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Role of a Cysteine Synthase in *Staphylococcus aureus*

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The gram-positive human pathogen *Staphylococcus aureus* is often isolated with media containing potassium tellurite, to which it has a higher level of resistance than *Escherichia coli*. The *S. aureus cysM* gene was isolated in a screen for genes that would increase the level of tellurite resistance of *E. coli* DH5α. The protein encoded by *S. aureus cysM* is sequentially and functionally homologous to the *O*-acetylserine (thiol)-lyase B family of cysteine synthase proteins. An *S. aureus cysM* knockout mutant grows poorly in cysteine-limiting conditions, and analysis of the thiol content in cell extracts showed that the *cysM* mutant produced significantly less cysteine than wild-type *S. aureus* SH1000. *S. aureus* SH1000 cannot use sulfate, sulfite, or sulfonates as the source of sulfur in cysteine biosynthesis, which is explained by the absence of genes required for the uptake and reduction of these compounds in the *S. aureus* genome. *S. aureus* SH1000, however, can utilize thiosulfate, sulfide, or glutathione as the sole source of sulfur. Mutation of *cysM* caused increased sensitivity of *S. aureus* to tellurite, hydrogen peroxide, acid, and diamide and also significantly reduced the ability of *S. aureus* to recover from starvation in amino acid- or phosphate-limiting conditions, indicating a role for cysteine in the *S. aureus* stress response and survival mechanisms.

Cysteine is an essential amino acid that performs vital functions in the catalytic activity and structure of many proteins. Cysteine residues are required for essential and ubiquitous proteins with iron-sulfur (Fe-S) clusters, including cytochromes and aconitase (4, 21). In many organisms, the cysteine-containing molecules glutathione and thioredoxin play a major role in maintaining an intracellular reducing environment and protection against oxidative stress (6, 14, 23, 51). The formation of disulfide bonds between cysteine residues is the critical step in the activation of bacterial transcriptional regulators such as OxyR (8, 71) and the molecular chaperone Hsp33 (2, 34). Disulfide bonds are also needed for proper folding and stability of some proteins, particularly those found in extracytoplasmic compartments (68).

In bacteria, cysteine is synthesized from serine by incorporation of sulfide or thiosulfate. Sulfide is obtained from the transport and reduction of inorganic sulfate or from organic sulfonate compounds such as taurine (61, 62). The final step in cysteine biosynthesis is catalyzed by either *O*-acetylserine (thiol)-lyase A or *O*-acetylserine (thiol)-lyase B, encoded by the genes *cysK* and *cysM*, respectively (19, 30, 31). The CysK and CysM proteins from *Escherichia coli* are 43% identical. CysK synthesizes cysteine from *O*-acetylserine and sulfide, while the CysM protein differs in that it can also utilize thiosulfate instead of sulfide. The reaction between *O*-acetylserine and thiosulfate produces *S*-sulfocysteine, which is converted into cysteine by an as yet uncharacterized mechanism (44).

It has been proposed that the *O*-acetylserine (thiol)-lyase B isozyme is preferentially used during growth in anaerobic growth conditions (37). In *E. coli*, cysteine can be used to donate the sulfur moiety for methionine biosynthesis in a set of reactions known as the trans-sulfuration pathway. This pathway can be reversed in *Bacillus subtilis*, which can therefore use methionine as its sole source of sulfur (24). The genes involved in cysteine biosynthesis and sulfur assimilation in *E. coli* and *Salmonella enterica* serovar Typhimurium have been well characterized (reviewed in reference 37). More recently, cysteine biosynthesis has been studied in the gram-positive bacteria *B. subtilis* (24, 63) and *Lactococcus lactis* (18), and also in the archaeon genus *Methanosarcina* (5, 36). In contrast, cysteine biosynthesis and sulfur assimilation in the gram-positive *Staphylococcus aureus* have not been well studied.

S. aureus is a medically important human pathogen capable of causing a variety of infections, ranging from minor skin and wound infections to life-threatening diseases (41). Staphylococci are often isolated and identified with growth media containing potassium tellurite (K_2TeO_3), to which they have a higher level of resistance than many other bacteria. Although tellurite has been used in health laboratories and the food industry for over 80 years as a selective agent for the isolation of pathogens, the mechanism of tellurite resistance in staphylococci is poorly understood (3, 42, 69, 70).

We initially isolated a cysteine synthase homologue in a screen for *S. aureus* genes that would confer increased tellurite resistance on *E. coli*. In this paper we show that this locus is functionally homologous to CysM and is involved in stress resistance. In addition, we investigated the cysteine biosynthetic pathways of *S. aureus* and its ability to grow with different sources of sulfur.

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MATERIALS AND METHODS

Media and growth conditions. *S. aureus* and *E. coli* strains, plasmids, and oligonucleotides are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB)

TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or primer	Genotype or description	Reference or source
E. coli		
DH5α	ϕ80 Δ*(lacZ)M15* Δ*(argF-lac)U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1*	52
XLOLR	Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 recA1 gyrA96 thi-1 relA1* [F′ *proAB lacI*^{q} *Z*Δ*M15* Tn*10* (Tet^r)] λ^r Su^-	Stratagene
S. aureus		
8325-4	Wild-type strain cured of prophages	Lab stock
RN4220	Restriction-deficient transformation recipient	Lab stock
SH1000	Functional *rsbU*$^+$ derivative of 8325-4	26
J96	SH1000 *cysM*::*tet*	This study
SMH2052	SH1000 *cysJ*::Tn*917*	28
J62	SH1000 *cysM-lacZ*	This study
J106	J96/pJIM80	This study
J108	J96/pMK4	This study
J116	SH1000/pMK4	This study
Plasmids		
pOB	pGEM3Zf(+) cloning vector	26
pAZ106	Promoterless *lacZ ery*^{r} insertion vector	35
pDG1513	Tet^r cassette vector	25
pBK-CMV	Kan^r phagemid recircularised from ZAP Express vector	Stratagene
pMK4	*E. coli* — *S. aureus cat*^{r} shuttle vector	55
pJIM27	pBK-CMV with *S. aureus cysM* (543551–545725)[a]	This study
pJIM28	pBK-CMV with *S. aureus cysM* (545746–543385)	This study
pJIM29	pBK-CMV with *S. aureus cysM* (545725–543551)	This study
pJIM30	pBK-CMV with *S. aureus cysM* (545725–543994)	This study
pJIM31	pBK-CMV containing random 2-kb fragment of *S. aureus* DNA	This study
pJIM62	4.8-kb fragment containing *cysM* in pOB	This study
pJIM64	pJIM62 containing *cysM*::*tet* cassette insertion	This study
pJIM44	6-kb fragment in pAZ106 forming *cysM-lacZ* fusion	This study
pJIM80	*cysM* locus in pMK4 for complementation	This study
Primers[b]		
JKL48	GTCGTTGAATTCTTATAGCAG	
JKL49	CCAGCGAATTCCTGAACGTG	
JKL7	CTTGGATCCAACGTCAAACAGAATCTAGACGCGG	
JKL22	CGCTCTTGGCGAATTCTTTCGGG	
T3	ATTAACCCTCACTAAAG	
T7	AATACGACTCACTATAG	

[a] Coordinates of fragment in *S. aureus* N315 genome database (38).
[b] Restriction sites are underlined.

medium at 37°C. *S. aureus* was grown at 25°C or 37°C with shaking at 250 rpm in brain heart infusion (BHI) (Oxoid), tryptic soy broth (TSB) (Difco), or chemically defined medium (CDM) (32, 66). For growth on solid media, 1% wt/vol agar was added. To measure the sulfur requirements of *S. aureus* strains on CDM agar plates, CDM was prepared without L-cysteine. This medium contains 2 mM $MgSO_4$. L-Cystine, glutathione, sodium sulfate, 4-nitrocatechol sulfate, sodium sulfite, sodium sulfide, sodium thiosulfate, ethanedisulfonate, or benzenesulfonate was added at a concentration of 500 μM. For anaerobic growth, plates were incubated in an anaerobic growth cabinet (MK3 Anaerobic Incubator, Don Whitley Systems). For liquid growth tests, L-cysteine or sodium thiosulfate was added at concentrations up to 500 μM, and cultures were inoculated from an overnight preculture to an optical density at 600 nm (OD_{600}) of 0.005. When included, antibiotics were added at the following concentrations: ampicillin, 100 mg liter^{-1}; chloramphenicol, 5 mg liter $^{-1}$, erythromycin, 5 mg liter^{-1}; lincomycin, 5 mg liter $^{-1}$; kanamycin, 50 mg liter $^{-1}$; and tetracycline, 5 mg liter $^{-1}$.

