Molecular characterization of a chromosomal locus in *Staphylococcus aureus* that contributes to oxidative defence and is highly induced by the cell-wall-active antibiotic oxacillin

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Previous studies employing two-dimensional gel electrophoresis and N-terminal protein sequencing have shown elevated synthesis of the enzyme methionine sulfoxide reductase (MsrA) in *Staphylococcus aureus* in response to cell-wall-active antibiotics. In the present study, the *S. aureus* *msrA* gene was cloned, overexpressed, purified as His-tagged MsrA and shown to have methionine sulfoxide reductase activity. The transcription of *msrA* was studied by assaying β -galactosidase activity in an *msrA* promoter::*lacZ* fusion strain and by Northern blot analysis. Transcription of *msrA* was increased by oxacillin; but not by a variety of other stresses including H₂O₂. Northern blot analysis revealed that the size of the *msrA* transcript was 2.3 kb, considerably larger than the 531 nt *msrA* ORF. The *msrA* transcription start site was mapped 25 nt upstream of the *msrA* start codon. Computer analysis from database sequences indicated at least three additional ORFs downstream of *msrA*. The deduced amino acid sequences of two of these three ORFs showed significant sequence homologies to PilB, and enzyme IIA of the phosphotransferase system, respectively. The third ORF could not be identified by homology searches. Northern blot hybridization with probes specific to the *msrA* downstream region indicated that the *S. aureus* *msrA* was transcribed as part of a polycistronic message. Interestingly, purified *S. aureus* PilB was shown to possess ~28-fold higher methionine sulfoxide reductase activity than the MsrA. An insertional knockout mutation in the first gene of this operon resulted in increased susceptibility of the mutant to H₂O₂ compared to the parent strain, but not to oxacillin.

Keywords: PilB, oxidative stress, MsrA, antibiotic stress

INTRODUCTION

Methionine residues are readily oxidized to methionine sulfoxide, Met(O), by several biologically synthesized molecular species such as H₂O₂, hydroxyl radicals, hypochlorous acid, chloramines and peroxynitrites (Moskovitz *et al.*, 1997, 1998, 1999). In most instances, oxidation of methionine residues in proteins severely affects their biological functions. Loss of function has been shown to be effectively restored by the reduction of

Met(O) back to methionine. Reduction of Met(O) is mediated by the enzyme peptide methionine sulfoxide reductase, MsrA (Abrams *et al.*, 1981; Moreno & Pryor, 1992; Vogt, 1995; Wizemann *et al.*, 1996; Moskovitz *et al.*, 1996, 1997, 1998; Sun *et al.*, 1999). Thus, it has been proposed that the methionine residues act as antioxidant defence molecules and prevent oxidative damage to an organism (Levine *et al.*, 1999).

Interestingly, increased expression of MsrA has been observed under different environmental conditions and studies on *msrA* mutants in various organisms have provided evidence that MsrA functions beyond its role in oxidative stress. In *Escherichia coli*, MsrA synthesis

Abbreviations: MBC, minimal bactericidal concentration; Met(O), methionine sulfoxide.

was increased about threefold in stationary phase (Moskovitz *et al.*, 1995), suggesting it to be a stationary-phase response protein. In addition, an increase in pH of the growth medium has been reported to cause increased *msrA* expression in *Streptococcus gordonii* (Vriesema *et al.*, 2000). MsrA synthesis was also induced in a rabbit model of infective endocarditis (Kili *et al.*, 1999). The MsrA proteins have been shown to contribute to the maintenance of adhesins in three major pathogens, viz., *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* and *E. coli* (Wizemann *et al.*, 1996), and to be a virulence determinant of the plant pathogen *Erwinia chrysanthemi* (Hassouni *et al.*, 1999).

Although MsrA was determined to have a role in the virulence of *Staphylococcus aureus* (Mei *et al.*, 1997), its precise role in this bacterium has not been elucidated. We recently identified a *S. aureus* MsrA homologue to be induced in response to cell-wall-active antibiotics (Singh *et al.*, 2001). In this paper, we report on the cloning and molecular characterization of the chromosomal locus that encodes *S. aureus* MsrA. Northern blot analysis indicates that the *S. aureus msrA* gene is transcribed as a polycistronic message with three additional downstream genes. An insertional *msrA* knock-out mutation increased the sensitivity of the bacterium to oxidative stress conditions.

