036–6042.
47. **Yoshida, A., and H. K. Kuramitsu.** 2002. Multiple. *Streptococcus mutans* genes are involved in biofilm formation. Appl. Environ. Microbiol. **68:**6283–6291.
48. **Ziebandt, A. K., H. Weber, J. Rudolph, R. Schmid, D. Hoper, S. Engelmann, and M. Hecker.** 2001. Extracellular proteins of *Staphylococcus aureus* and the role of SarA and sigma B. Proteomics **1:**480–493.