Screen for tellurite-resistant clones. A phagemid library of *S. aureus* 8325-4 genomic DNA was prepared by mass excision from a λZAP Express (Stratagene) library made previously (20). The pBK-CMV phagemids are recircularized in *E. coli* XLOLR cells, have a kanamycin resistance marker, and contain random inserts of up to 12 kb of partially *Sau*3A-digested *S. aureus* DNA. To determine the MIC of tellurite, an overnight culture was diluted in phosphate-buffered saline (PBS), and a 10-μl inoculum containing approximately 5×10^4 bacteria was spotted onto LB agar containing different concentrations of K_2TeO_3. The MIC of tellurite was defined as the lowest concentration of K_2TeO_3 at which there was no bacterial growth. The MIC of tellurite for *E. coli* XLOLR and DH5α was found to be 200 nM and 1 μM, respectively; 10^5 random XLOLR clones were plated onto LB plates containing kanamycin and 1 μM K_2TeO_3. Several kanamycin- and tellurite-resistant colonies were obtained. Phagemid DNA was purified from these colonies and transformed into *E. coli* DH5α to verify that the increased tellurite resistance was plasmid linked.

To determine the maximum level of tellurite resistance, the clones were streaked out on LB containing up to 1 mM K_2TeO_3. The inserts of plasmids conferring tellurite resistance were sequenced with the Big Dye dideoxy terminator cycle sequencing kit and an ABI 373A DNA sequencer according to the manufacturer's instructions (Applied Biosystems) with oligonucleotide primers complementary to the T3 and T7 promoter sequences of pBK-CMV. Sequences were compared to the *S. aureus* N315 genomic DNA sequence (38) and investigated with the NCBI-BLAST homology search program (http://www.ncbi.nlm.nih.gov/BLAST/) (1).

Construction of strains and plasmids. DNA manipulations and gel electrophoresis were carried out according to methods described in Sambrook et al. (52). To construct the *S. aureus cysM*::*tet* knockout, a 4.8-kb PCR fragment that

is flanked by *Eco*RI restriction sites was amplified with primers JKL48 and JKL49. This fragment was restricted with *Eco*RI and ligated with *Eco*RI-cut pOB (27), creating pJIM62. A unique *Cla*I site 508 bp downstream from the putative start codon of the *cysM* open reading frame was used to insert a 2.1-kb *Cla*I fragment containing a tetracycline resistance cassette from pDG1513 (25), creating pJIM64.

To construct the single-crossover chromosomal *cysM-lacZ* fusion, a 6-kb PCR fragment was amplified with primers JKL7 and JKL22, which contain *Bam*HI and *Eco*RI restriction sites, respectively. The cut fragment was ligated into the *lacZ* fusion vector pAZ106 (35) digested with *Bam*HI and *Eco*RI, creating pJIM44, in which the *lacZ* gene is fused 300 bp downstream of the putative *cysM* start codon. To complement the *cysM*::*tet* mutant, a 3-kb *Eco*RI-*Pst*I fragment from pJIM62 was ligated into the shuttle vector pMK4 (55) cut with *Eco*RI and *Pst*I, creating pJIM80. The insert begins 108 bp upstream from the start codon of the *hsp33* gene upstream of *cysM* and ends 682 bp downstream from the start codon of the *folP* gene downstream of *cysM*. Transformation into *S. aureus* RN4220 was performed as described by Schenk and Ladagga (53), selecting for tetracycline resistance, erythromycin resistance, and lincomycin resistance (for pJIM64), erythromycin resistance and lincomycin resistance (for pJIM44), or chloramphenicol resistance (for pJIM80 and pMK4) colonies.

Phage transduction into recipient strains was performed as described by Novick (48) with ϕ11 as the transducing phage. J96 (SH1000 *cysM*::*tet*) was isolated after transduction of an integrated RN4220 transformant of pJIM64 into *S. aureus* strain SH1000 (26), selecting for tetracycline-resistant, erythromycin-sensitive colonies. J62 (SH1000 *cysM-lacZ*) was isolated as an erythromycin-resistant, lincomycin-resistant colony after transduction of an integrated transformant of pJIM44. Southern blotting was used to verify the location and correct integration of DNA at chromosomal loci. J106 (J96/pJIM80), J108 (J96/pMK4), and J116 (SH1000/pMK4) were isolated as chloramphenicol-resistant colonies after transduction into SH1000 from RN4220 transformed with pJIM80 or pMK4. The presence of pJIM80 or pMK4 was confirmed by PCR with forward and reverse universal primers which are complementary to regions that flank the pMK4 polylinker. Strain SMH2052 was obtained from a random mutagenesis study (28) and contains a Tn*917* transposon insertion 655 bp downstream of the putative *cysJ* start codon.

Analysis of thiols from *S. aureus* and *E. coli*. *S. aureus* and *E. coli* cultures were grown in TSB medium for thiol analysis. *E. coli* carrying pJIM31 was isolated as a kanamycin-resistant, tellurite-sensitive colony and used as a neutral control in comparison with pJIM27 carrying *S. aureus cysM*. Washed cell pellets (100 to 250 mg) were resuspended in 1 ml of 50% vol/vol acetonitrile in Tris-HCl buffer (20 mM, pH 8.0), containing 2 mM monobromobimane (Calbiochem) and incubated at 60°C for 15 min in the dark. Control samples were treated with 5 mM *N*-ethylmaleimide for 10 min under the same conditions before the addition of monobromobimane (to 2 mM). The cellular debris was removed by centrifugation, and the samples were diluted in 10 mM aqueous methane sulfonic acid for reverse-phase high-pressure liquid chromatography (HPLC) analysis or to be kept frozen for future analyses. Thiol standards were prepared as described (17). The amount of thiol in the supernatant fraction is expressed on the basis of the dry weight of the residual cell pellet from each extract, which was determined by drying the cell pellet in an oven (80°C) until a constant weight was obtained. These residual dry weights were found to be 70 to 80% of the dry weight obtained for cells that had not been extracted.

HPLC analysis of thiol-bimane derivatives. Duplicate samples of cell extracts were routinely analyzed for thiols as their bimane derivatives by at least two different HPLC protocols (45). The chromatographic conditions used in these protocols, the sources for reagents, the preparation of thiol-bimane standards, and the HPLC equipment used have been described in detail elsewhere (17). Briefly, in the HPLC trifluoroacetic acid-methanol method (45), a reverse-phase column with trifluoroacetic acid-water and methanol gradients was used to separate most low-molecular-weight thiol derivatives normally encountered in biological extracts but not those of highly charged thiols such as coenzyme A. To confirm the identity and amounts of thiols found by the trifluoroacetic acid method, the coenzyme A method was used, which constituted a tetrabutylammonium phosphate (TBAP) ion-pairing protocol designed for the separation of coenzyme A-bimane derivatives (17). This method used a C8 RP column (C8 Symmetry, 3.9 by 150 mm; Waters) at a flow rate of 1.0 ml min^{-1}. The chromatographic protocol employed solvents and gradients as follows: solvent A, 10% (vol/vol) methanol, 0.25% (vol/vol) acetic acid, and 10 mM TBAP, pH 3.4; solvent B, 90% (vol/vol) methanol, 0.25% (vol/vol) acetic acid, and 10 mM TBAP. At time zero, 10% B; 15 min at 25% B; 30 min at 50% B; 40 min at 75% B; 45 min at 100% B, wash, equilibrate, and reinject.