METHODS

Bacterial strain, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *S. aureus* and *E. coli* cells were grown in tryptic soy broth/agar (TSB/TSA; Difco) and Luria-Bertani broth/agar, respectively, at 37 °C in a shaking incubator (250 r.p.m.) unless stated otherwise. When needed, ampicillin (50 µg ml⁻¹), kanamycin (30 µg ml⁻¹ in the case of *E. coli*; 100 µg ml⁻¹ in the case of *S. aureus*), erythromycin (20 µg ml⁻¹) and chloramphenicol (15 µg ml⁻¹) were added to the growth medium.

DNA manipulations. Plasmid DNA was isolated using the Qiaprep kit (Qiagen Inc.); chromosomal DNA was isolated using a DNAzol kit (Molecular Research Center). The *Pfu* DNA polymerase, restriction enzymes and modification enzymes were purchased from Promega. DNA manipulations, Southern blot and Northern blot analysis were carried out as described by Sambrook *et al.* (1989). PCR was performed with the GeneAmp PCR system (Perkin-Elmer). Oligonucleotide primers were obtained from Integrated DNA Technology.

RNA analysis. To confirm oxacillin-induced synthesis of MsrA overnight cultures of *S. aureus* were diluted 100-fold in 25.0 ml of fresh TSB and grown to an OD₆₀₀ of 0.3. At this point, 1.2 µg ml⁻¹ oxacillin (a concentration that was found to be optimum for MsrA induction in our previous study; Singh *et al.*, 2001) was added and the cells were allowed to grow for 1 h. Fifteen millimolar H₂O₂ was added to one of the *S. aureus* RN450 cultures to generate conditions comparable to oxacillin stress, as indicated by the degree of growth inhibition. The culture grown for 1 h without the addition of H₂O₂ or oxacillin after reaching a culture density of 0.3 at OD₆₀₀ was used as a control. Cells were harvested by centrifugation, resuspended in 20 mM Tris/HCl buffer (pH 7.5) with 145 mM NaCl, and were lysed by the addition of 50 µg lysostaphin for 10 min at room temperature. Total RNA was subsequently

isolated using a Qiagen RNeasy mini kit. Equal amounts of RNA (10 µg) from each sample were run on a 1.2% denaturing agarose gel and transferred to a nylon membrane. The construct pRSETa-*msrA* was digested with *Bam*HI and *Hind*III; the *msrA* gene fragment was gel-purified, radio-labelled using the Prime-a-Gene labelling system (Promega) in the presence of [α -³²P]dCTP (specific activity > 3000 Ci mmol⁻¹ [111000 Bq], ICN Pharmaceuticals) and used to probe the membrane.

Determination of *msrA* promoter activity under different environmental conditions. Construction of an *msrA* promoter::*lacZ* reporter strain has been previously described (Singh *et al.*, 2001). In this construct, a 1.3 kb DNA fragment starting 44 nt downstream and going upstream of the *msrA* gene was cloned in the correct orientation in front of the promoterless *lacZ* gene of the vector pAZ106 (Chan *et al.*, 1998) and subsequently fused in the chromosome of *S. aureus* RN450. β -Galactosidase activity in the reporter strain (control and oxacillin-treated) was assayed using ONPG as the substrate (Miller, 1972).

Primer extension reaction. The primer extension reaction was carried out as described previously (Xiong & Jayaswal, 1998). Total RNA was isolated from the oxacillin-stressed cells of *S. aureus* and 5.0 µg of this RNA was reverse-transcribed using primer P1 (5'-CACCAGAAACATCCTCCTGC-3') in the presence of radiolabelled dATP (α -³²P, specific activity > 3000 Ci mmol⁻¹). A DNA sequencing ladder was generated using a 70770 Sequenase DNA sequencing kit (version 2.0; USB), primer P1 and an appropriate DNA fragment; the ladder was electrophoresed alongside the product of the primer extension reaction to determine the position of the transcription start site.

Overexpression and purification of *S. aureus* MsrA and PilB for determination of methionine sulfoxide reductase activity.

The *msrA* ORF was PCR amplified using *S. aureus* RN450 genomic DNA as the template, a 5' sense primer (P2) containing a *Bam*HI site (underlined) (5'-GGATCCATGACAAAAGAATATGCAAC-3') and a 3' reverse complement primer (P3) containing a *Hind*III site (underlined) (5'-AAGCTTAGCATTGATTCCCCCAATGTG-3'). The amplified *msrA* gene was cloned in-frame at the *Bam*HI and *Hind*III sites of the overexpression vector pRSETa (Invitrogen). *E. coli* strain BLR(DE3)pLysS (Novagen) was subsequently transformed with the construct pRSETa-*msrA*. The resulting transformants were grown in LB containing ampicillin (50 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), and tetracycline (12 µg ml⁻¹) to an OD₆₀₀ of 0.4 and induced for the synthesis of His-tagged MsrA by the addition of 2.5 mM IPTG for 2.5 h. The induced culture was harvested, resuspended in 50 mM Tris/HCl buffer (pH 7.5), sonicated, and centrifuged. The supernatant was applied to a nickel-charged-agarose-affinity-column and eluted with 400 mM imidazole using the Xpress Purification system (Invitrogen). Fractions containing the overexpressed His-tagged MsrA were pooled, dialysed, and concentrated against 50 mM Tris/HCl, 50 mM NaCl, 1 mM DTT, pH 7.5. The methionine sulfoxide reductase activity of the purified protein was assayed using 1 mM Dabsyl-Met(O) and 20 mM DTT in 50 mM Tris/HCl (pH 7.5) following incubation at 37 °C for 30 min, as described by Moskovitz *et al.* (1997). The *pilB* ORF was amplified using *S. aureus* RN450 genomic DNA as the template, a 5' sense primer (P4) containing a *Bam*HI site (underlined) (5'-GGATCCATGCTTAAAAAAGATAAA-3'), and a 3' reverse complement primer (P5) containing a *Hind*III site (underlined) (5'-AAGCTTCTTATCAAAATGTGATATTA-3'). The amplified *pilB* gene was cloned in-frame at *Bam*HI and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics*	Reference
Bacteria		
<i>S. aureus</i> RN450	A laboratory strain of <i>S. aureus</i> cured of all the prophages	Novick (1991)
<i>S. aureus</i> RN4220	A restriction-minus derivative of <i>S. aureus</i> RN450	Kreiswirth <i>et al.</i> (1983)
<i>S. aureus</i> COL	Homogeneous in methicillin-resistance expression	Pfeltz <i>et al.</i> (2000)
<i>S. aureus</i> RN450: <i>msrA</i>	<i>S. aureus</i> RN450 with mutation in the <i>msrA</i> gene (Kan ^R)	This study
<i>S. aureus</i> COL: <i>msrA</i>	<i>S. aureus</i> COL with mutation in the <i>msrA</i> gene (Kan ^R)	This study
MC1	<i>S. aureus</i> RN450: <i>msrA</i> with construct pCU1- <i>msr1</i> (Kan ^R Cam ^R)	This study
MC2	<i>S. aureus</i> RN450: <i>msrA</i> with construct pCU1- <i>msr2</i> (Kan ^R Cam ^R)	This study
<i>S. aureus</i> PmsrA:: <i>lacZ</i>	<i>S. aureus</i> RN450; a reporter strain for the <i>msrA</i> promoter (Erm ^R)	Singh <i>et al.</i> (2001)
<i>E. coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'(traD36 proAB⁺ lacI^aΔM15)</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> BLR(DE3)pLysS	An overexpression strain of <i>E. coli</i> (Cam ^R Tet ^R)	Novagen
Plasmids		
pRSETa	An <i>E. coli</i> overexpression vector (Amp ^R)	Invitrogen
pRSETa- <i>msrA</i>	The <i>msrA</i> ORF cloned in-frame in pRSETa at <i>Bam</i> HI and <i>Hind</i> III sites (Amp ^R)	This study
pRSETa- <i>pilB</i>	The <i>pilB</i> ORF cloned in-frame in pRSETa at <i>Bam</i> HI and <i>Hind</i> III sites (Amp ^R)	This study
pTZ18R	Cloning vector for <i>E. coli</i> (Amp ^R)	Mead <i>et al.</i> (1986)
pCU1	Shuttle vector (Amp ^R in <i>E. coli</i> and Cam ^R in <i>S. aureus</i>)	Augustin <i>et al.</i> (1992)
pTZ- <i>msr</i>	A 2.1 kb DNA fragment encompassing the <i>msrA</i> gene (Amp ^R)	This study
pTZ-MK	A kanamycin-resistance cassette inserted in the middle of the construct pTZ- <i>msr</i> (Amp ^R , Kan ^R)	This study
pCU1- <i>msr1</i>	The 1110 bp DNA fragment containing the <i>msrA</i> gene under its own promoter	This study
pCU1- <i>msr2</i>	The 2767 bp DNA fragment containing the <i>msrA</i> operon	This study