Stress resistance and starvation survival assays. Determination of the MIC of tellurite for *S. aureus* strains was done in the same way as for *E. coli* strains (see above) except with BHI medium instead of LB. Hydrogen peroxide resistance assays were carried out as described by Watson et al. (66), with the following modifications: cells were grown in amino acid-limiting CDM (1%, wt/vol, glucose) to exponential phase (OD_{600} = 0.1). Following the addition of H_2O_2 to a final concentration of 10 mM and incubation, cells were serially diluted in PBS containing catalase at 10 mg ml^{-1}, and viability was assessed by overnight growth on BHI agar. Liquid tellurite resistance assays were performed in the same way except with K_2TeO_3 at a final concentration of 200 mM instead of H_2O_2 and serial dilution in PBS.

Acid resistance assays were performed by growing cells to exponential phase in BHI, harvesting, and resuspension in BHI acidified to pH 2 with HCl. Cells were serially diluted in 4× PBS and viability was determined on BHI agar. Disk diffusion assays were performed as follows: 5 ml of BHI top agar (0.7%, wt/vol) was seeded with 5 μl of an exponential-phase *S. aureus* BHI culture (OD_{600} = 0.2), and used as an overlay on a BHI agar plate. Sterile 13-mm antibiotic disks were placed on top of the overlay, and either 20 μl of 500 mM diamide, 35 μl of 2 M methyl viologen, or 20 μl of K_2TeO_3 was added to the disk. Zones of growth inhibition were measured after 24 h of incubation at 37°C. Starvation survival experiments were performed in amino acid-limiting, glucose-limiting, or phosphate-limiting CDM (32, 66); 50-ml cultures were grown for 24 h with shaking at 37°C, then kept static at 25°C. Samples were serially diluted and viability was assessed by growth on BHI agar. The results presented here are representative of three independent experiments that showed less than 10% variability.

β-Galactosidase assays. Expression of *cysM-lacZ* in *S. aureus* was measured in BHI cultures of J62 grown with shaking at 37°C. Cultures were inoculated from exponential-phase precultures to an OD_{600} of 0.001. To test for induction of *cysM-lacZ*, subinhibitory concentrations of diamide (200 μM), methyl viologen (25 μM), or K_2TeO_3 (5 μM) were added after 2 h of growth. Levels of β-galactosidase activity were measured as described previously (27) with 4-methylumbelliferyl-β-D-galactosidase as the substrate. Assays were performed in duplicate, and the values were averaged. The results presented here are representative of two independent experiments that showed less than 10% variability.

RESULTS

***S. aureus* cysteine synthase gene confers increased tellurite resistance on *E. coli*.** The MIC of tellurite for *S. aureus* 8325-4 and *S. aureus* SH1000 (an $rsbU^+$ derivative of 8325-4) (26) was 6 mM and 7 mM, respectively. In contrast, the MIC of tellurite for *E. coli* XLOLR and DH5α was 200 nM and 1 μM, respectively. A phagemid library of *S. aureus* 8325-4 DNA was used to screen for genes conferring increased tellurite resistance on *E. coli*. The library was prepared by mass excision from a λZAP Express library made previously by Foster (20); 10^5 clones were screened on the basis of their ability to enable growth of *E. coli* XLOLR on LB agar containing 1 μM K_2TeO_3.

Phagemid DNA was purified from nine apparently tellurite-resistant colonies and transformed into *E. coli* DH5α to verify that the increased tellurite resistance was plasmid linked. Four clones conferred increased tellurite resistance on *E. coli* DH5α (pJIM27 to pJIM30, Fig. 1). All four clones contained an overlapping region with only one intact open reading frame (SA0471), encoding a putative protein of 310 amino acid residues, with 45% amino acid identity to CysK of *E. coli* and 35% amino acid identity to CysM of *E. coli*. Downstream of SA0471 is a putative Rho-independent terminator (17 to 47 bases from the stop codon). Plasmids pJIM28 to pJIM30 enabled *E. coli* DH5α to grow on LB medium containing up to 10 μM tellurite. pJIM27, which carries the same insert but in the opposite orientation, so that the *cysM* gene is downstream of the pBK-CMV *lac* promoter, conferred resistance up to 500 μM tellurite.

High levels of glutathione are produced by *E. coli* carrying an *S. aureus* cysteine synthase. The thiol content of cultures of *E. coli* carrying pJIM27 was analyzed by separating the bimane derivatives of low-molecular-weight thiols in cell extracts by

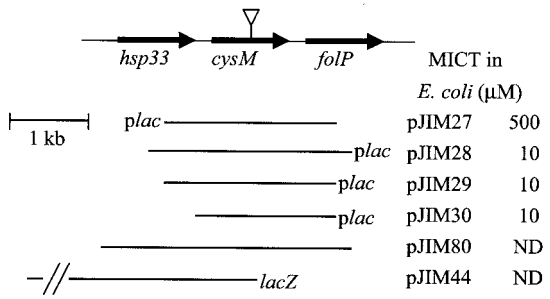

FIG. 1. *S. aureus cysM* region and plasmids conferring tellurite resistance in *E. coli*. The inserts in pJIM27, pJIM28, pJIM29, and pJIM30 are shown in relation to the *lac* promoter on the pBK-CMV vector. The MIC of tellurite (MICT) of each clone is shown. The MIC of tellurite for *E. coli* DH5α is 1 μM. The inverted triangle shows the position of the tetracycline resistance cassette insertion in *S. aureus* J96 (*cysM*::*tet*). Plasmids pJIM80, used for complementation, and pJIM44, used to make *S. aureus* J62 (*cysM-lacZ*), are shown. ND, not determined.

reverse-phase HPLC. *E. coli* containing pJIM31, a pBK-CMV derivative that did not confer increased tellurite resistance, was used as a control. Extracts from both strains gave a peak corresponding to glutathione (Fig. 2). The amount of glutathione in these cultures was quantified, and in *E. coli*/pJIM27 cultures the amount of glutathione was significantly higher throughout growth compared to the control strain. A culture of *E. coli*/pJIM31 at an OD_{600} of 3.9 contained 1.1 nmol of glutathione per mg of dry cell weight. In contrast, a culture of *E. coli*/pJIM27 at a similar OD_{600} of 3.5 produced 9.93 nmol of glutathione per mg of dry cell weight.

Cysteine synthase proteins and molecules containing cysteine, including glutathione, have previously been shown to be involved in tellurite resistance in *E. coli* (15, 49, 59, 60). Tellurite resistance in *S. aureus* is uncharacterized, and furthermore, little is known about cysteine biosynthesis and its importance in this bacterium. It was therefore of interest to investigate the role of this cysteine synthase locus in *S. aureus*.