* Kan^R, kanamycin-resistant; Cam^R, chloramphenicol-resistant; Erm^R, erythromycin-resistant; Tet^R, tetracycline-resistant; Amp^R, ampicillin-resistant.

*Hind*III sites of pRSETa, overexpressed, purified, and assayed for methionine sulfoxide reductase activity essentially as described above for MsrA.

Construction of an *msrA* knockout mutant in *S. aureus*. To create an *msrA* null mutant, primers P1 and P6 (5'-AAACCACTACCGAATCGTCG-3') were used to amplify a 1.3 kb DNA fragment, using *S. aureus* RN450 chromosomal DNA as the template, which represented the left flanking region of the *msrA* gene starting 44 nt downstream of the *msrA* start codon. Primers P7 (5'-CAGGAGGATGTTTCTGGTG-3') and P8 (5'-GCCGCCACTTTCTTTAGGTC-3') were used to amplify an 831 bp fragment which represented the right flanking region of the *msrA* gene starting 25 nt downstream of the *msrA* start codon. These two fragments were ligated together in vector pTZ18R (Mead *et al.*, 1986) resulting in the construct pTZ-*msr*, which simultaneously generated a unique *Eco*RI restriction site between the ligated fragments. A 1.5 kb kanamycin gene cassette was inserted into this *Eco*RI site, resulting in the construct pTZ-MK. The vector pTZ18R cannot replicate in Gram-positive bacteria; thus pTZ-MK was used as a suicidal construct to transform *S. aureus* RN4220 cells by electroporation (Schenk & Laddaga, 1992). Selection of the transformants on kanamycin plates led to the integration of the entire construct into the chromosome. Phage-80% was propagated on these transformants and used to resolve the mutation in the *msrA* gene in the *S. aureus* strains RN450 and COL by performing transductional outcrosses, as described by Novick *et al.* (1986).

Determination of the sensitivity of the *msrA* mutant to oxacillin and H₂O₂. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for the *S. aureus* strains were determined as described by Pfeltz *et al.* (2000) using 96-well microtitre plates with serial dilutions of oxacillin (1000 to 50 µg ml⁻¹) and H₂O₂ (12 to 0.125 mM) in TSB. The overnight bacterial cultures were diluted to a final concentration of 1 × 10⁶ cfu ml⁻¹ in each well and the plates were incubated at 37 °C for 48 h. In H₂O₂ sensitivity studies, the *msrA* mutant was complemented in *trans* with the *msrA* gene on the shuttle vector pCU1 (Augustin *et al.*, 1992). To clone the entire *msrA* gene into pCU1, a 1110 bp fragment was amplified using the primers P8 and P9 (5'-CAAATCCATTGGTGTCG-3') starting 254 bp upstream of the *msrA* start codon, thus representing the entire *msrA* gene. This construct, pCU-*msr1*, was transferred to *S. aureus* RN4220 by electroporation and subsequently transduced into the *msrA* mutant strain resulting in strain MC1. The *msrA* mutant was also complemented with a 2767 bp PCR-amplified fragment and cloned in the vector pCU1 using primers P9 and P10 (5'-CGTCTTTTCTTCACTACCTC-3'), again starting 254 bp upstream of the *msrA* start codon, resulting in strain MC2. To maintain identical conditions during the H₂O₂ tolerance study, the wild-type and the *msrA* mutant were transformed with the empty plasmid pCU1. Subsequently, the parent and the mutant transformed with pCU1, MC1 and MC2 were diluted to 2.5 × 10⁷ cfu ml⁻¹ from their respective overnight cultures in 100 ml fresh TSB containing 10 µg chloramphenicol ml⁻¹ and

8 mM H_2O_2 . The number of c.f.u. in each culture was determined with respect to time.

RESULTS

Overexpression and purification of MsrA and PilB and determination of the methionine sulfoxide reductase activity

The *S. aureus* *msrA* and *pilB* genes were cloned in the pRSETa vector and overexpressed in *E. coli* from a T7 promoter by induction with IPTG. The purified proteins appeared to be > 95 % homogeneous on an SDS-polyacrylamide gel. Enzymic activity determination showed that the purified His-tagged MsrA could reduce Dabsyl-Met(O) to 64513 pmol of Dabsyl-Met (mg protein)⁻¹ min⁻¹. Interestingly, purified PilB could reduce the same substrate to 1811929 pmol of Dabsyl-Met (mg protein)⁻¹ min⁻¹, an approximately 28-fold higher specific activity than the MsrA.

Analysis of *msrA* expression

The expression of the *msrA* gene was studied at the transcriptional level to complement the observation of induced MsrA synthesis in oxacillin-stressed *S. aureus* cells (Singh *et al.*, 2001). In Northern blot analysis, only a faint band corresponding to the *msrA* transcript could be detected in the lanes representing RNA from the non-stressed control or H_2O_2 -treated RN450 cells (Fig. 1, lanes 1 and 2). However, the level of *msrA* transcript in the RNA from oxacillin-treated RN450 cells was higher, as a significantly darker band was observed (Fig. 1, lane 3). The size of the *msrA* transcript on the Northern blot was determined to be ~2.3 kb (Fig. 1), which is

Fig. 1. Northern blot hybridization of the *msrA* gene with the total RNA isolated from the *S. aureus* cells and separated on a 1.2 % denaturing agarose gel. Lane 1, total RNA from *S. aureus* RN450 grown in TSB for 1 h after reaching an OD_{600} of 0.3. Lanes 2 and 3, total RNA from *S. aureus* RN450 cells stressed with 15 mM H_2O_2 and 1.2 μg oxacillin ml^{-1} , respectively, for 1 h after reaching an OD_{600} of 0.3. The arrow indicates the transcript reacting with the *msrA* gene probe.

Fig. 2 (a) Expression of β -galactosidase in the *S. aureus* RN450 *PmsrA::lacZ* reporter strain. The growing cultures of the reporter strain were collected at different culture densities and stressed with oxacillin for 1 h. The oxacillin concentrations used were 1.2, 2.4, 3.6, 4.8, 6.0 and 8.0 μg ml^{-1} for cells at culture densities of 0.3, 0.6, 0.9, 1.2, 1.5 and 2.0 at OD_{600} respectively. The control cultures were allowed to grow for 1 h without oxacillin. The bars represent the mean β -galactosidase activity (specific activity in terms of OD_{600}) of three independent experiments $\pm$ SE: shaded bars represent the values for oxacillin-induced cells; open bars represent the values for the non-induced control cells. (b) Expression of β -galactosidase in the *PmsrA::lacZ* reporter strain grown to an OD_{600} of 0.3 and subsequently exposed to different environmental stresses for 1 h. The bars represent the mean values of three independent experiments $\pm$ SE.

considerably larger than the 531 nt *msrA* ORF. Determination of β -galactosidase activity in an *msrA* promoter::*lacZ* reporter strain revealed a significant increase in β -galactosidase activity when actively growing cultures of this reporter strain were exposed to oxacillin (Fig. 2a, b); however, other stress conditions had no appreciable effect when applied for 1 h (Fig. 2b). These studies also revealed that under normal physiological conditions the *msrA* promoter is maximally active during late exponential phase (Fig. 2a). In addition, the oxacillin-induced *msrA* promoter activity

Fig. 3. Mapping of the transcription start site of the *msrA* gene. The -10 sequences, the transcription start site (+1) and RBS are indicated in the non-coding strand of the *S. aureus* *msrA* gene.

was apparent in growing cells from early exponential to stationary phase (Fig. 2a). Detection of no apparent β -galactosidase activity in the reporter strain exposed to 46 °C might be due to the inactivation of the enzyme at this elevated temperature, rather than to suppression of *msrA* promoter activity (Fig. 2b).