Search for cysteine biosynthetic genes in the *S. aureus* genome. The process of sulfur assimilation has been shown to involve many genes and enzymatic reactions in bacteria such as

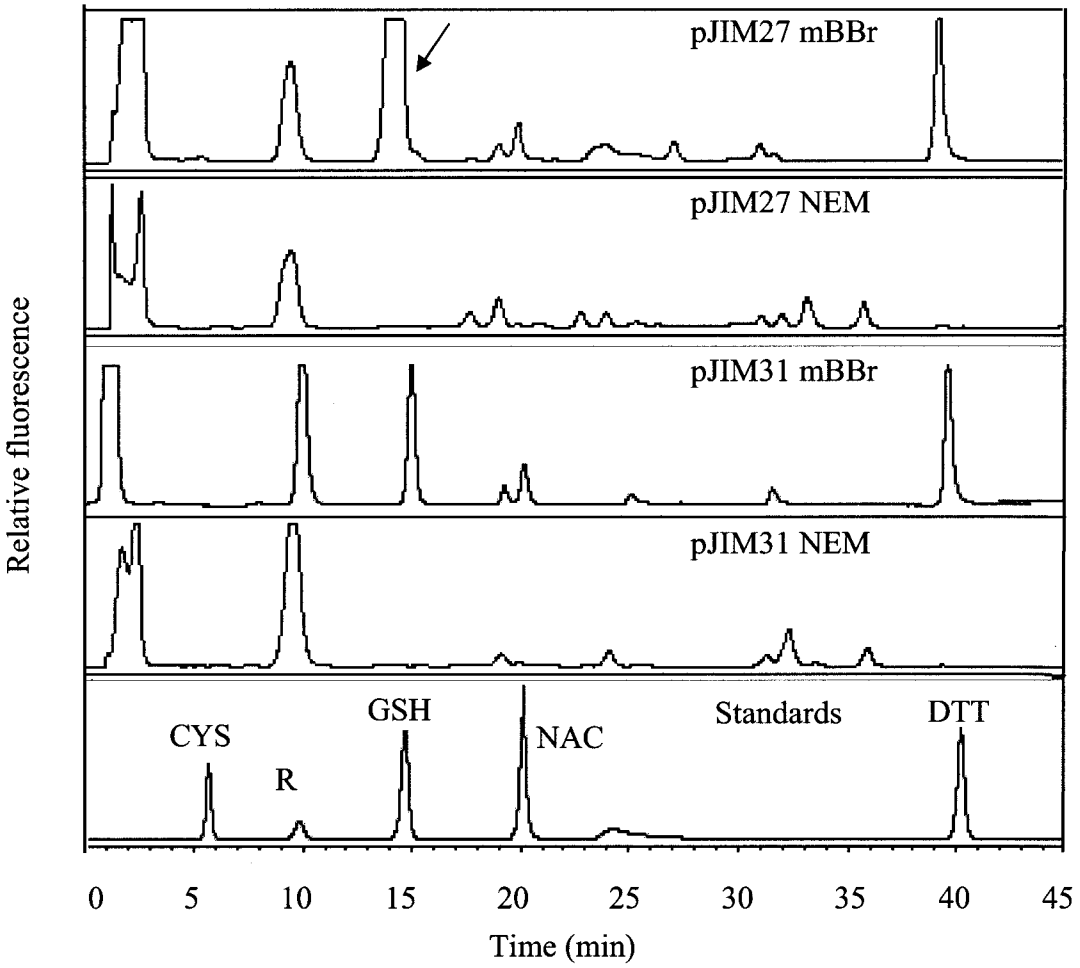

FIG. 2. Reverse-phase HPLC of *E. coli* thiols. Traces shown are from extracts of *E. coli*/pJIM27 and *E. coli*/pJIM31 (control) cultures grown in TSB to an OD_{600} of 1.7 and 2.0, respectively. Arrow points to the glutathione peak. CYS, cysteine; GSH, glutathione; NAC, *N*-acetylcysteine; DTT, dithiothreitol; R, peak produced by chemical reagents; NEM, *N*-ethylmaleimide; mBBr, monobromobimane.

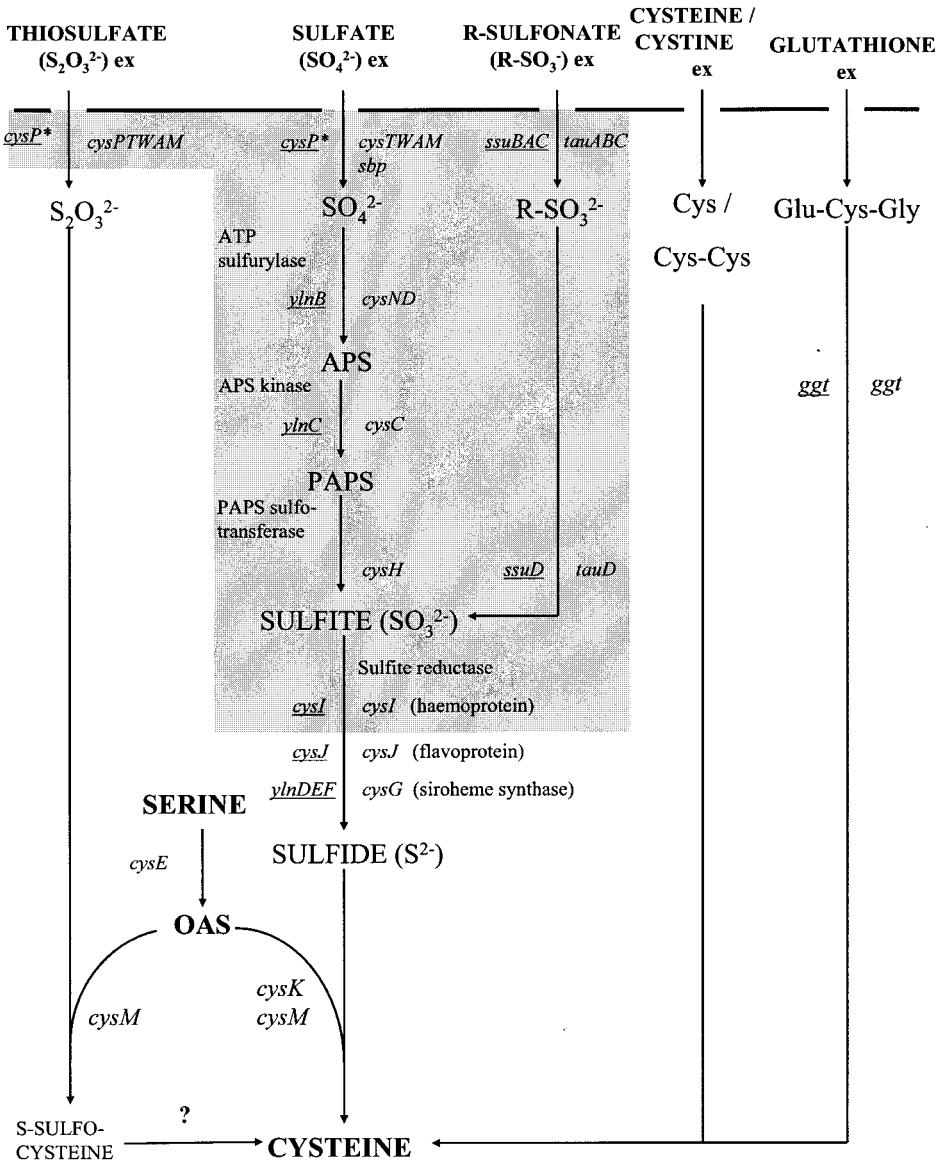

FIG. 3. Sulfur assimilation and cysteine biosynthesis in *E. coli* and *B. subtilis*. Genes involved in uptake and assimilation of inorganic and organic sulfur sources in *E. coli* and *B. subtilis* are shown. *B. subtilis* genes are underlined. The shaded region covers genes for which no apparent homologue can be found in the *S. aureus* N315 genome sequence. Note that *E. coli*, *B. subtilis*, and *S. aureus* all encode *cysE*, *cysK*, and *cysM* homologues. Ex, external sulfur source. Cysteine, cystine, and glutathione may be used as organic sources of sulfur. APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; OAS, *O*-acetylserine. *cysP**, the *B. subtilis* CysP sulfate/thiosulfate transporter is distinct from the *E. coli* CysP thiosulfate-binding protein.