Mapping of the *msrA* transcription start site and further nucleotide sequence analysis

The transcription start site of the *msrA* transcript was mapped, as we observed a larger than expected *msrA* transcript (~ 2.3 kb) in Northern blot analysis. The primer extension studies revealed the transcription start site of this 2.3 kb transcript to be located 25 nt upstream of the *msrA* start codon. The potential -10 , -35 sequences (-35 sequences not shown in the figure) and ribosome-binding site (RBS) were also identified (Fig. 3). In subsequent computer analysis of the database sequence (<http://www.genome.ou.edu/staph.html>) and the complete genome sequences of two *S. aureus* strains (Kuroda *et al.*, 2001), the presence of at least three

additional ORFs downstream of *msrA* has been identified. The deduced amino acid sequences of the first of these three ORFs showed $> 50\%$ homology to the deduced protein product of *yppQ* (a *pilB* family homologue, as per the database entry) of *Bacillus subtilis*, and to the C-terminus of the MsrA proteins of *S. gordonii* (Vriesema *et al.*, 2000) and *S. pneumoniae* (Wizemann *et al.*, 1996). The deduced amino acid sequence of the protein product of the second of these three ORFs has been identified as enzyme IIa of the phosphotransferase system (PTS) (Kuroda *et al.*, 2001). The identity of the third ORF could not be established based on homology searches. The RNA blot described above (Fig. 1) was probed again with either the *pilB* cloned in pRSETa-*pilB* or a PCR-amplified fragment using primers P11 (5'-CGGAAAAGGCAAAGAAGTTC-3') and P12 (5'-TTGGTCTTGATTGCTTGCTTC-3'). These primers amplified a 968 bp fragment starting 17 nt downstream of the gene encoding PTS enzyme IIa and going further downstream. In these experiments, we observed hybridization of the same band (data not shown) that hybridized with the *msrA* probe. Additionally, there is no apparent promoter upstream of any of these three ORFs. These findings suggest that the *S. aureus* *msrA* gene is transcribed as part of a polycistronic message.

The *S. aureus* *msrA* mutant showed increased sensitivity to oxidative stress but remained resistant to oxacillin

Considering that the *S. aureus* MsrA homologue is upregulated in response to oxacillin, an *msrA* mutant was created by site-directed mutagenesis in *S. aureus* strain RN450; the mutation was confirmed by Southern blot and PCR analysis (data not shown). However, the oxacillin resistance of the mutant remained unchanged compared to the wild-type parent (Table 2). As RN450 is an oxacillin-sensitive strain, the mutation was transduced into the highly oxacillin-resistant *S. aureus* strain COL. Again, oxacillin resistance in the *S. aureus* COL *msrA* mutant remained unchanged in comparison to wild-type COL (Table 2).

Since MsrA proteins have been shown to contribute to the oxidative stress tolerance of an organism, the sensitivity of the *msrA* mutant to H_2O_2 was investigated. MIC and MBC determinations revealed that the *msrA* mutants of strains RN450 or COL were more sensitive to H_2O_2 compared to their respective wild-type parents (Table 2). In addition, the *msrA* mutant of strain RN450 complemented with the *msrA* gene alone (MC1) or with the entire *msrA* operon (MC2) showed higher H_2O_2 tolerance than the parent (Table 2). On agar, an identical zone of inhibition around discs soaked with 10 μ l 3% H_2O_2 was observed for all four strains; however, 48 h after incubation a few colonies were apparent in the vicinity of the disc for the parent, MC1 and MC2, but not for the *msrA* mutant.

Growth kinetics were determined for all four strains in liquid cultures containing 8 mM H_2O_2 . The mutant

Table 2. Susceptibilities of the *S. aureus* wild-type parents, *msrA* mutants, MC1, and MC2 to H₂O₂ and oxacillin

ND, Not determined; MIC, the lowest oxacillin or H₂O₂ concentration that did not allow any bacterial growth; MBC, the lowest oxacillin or H₂O₂ concentration that killed the entire population of bacterial inoculum (> 99.9%).

Strains	Oxacillin		H ₂ O ₂	
	MIC (µg ml ⁻¹)	MBC (µg ml ⁻¹)	MIC (mM)	MBC (mM)
RN450	0.375	0.375	0.5	0.5
RN450: <i>msrA</i>	0.375	0.375	0.25	0.25
MC1	ND	ND	1.0	2.0
MC2	ND	ND	1.0	2.0
COL	400	400	1.0	2.0
COL: <i>msrA</i>	400	400	0.5	1.0

Fig. 4. Growth of *S. aureus* RN450 transformed with pCU1 (◆), RN450:*msrA* mutant transformed with pCU1 (◇), and RN450:*msrA* mutant complemented with pCU1-*msr1* (MC1; ●) and pCU1-*msr2* (MC2; ○), in TSB containing 8 mM H₂O₂. In each case, the overnight culture was used to dilute 100 ml fresh TSB to 2.5 × 10⁷ c.f.u. ml⁻¹ before the addition of H₂O₂ (to a final concentration of 8 mM; 0 h).

bacteria were almost completely killed under these conditions, whereas the parent bacteria were able to grow after a lag phase (Fig. 4). However, the two complemented clones responded more efficiently and were able to grow more rapidly than the parent (Fig. 4). A similar response was observed in liquid culture of these strains in the presence of 8 mM methyl viologen (paraquat), where the growth of the mutant was slower compared to that of the parent and the two *msrA*-complemented strains (data not shown).

DISCUSSION

Methionine sulfoxide reductases reduce protein-bound or free Met(O) to methionine and also play an important role in the expression of adhesins (Wizemann *et al.*,

1996; Moskovitz *et al.*, 1997, 1998, 1999). *S. aureus* MsrA was recently observed to be upregulated by cell-wall-active antibiotics (Singh *et al.*, 2001). In this study, the chromosomal locus that encodes the *S. aureus* MsrA has been characterized at the molecular level.

The active sites of the MsrA enzymes of *E. coli* and *Bos taurus* have been mapped and shown to possess an identical active site, GCFWG (Lowther *et al.*, 2000; Moskovitz *et al.*, 2000). Comparative analysis of the amino acid sequences indicates that in the case of *S. aureus* MsrA the characteristic MsrA active site differs at one amino acid: GCFWC in place of GCFWG. However, purified *S. aureus* MsrA was shown to possess methionine sulfoxide reductase activity, which confirms the identity of the oxacillin-induced protein as an authentic MsrA, despite a modified active site. Although the active site of the MsrA in *B. subtilis* has not been mapped, amino acid analysis indicates that the active site of *B. subtilis* MsrA (Hayes *et al.*, 1998) is also GCFWC. In this context, very high methionine sulfoxide reductase activity for the protein product of the second gene of the *msrA* operon is quite interesting as the deduced protein shows > 50% homology to the family of PilB transcriptional repressors, as per the database entry. However, the precise role of these proteins in the regulation of bacterial transcription is not yet known. More significantly, this PilB homologue was over 50% homologous to the C-terminus of *S. gordonii* (Vriesema *et al.*, 2000) and *S. pneumoniae* (Wizemann *et al.*, 1996) MsrA proteins, as well as those of several other bacterial species. In addition, NCBI database searches indicate that in numerous bacteria the gene encoding this protein is present just downstream of the *msrA* gene, in an operon-like organization. This indicates that the presence of a *pilB*-like gene (along with *msrA*) is conserved across many bacterial species, either as a separate gene entity or as an integral part of a larger *msrA*. However, to date, none of the PilB proteins that are expressed separately have been reported to possess MsrA-like functions. Interestingly, in the case of *S. aureus*, this

protein shows 28-fold higher methionine sulfoxide reductase activity, but it lacks the characteristic active site GCFWG of the MsrA proteins. This indicates that the methionine sulfoxide reductase activity of PilB occurs via different, unknown mechanisms.

We previously observed that cell-wall-active antibiotics induced the *S. aureus* *msrA* operon (Singh *et al.*, 2001). Studies employing a reporter strain with the *msrA* promoter cloned upstream of a promoterless *lacZ* gene and fused in the chromosome of the *S. aureus* strain RN450 revealed that the *msrA* promoter was not inducible by environmental stresses other than oxacillin. Increased expression of *msrA* has been reported upon an increase in pH of the growth medium in *S. gordonii* (Vriesema *et al.*, 2000); however, we did not see any significant increase in the *msrA* promoter activity in response to pH upshift or downshift in this study. Maximum *msrA* promoter activity in late-exponential and stationary phase is consistent with the observation of a threefold increase in the production of MsrA during this stage of growth in *E. coli* (Moskovitz *et al.*, 1995). Furthermore, as in *E. coli*, H₂O₂ did not induce the expression of the *S. aureus* *msrA* at the transcriptional level. This suggests that *msrA* expression is not under the control of the *oxyR* regulon that controls the oxidative stress response in bacteria (Moskovitz *et al.*, 1995). Although *S. aureus* does not have an OxyR homologue it does have proteins like PerR, which perform similar functions such as regulating the transcription of the gene encoding oxidative stress resistance proteins (Horsburgh *et al.*, 2001).