E. coli and *B. subtilis* (reviewed in references 24 and 37). All of the published *S. aureus* genome sequences contain cysteine synthase homologues (38; http://www.genome.ou.edu/staph.html, http://www.tigr.org), but auxotrophy for cysteine has been reported in some *S. aureus* strains (16).

A systematic search of the *S. aureus* 8325 and *S. aureus* N315 genome sequences was performed, looking for homologues of genes known or thought to be involved in bacterial cysteine biosynthesis and sulfur assimilation. Neither sequence contained any homologues of the *E. coli* or *B. subtilis* genes required for sulfate or thiosulfate uptake, reduction of sulfate to sulfite, or uptake and reduction of organic sulfonates (Fig. 3). The *S. aureus* N315 genome encodes homologues of the *B. subtilis* proteins CysE (SA0487, 64% amino acid identity); CysJ (SA2413, 50% identity); YlnD (SA2186, 42% identity); YlnE (SA2189, 21% identity); YlnF (SA2186, 35% identity); and the γ-glutamyl peptidase encoded by *ggt* (SA0202, 37% identity). The *S. aureus* N315 genome also has three cysteine synthase gene homologues (Table 2), including SA0471, which corresponds to the gene conferring increased tellurite resistance carried on pJIM27 to pJIM30.

Growth of *S. aureus* with different sulfur sources. The genomic data suggested that *S. aureus* potentially can use sulfide or thiosulfate to produce cysteine, but does not have any

TABLE 2. Cysteine synthase homologues in *S. aureus* N315

S. aureus N315 gene no.	N315 coordinates (no. of amino acids)	% identity[a] to CysK/CysM	Open reading frames[b]	
			Upstream	Downstream
SA0112	128772–129752 (327)	33/35	*sirABC* (Fe^{3+} uptake)	Putative ornithine cyclodeaminase
SA0418	479707–480612 (302)	36/33	*metB* (cystathionine γ-synthase)	Putative Na^+-dependent transporter
SA0471	544265–545197 (311)	45/35	*hsp33* (redox-sensitive chaperone)	*folPBK* (folic acid biosynthesis)

[a] Amino acid identity with CysK and CysM from *E. coli* based on SWISSPROT sequence P11096 for CysK and SWISSPROT sequence P16703 for CysM.
[b] Putative function based on homology to known sequences.

of the recognized bacterial systems for the uptake and reduction of sulfate or sulfonates. A strain carrying a knockout mutation in the SA0471 cysteine synthase locus was constructed (J96). In addition, strain SMH2052, which has a transposon insertion in the *cysJ* homologue, was isolated in a random mutagenesis study (28). To investigate cysteine biosynthesis and the role of these two genes in *S. aureus*, the ability of SH1000, J96 (*cysM*), and SMH2052 (*cysJ*) to grow with different sources of sulfur was tested (Table 3). None of the three strains could grow on chemically defined medium (CDM) agar plates lacking cysteine (note that CDM contains $MgSO_4$ and methionine at final concentrations of 2 mM and 200 μM, respectively) or with the addition of sulfate, sulfite, ethanedisulfonate, or benzenesulfonate. All three strains could grow with cysteine, cystine, or glutathione as the sole sulfur source. In the presence of sodium sulfide, strains SH1000 and SMH2052 grew normally, while J96 (*cysM*) colonies grew poorly, only appearing after 3 days of incubation. In anaerobic conditions, the results for each strain were the same as were found in aerobic conditions (data not shown). In addition, a number of other *S. aureus* strains, including Newman and COL, were unable to utilize sulfate as a sole sulfur source (data not shown).

***S. aureus* SH1000 requires *cysM* to utilize thiosulfate as the sole sulfur source.** Significantly, SH1000 and SMH2052 (*cysJ*), but not J96 (*cysM*), could grow with sodium thiosulfate as the sole sulfur source. Plasmid pJIM80, which carries the *S. aureus cysM* gene in the shuttle vector pMK4, was constructed. The introduction of pJIM80 into J96 (*cysM*) complemented the *cysM* mutation, enabling growth to wild-type levels in CDM broth containing 500 μM thiosulfate, whereas the addition of pMK4 into J96 (*cysM*) did not (Fig. 4A). *cysM* mutants of *E. coli* are unable to grow on thiosulfate, as CysK cannot utilize thiosulfate as a substrate. The growth and complementation data demonstrated that the gene inactivated in *S. aureus* J96 encodes a cysteine synthase functionally homologous to *E. coli* CysM.

TABLE 3. Growth of *S. aureus* strains on different sulfur sources[a]

Sulfur source added	Growth		
	SH1000	J96 (*cysM*)	SMH2052 (*cysJ*)
None (SO_4^{2-} + methionine)	−	−	−
L-Cystine	+	+	+
L-Cysteine	+	+	+
Glutathione	+	+	+
4-Nitrocatechol sulfate	−	−	−
Sodium sulfate	−	−	−
Sodium sulfide	+	+/−	+
Ethanedisulfonate	−	−	−
Benzenesulfonate	−	−	−
Sodium thiosulfate	+	−	+

[a] Strains were grown on CDM agar containing 2 mM $MgSO_4$ and 200 μM methionine. Different sulfur sources were added at 500 μM. +, normal growth (colony diameter, 2 to 3 mm) after 24 h at 37°C; −, no growth; +/−, poor growth (colony diameter up to 1 mm).

***S. aureus* J96 (*cysM*) shows reduced growth in cysteine-limiting conditions.** After 32 h of growth in CDM broth containing 200 μM cysteine, the OD_{600} of SH1000 and J96 (*cysM*) cultures was 12 and 10.5, respectively (Fig. 4B). However, at lower cysteine concentrations, J96 (*cysM*) cultures grew to a lower density than wild-type SH1000. After 32 h of growth in the presence of 10 μM cysteine, the OD_{600} of SH1000 and J96 (*cysM*) cultures was 4.9 and 0.8, respectively. A similar growth defect could be seen with cystine as the sulfur source (data not shown).

Analysis of the thiol content of *S. aureus*. The thiol contents of *S. aureus* SH1000 and J96 (*cysM*) were analyzed (Fig. 5). Analysis was performed on extracts of cultures at stages throughout the growth phase. SH1000 wild-type extracts gave peaks corresponding to cysteine and coenzyme A and also peaks at around 16 min and 33 min which represent uncharacterized *S. aureus* thiol components that did not correspond to any of the known standards. HPLC traces from J96 (*cysM*) cultures were essentially the same except for the reduction in the size of the peak corresponding to cysteine. When quantified, it was found that, whereas the cysteine concentrations in SH1000 extracts are maintained at around 0.5 nmol per mg of dry cell weight, in J96 (*cysM*) cultures the cysteine concentration falls to undetectable levels at an OD_{600} of 1.4 and above (Fig. 5 and data not shown). In contrast, the uncharacterized thiol-containing compounds remained constant.

The *cysM* mutant is sensitive to tellurite, oxidative, and disulfide stress. Since the *S. aureus cysM* gene conferred increased tellurite resistance in *E. coli*, its effect on the same in *S. aureus* was tested. The MIC of tellurite for J96 was 5 mM, lower than that of SH1000 (7 mM). In liquid assays, when exponential-phase cells were challenged with 200 mM tellurite, J96 (*cysM*) cultures showed a 15-fold reduction in viability compared to SH1000 after 6 h (Fig. 6A).

S. aureus survives a diverse range of stresses during its life cycle (9), and the role of *cysM* in the response to other stresses was investigated. The viability of J96 (*cysM*) was reduced 45-fold in comparison to SH1000 6 h after the addition of 10 mM H_2O_2 (Fig. 6B). J96 (*cysM*) was also more sensitive to acid stress, its viability reduced 30-fold after 4 h at pH 2 (Fig. 6C). In disk diffusion assays, growth of J96 (*cysM*) was significantly

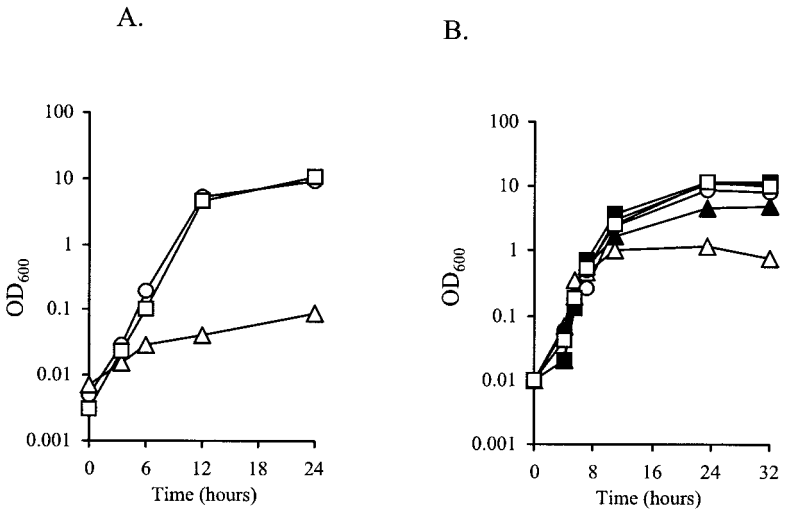

FIG. 4. (A) Growth of *S. aureus* strains in CDM broth containing 500 μM sodium thiosulfate as the sole sulfur source. Squares, J116 (SH1000 wild-type carrying pMK4); circles, J106 [J96 (*cysM*) carrying pJIM80]; triangles, J106 [J96 (*cysM*) carrying pMK4]. (B) Growth of wild-type *S. aureus* SH1000 (solid symbols) and J96 (*cysM*) (open symbols) in CDM broth with various cysteine concentrations. Squares, 200 μM cysteine; circles, 50 μM cysteine; triangles, 10 μM cysteine. Cultures were grown with shaking at 37°C. Results are representative of at least two independent experiments.

more inhibited than that of SH1000 by 1 M diamide, a specific thiol oxidant that causes disulfide stress. This diamide sensitivity could also be complemented by pJIM80 (Fig. 6D). However, J96 (*cysM*) was no more sensitive to methyl viologen than SH1000 (data not shown).

To determine whether the *S. aureus cysM* gene was induced by stress, *cysM-lacZ* fusion strain J62 was constructed. Expression of *cysM-lacZ* reached a sharp peak in the postexponential/early stationary phase of growth in BHI medium (Fig. 7). The addition of diamide, methyl viologen, hydrogen peroxide, or tellurite during the early exponential phase of growth (OD = 0.05) did not lead to early induction of *cysM-lacZ* expression (data not shown). A slight increase in the level of expression was observed in stationary phase following addition of 5 μM tellurite (Fig. 7).

The *cysM* locus affects starvation survival but not exoprotein production and pathogenicity. Genes involved in stress resistance have also been shown to affect the viability of *S. aureus* during long-term starvation conditions (10, 11, 66, 67). In amino acid-limiting CDM containing 1% (wt/vol) glucose, the viability of J96 (*cysM*) was reduced 1,000-fold in comparison to SH1000 after 27 days, and in phosphate-limiting CDM, J96 (*cysM*) lost all viability after 21 days (Fig. 8). There was no difference in the viability of J96 and SH1000 grown in glucose-limiting CDM (data not shown).

The ability of *S. aureus* to respond to and survive various stresses is vital for its ability to successfully colonize tissues and evade host defense mechanisms, but despite being sensitive to the various stress conditions described above, J96 (*cysM*) was not attenuated in a mouse lesion model of pathogenicity (7), was no different from SH1000 in hemolytic activity on rabbit or sheep blood plates, and produced the same profile on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of exoprotein extracts (data not shown).

DISCUSSION

Role of *cysM* in tellurite resistance in *E. coli* and *S. aureus*. The use of tellurite in selective media for *S. aureus* was first described by Ludlam in 1949 (42). In Baird-Parker medium, a mixture of egg yolk and tellurite is used to isolate and identify coagulase-positive staphylococci from food (3). *S. aureus* colonies are distinguished by a zone of clearing in the egg yolk due to lecithinase activity and a shiny black appearance. The black color of bacteria grown on tellurite media is caused by deposits of mostly elemental tellurium produced by the reduction of tellurite (58). The toxicity of tellurite is thought to come from its strong oxidizing ability (58, 60). Genes associated with tellurite resistance are found in many pathogenic bacteria and can be plasmid borne, such as the *klaABC* operon found on IncP-type plasmids (50).

CysK from *Bacillus stearothermophilus* and *Rhodobacter sphaeroides* confers tellurite resistance when introduced into *E. coli* and *Paracoccus denitrificans*, respectively (49, 64). The exact mechanism of CysK-mediated tellurite resistance is not known. Cysteine residues are known to be important in the function of many metal-binding proteins (22). The *E. coli* tellurite resistance determinants TehA and TehB each contain three cysteine residues, and replacement of these cysteines with alanine residues by site-directed mutagenesis leads to a decrease in tellurite resistance (15). In addition, the thiol redox enzymes (glutathione reductase and thioredoxin reductase) and their metabolites (glutathione, glutaredoxin, and thioredoxin), which all contain cysteine residues, have been shown to be involved in tellurite resistance (59, 60). Turner et al. (60) suggested that reduced thiols cause reduction of TeO_3^{2-} to Te^0, possibly via an intermediate telluro-ether disulfide bond (RS-Te-SR), and that this, in combination with the action of other components, contributes to tellurite resistance.