Interestingly, oxacillin induced the expression of *S. aureus* *msrA* but a mutation in the *msrA* gene did not affect the oxacillin MIC either in an oxacillin-sensitive or in an oxacillin-resistant staphylococcal strain. However, the *msrA* mutant showed higher sensitivity to H₂O₂ compared to the wild-type parent in the MIC determinations, although there was no appreciable difference in the growth kinetics of the mutant and the parent in liquid culture containing 4 mM H₂O₂ or paraquat. The increased sensitivity of the mutant was clearly observed in the presence of 8 mM H₂O₂, a concentration considered lethal in most other bacteria. At this concentration *S. aureus* was able to grow after a considerably longer lag; however, the *msrA* mutant failed to grow at all. For Northern blot experiments 15 mM H₂O₂ was added to actively growing cultures (OD₆₀₀ of 0.3) where the bacteria showed higher tolerance, which is believed to be due to increased cell density. In these H₂O₂ tolerance studies the *msrA* mutant, complemented either with the *msrA* gene alone or with the entire *msrA* operon, responded more efficiently than even the wild-type parent. This is probably because of the presence of *msrA* on a high copy plasmid. The oxidative stress tolerance of *msrA* mutants of *E. coli* and *B. subtilis* were comparable to their respective wild-type parents in liquid cultures (Moskovitz *et al.*, 1995; Hayes *et al.*, 1998). In *E. coli* increased sensitivity to H₂O₂ could be seen in *msrA* mutants only when grown on agar (Moskovitz *et al.*,

1995). The authors concluded that the unaffected oxidative stress tolerance of the mutant in liquid culture was probably due to the overriding effects of other protective mechanisms (Hayes *et al.*, 1998). However, the role of MsrA in oxidative stress is visible in liquid culture, which may suggest that MsrA is more important in oxidative stress resistance in *S. aureus* than it is in *E. coli*. In addition, a recent GenBank search reveals the presence of another locus in the *S. aureus* genome that encodes an MsrA-like protein (GI no. 12656489). This protein has also been identified in whole-genome sequence analyses of two methicillin-resistant *S. aureus* strains (GI no. 13701158) (Kuroda *et al.*, 2001). The protein is 50 % homologous to the MsrA reported in the present study and contains the MsrA active site GCFWC; however, the genes encoding these two staphylococcal MsrA proteins share no homology with each other at the nucleotide level. It is also quite likely that this additional MsrA protein accounts for the partial tolerance of our *S. aureus* *msrA* mutant to H₂O₂, but the bacterium may need both *msrA* genes for full-scale recovery under more acute oxidative stress conditions.

A mutant with reduced adherence to type II lung epithelial and human vein endothelial cells was identified in *S. pneumoniae*. The protein encoded by the disrupted gene displayed > 40 % identity with the MsrA of *E. coli* and > 50 % identity with the C-terminal region of gonococcal PilB (Taha *et al.*, 1988, 1992; Wizemann *et al.*, 1996). In addition, an *Erwinia chrysanthemi* *msrA* mutant was less motile on solid medium (Hassouni *et al.*, 1999). A role for MsrA has also been observed in the expression of adhesins in *S. pneumoniae*, *N. gonorrhoeae* and *E. coli* (Wizemann *et al.*, 1996). It has been argued that MsrA has a special role in repairing surface layers (Hassouni *et al.*, 1999). However, as MsrA is cytoplasmic and the pilins or adhesins are located outside the cell, the authors speculate that MsrA also functions as a repair system and as a regulator for the production of the extracellular appendices (Wizemann *et al.*, 1996). As MsrA and PilB have been speculated to repair the cell surface (Wizemann *et al.*, 1996), induction of the *msrA* operon by antibiotics that interfere with cell-wall synthesis might be an attempt by the bacterium to protect its cell surface. However, the roles of MsrA and PilB in the oxidative stress response and cell surface repair need further investigation. Experiments are currently under way to determine the precise roles of the individual ORFs of the *msrA* locus.

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