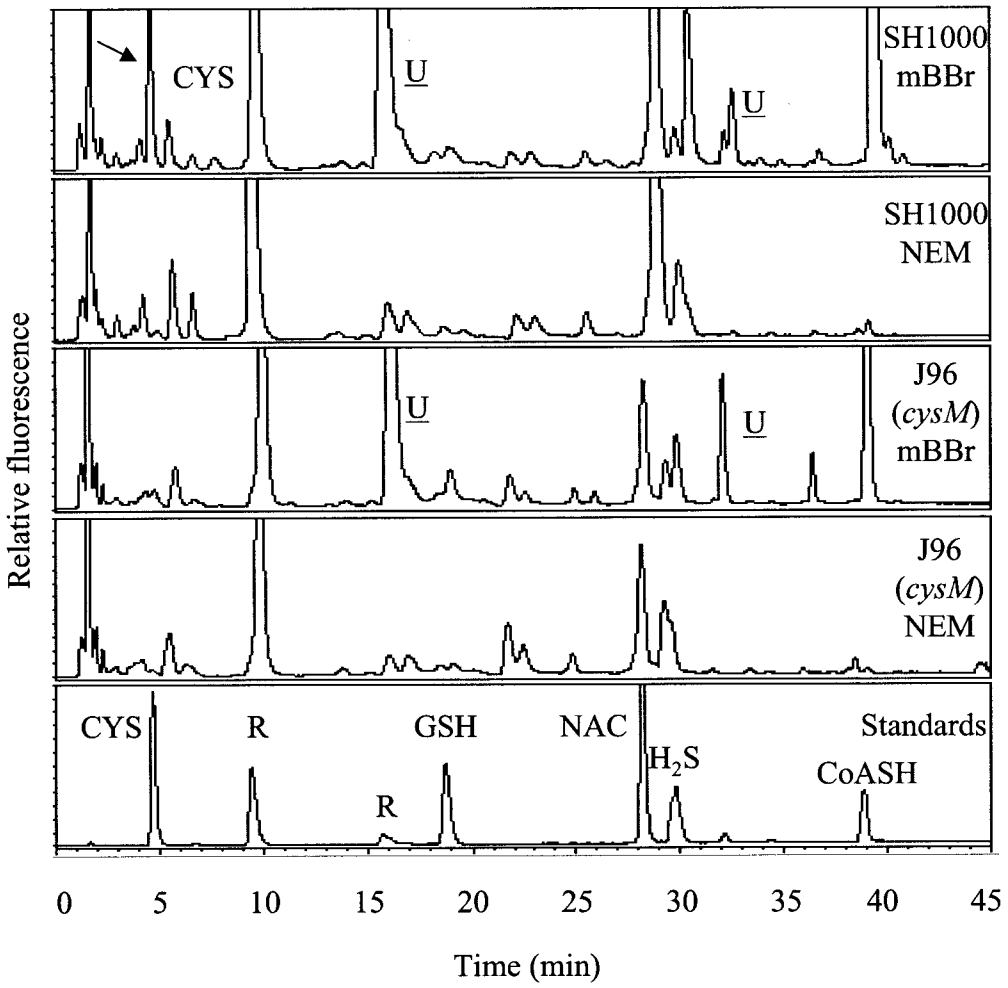

FIG. 5. Reverse-phase HPLC of *S. aureus* thiols. Extracts of *S. aureus* SH1000 and J96 (*cysM*) cultures grown in TSB medium to an OD_{600} of 1.5 were reacted with monobromobimane (mBBr) and separated by reverse-phase HPLC. These extracts are representative of all stages of growth. Control samples were reacted with *N*-ethylmaleimide (NEM) and monobromobimane. To identify peaks, bimane derivatives of standard thiols were used. CYS, cysteine; GSH, glutathione; NAC, *N*-acetylcysteine; CoASH, reduced coenzyme A; U, unidentified components; R, peaks produced by reagents. The arrow points to the cysteine peak found in SH1000 but absent in J96 (*cysM*) samples.

E. coli cells carrying the *S. aureus cysM* gene produce a large amount of glutathione (γ-glutamyl-cysteinyl-glycine), although the level of cysteine is not greatly higher than in the control cultures. Glutathione protects many organisms from oxidative toxicity by functioning as a slowly autooxidizing reserve of cysteine and as a cofactor in the detoxification of products formed from oxygen reactions (6, 14, 51). The production of glutathione is limited by the availability of cysteine, and therefore it is likely that expression of the *S. aureus cysM* leads to increased levels of cysteine, which in turn is incorporated into glutathione by *E. coli* glutathione synthetase.

In pJIM27, the *cysM* gene is downstream of the pBK-CMV *lac* promoter. Enhanced expression of the *S. aureus cysM* gene from the *lac* promoter may explain the much higher level of glutathione production and tellurite resistance conferred by pJIM27. It is possible that the increased tellurite resistance is because of a direct reaction of glutathione with TeO_3^{2-} or due to glutathione's reversing the effects of tellurite oxidation on other thiols in the cell. When grown on LB medium containing tellurite, colonies of *E. coli* carrying pJIM27 also had a grey-black appearance. *S. aureus* colonies grown on tellurite medium have a characteristic black color, suggesting that the *S. aureus* cells reduce tellurite. However, glutathione cannot be responsible for tellurite reduction in *S. aureus*, as it does not synthesize this compound (46). The *S. aureus cysM* mutant shows increased sensitivity to tellurite, suggesting that cysteine or a compound that requires cysteine for its synthesis other than glutathione contributes to tellurite resistance.

S. aureus SH1000 cultures were shown to contain cysteine, reduced coenzyme A, H_2S, and at least two more uncharacterized thiol compounds. In contrast to the wild type, cysteine levels were not maintained throughout growth in the *cysM* mutant. The level of the other thiols, however, was not significantly altered in the *cysM* mutant. Reduced coenzyme A has been shown to be the predominant low-molecular-weight thiol in *S. aureus* (12, 13), and its synthesis does not involve cysteine. It is possible that the cysteine molecule itself is involved in direct reduction of tellurite. Alternatively, protein or peptide

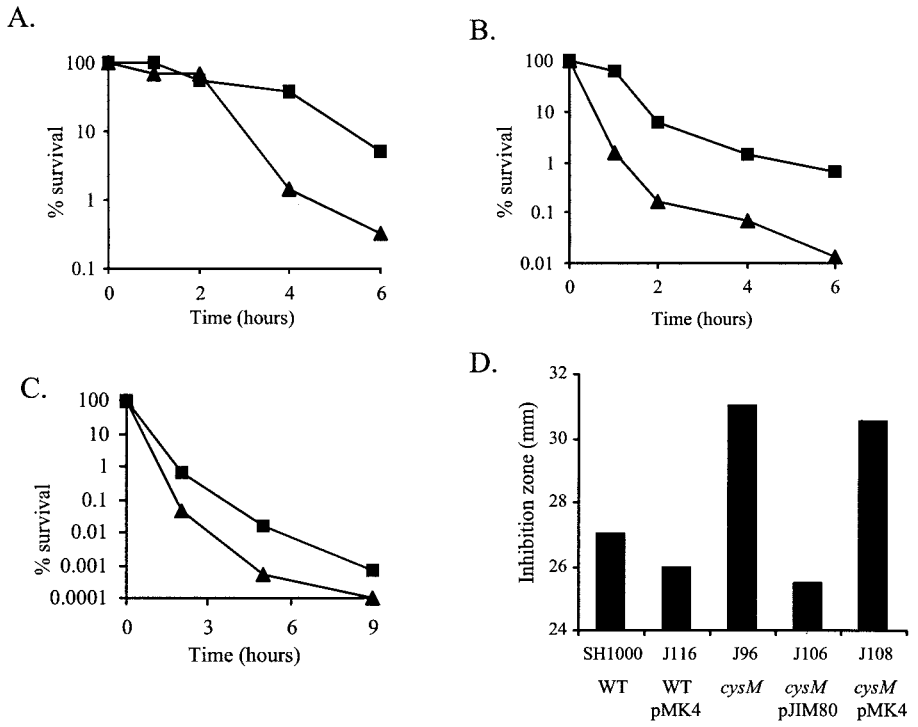

FIG. 6. Stress resistance assays. (A, B, and C) Viability of *S. aureus* SH1000 (squares) and J96 (*cysM*) (triangles) exponential-phase cells after challenge with (A) 200 mM K_2TeO_3, (B) 10 mM H_2O_2, and (C) hydrochloric acid to pH 2. (D) Disk diffusion assays with 1 M diamide. The sensitivity of J96 (*cysM*) can be complemented with pJIM80 but not the control pMK4 plasmid.

synthesis may be affected by the absence of cysteine and that other specific tellurite-reducing peptides or proteins are reduced. Also, it has recently been proposed that important proteins containing iron-sulfur clusters are the indirect targets of tellurite via the production of superoxide (57). Cysteine is important as a source of sulfur for the repair of oxidatively damaged iron-sulfur cluster proteins with crucial roles in metabolism (57).

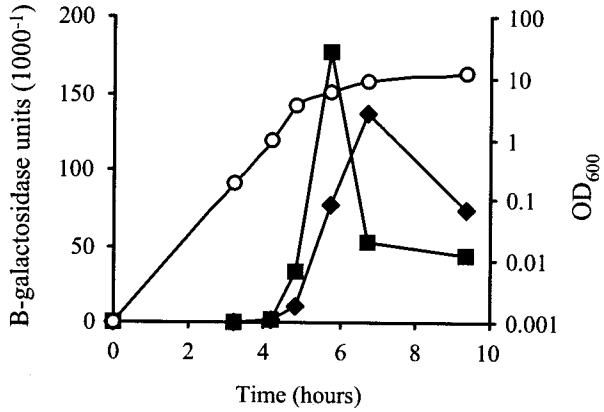

FIG. 7. Expression of *cysM-lacZ*. J62 (SH1000 *cysM-lacZ*) cultures were grown at 37°C with shaking in BHI medium. Squares, *cysM-lacZ* expression, no additions; diamonds, *cysM-lacZ* expression with addition of 5 μM K_2TeO_3 at 2 h; circles, OD_{600} of representative culture.

Role of *cysM* in *S. aureus* stress resistance and starvation survival. The observation that plasmid pJIM80 could complement the sensitivity of J96 (*cysM*) to diamide, a specific thiol oxidant, suggests that the increased sensitivity of J96 (*cysM*) is not due to any polar effects on genes downstream of *cysM*.

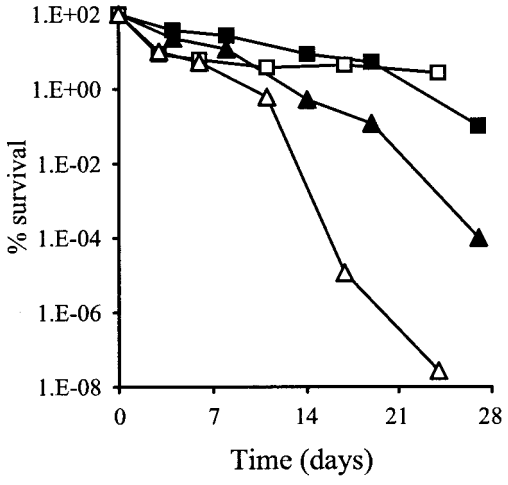

FIG. 8. Starvation survival recovery. Cultures of SH1000 (squares) and J96 (triangles) were grown with shaking for 24 h at 37°C in either amino acid-limiting (solid symbols) or phosphate-limiting (open symbols) CDM, and then kept static at 25°C. Samples were removed and serially diluted, and viability was assessed by growth on BHI plates.

Tellurite, hydrogen peroxide, acid, and diamide are all substances that can cause imbalance in the thiol redox status of the cytoplasm, or oxidative stress (6, 34, 40, 58). Cysteine residues in the cytoplasm are normally kept in a reduced state, but under oxidative conditions they form disulfide bonds, causing misfolding and inactivation of proteins (2).

In *S. aureus*, free cysteine, reduced coenzyme A, and other thiols may help maintain the thiol redox balance as well as thioredoxin and thioredoxin reductases, which are encoded by the *trxAB* genes. A defect in cysteine biosynthesis would also impair protein synthesis, which has been shown to be important in starvation survival of *S. aureus* (66). This could explain the more pronounced loss of viability in cultures of J96 (*cysM*) in phosphate- or amino acid-limiting conditions. Glucose limitation leads to the development of a stable survival state in 0.1 to 1% of *S. aureus* cells (66), and in these conditions, J96 (*cysM*) apparently obtains enough cysteine from the medium to enable it to remain viable. A recent study has shown that a number of *B. subtilis* genes are induced by diamide stress, including *cysK* (40). Although the *S. aureus cysM* gene is involved in resistance to diamide and other stresses, expression of *cysM* was not affected by addition of diamide, methyl viologen, or hydrogen peroxide, indicating differences in the stress responses of *B. subtilis* and *S. aureus*.

Cysteine biosynthesis and sulfur assimilation in *S. aureus*. In cysteine-limiting conditions, the growth of the *S. aureus cysM* mutant is significantly impaired compared to the wild-type SH1000. This suggests that CysM is the major cysteine synthase in *S. aureus*, since neither of the other two cysteine synthase homologues in the *S. aureus* genome can compensate for the mutation of *cysM*. It has been suggested that CysM-type cysteine synthases are preferentially used during anaerobic growth (37). In *S. aureus* it appears that CysM is required for growth with thiosulfate in both aerobic and anaerobic conditions.

In other bacteria, including *E. coli* and *B. subtilis*, sulfate or organic sulfonates are reduced to sulfide, a substrate for both CysK- and CysM-type cysteine synthases. It is likely that *S. aureus* cannot utilize sulfate or sulfonates as a sulfur source because its genome does not encode any of the genes required for the uptake and subsequent reduction of such compounds. Recent studies have shown that the genomes of the gram-positive lactic acid bacterium *Lactococcus lactis* and the gram-negative pathogen *Haemophilus influenzae* do not encode genes for sulfate uptake and reduction but do have cysteine synthase homologues (18, 65), just as in the genomes of several *S. aureus* strains. In addition, it has been shown that both *L. lactis* and *H. influenzae* are unable to grow with sulfate as the only sulfur source (18, 65). *S. aureus* can, however, use both thiosulfate and sulfide as a sulfur source. The use of thiosulfate is dependent on *cysM*, while growth on sulfide is impaired but not totally abolished in the J96 (*cysM*) mutant, suggesting that one or both of the other cysteine synthases can use sulfide as a substrate, but not as effectively as CysM.

S. aureus CysM is clearly an *O*-acetylserine (thiol)-lyase B-type protein, despite its having greater sequence homology to *E. coli* CysK, an *O*-acetylserine (thiol)-lyase A. In *E. coli*, thiosulfate is transported with the *cysTWA* permease in conjunction with a thiosulfate binding protein, CysP (21), but no homologues of these are present in the *S. aureus* genome. The ability of *S. aureus* SH1000 to use thiosulfate indicates the presence of an unknown thiosulfate uptake mechanism.

The evidence presented here suggests that many *S. aureus* strains can assimilate thiosulfate and sulfide but not sulfate. The physiological significance of this is unknown. Sulfate and sulfide are the major available forms of sulfur in soil and water, and both are also present in humans, although sulfide levels are presumably kept low due to its toxicity (47). Thiosulfate is present at least transiently in the environment and is present in humans, although usually at low concentrations (33, 39). It is likely that while in a human host, *S. aureus* gains most of its sulfur from organic sources and infrequently requires assimilation of inorganic sulfur. The inability of some *S. aureus* strains to use sulfate or sulfonates could be linked to the scarcity of these compounds in the environment in which *S. aureus* has evolved. It is interesting that *S. aureus* SH1000 can utilize the organic compounds cysteine, cystine, and glutathione, which are readily available in the mammalian cell environment (43).

S. aureus SH1000 cannot grow in CDM containing methionine and sulfate as the only sources of sulfur, showing that it is unable to perform the trans-sulfuration reaction which converts methionine into cysteine. Growth on cysteine, cystine, and glutathione suggests that *S. aureus* has, first, a transport mechanism for these molecules, and second, an ability to break down cystine (Cys-Cys) or glutathione to yield cysteine. No specific cysteine permease has been described, although the *S. aureus* genome sequence encodes many putative ABC-type transporters, one of which could transport cysteine, and several putative oligopeptide transporters, which could transport cystine or glutathione. In *E. coli*, γ-glutamyl peptidase cleaves glutathione to cysteinyl-glycine during the transport of an external amino acid, followed by breakdown of cysteinyl-glycine to yield cysteine (56). Although it does not synthesize glutathione, *S. aureus* can apparently import and metabolize glutathione, as has been shown for *Streptococcus mutans* (54) and *H. influenzae* (65). Interestingly, it has recently been reported that imported glutathione forms part of the oxidative stress resistance mechanism of *H. influenzae* (65). It is theoretically possible that glutathione may be used in a similar way by *S. aureus* to resist oxidative attack during infection of the host.

This study has shown that a link between metabolism and stress resistance is part of the overall complex physiology of *S. aureus* which allows it to inhabit so many different niches and be such a successful pathogen. Elucidation of the metabolic capabilities of *S. aureus* and an understanding of those important for growth of the organism in vivo may lead to the development of novel intervention strategies.

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