JOURNAL OF BACTERIOLOGY, July 1999, p. 3898–3903 Vol. 181, No. 13
0021-9193/99/$04.00+0

Characterization of the Major Superoxide Dismutase of *Staphylococcus aureus* and Its Role in Starvation Survival, Stress Resistance, and Pathogenicity

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Received 28 January 1999/Accepted 7 April 1999

A *Staphylococcus aureus* mutant (SPW1) which is unable to survive long-term starvation was shown to have a transposon insertion within a gene homologous to the *sodA* family of manganese-dependent superoxide dismutases (SOD). Whole-cell lysates of the parental 8325-4 strain demonstrated three zones of SOD activity by nondenaturing gel electrophoresis. The activities of two of these zones were dependent on manganese for activity and were absent in SPW1. The levels of SOD activity and *sodA* expression were growth-phase dependent, occurring most during postexponential phase. This response was also dependent on the level of aeration of the culture, with highest activity and expression occurring only under high aeration. Expression of *sodA* and, consequently, SOD activity could be induced by methyl viologen but only during the transition from exponential- to postexponential-phase growth. SPW1 was less able to survive amino acid limitation and acid stress but showed no alteration in pathogenicity in a mouse abscess model of infection compared to the parental strain 8325-4.

The aerobic environment is potentially toxic to life due to the high reactivity of oxygen. When oxygen becomes partially reduced, reactive oxygen species such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are often formed (17). Internal generation of these reactive species results in damage to DNA, proteins, and lipids (23). As a defense against these toxic effects, organisms have evolved superoxide dismutases (SODs) which detoxify $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2), which in turn is generally broken down to water by catalase. SODs are classified according to the metal ion cofactor required for activity; the copper-zinc type (Cu/Zn-SOD), the manganese type (Mn-SOD), the iron type (Fe-SOD), and the recently identified nickel type (Ni-SOD) (18, 26).

Escherichia coli has three SODs, which differ in their location and temporal expression. Both Fe- and Mn-SOD are present in the cytoplasm, where they protect DNA and proteins from oxidation. Expression of the Fe-SOD, encoded by *sodB*, is constitutive and therefore is thought to play a housekeeping role (21), while the levels of Mn-SOD, encoded by *sodA*, fluctuate, increasing in response to elevated levels of internal $O_2^{\cdot-}$ and upon changes in growth phase (13). The Cu/Zn-SOD, encoded by *sodC*, is located in the periplasm and protects the periplasmic and membrane constituents from exogenous superoxide (4, 24). To date, bacterial Cu/Zn-SOD has been identified only in gram-negative pathogens, where it is thought to protect against host defense mechanisms (4, 29, 38, 45). After engulfment of bacteria by professional phagocytes, the induction of highly microbiocidal reactive oxygen metabolites during the oxidative burst occurs, resulting in killing (2, 37).

Staphylococcus aureus is medically important as the cause of many nosocomial infections (42). The ability of *S. aureus* to survive in nutrient-limiting and stressful conditions contributes to its transmissibility in the hospital environment, which occurs primarily via direct contact (e.g., hand to wound), airborne carriage, and contact with surfaces such as indwelling devices (e.g., catheters). Recently we have characterized the starvation survival and stress responses of *S. aureus* and have identified several loci involved in these processes (7, 10, 11, 43, 44). During a screen for starvation survival mutants, a gene showing homology to the *sodA* family of SOD was identified. This study describes the characterization of the SOD and the demonstration of its role in stress resistance but not pathogenicity in a mouse abscess model of infection.

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MATERIALS AND METHODS

Cloning and sequencing of *sodA*. A plasmid, pSPW1, which contained chromosomal DNA flanking the *lacZ* proximal region of the Tn insertion within *S. aureus* SPW1, was generated in a previous study (43). The insert in pSPW1 (approximately 5 kbp) was excised, DIG labelled according to the protocol of Boehringer Mannheim GmbH (Germany), and used to probe a λ ZAP Express library of partial *Sau*3A digest (2 to 10 kb) of *S. aureus* 8325-4 genomic DNA (15). A clone containing a 3.3-kbp genomic DNA fragment spanning the SPW1 transposon insertion site was identified, and a stable phagemid (pSPW100) was excised from λ ZAP Express in *E. coli* XLOLR (Stratagene). A primer walking-based approach was used to sequence a 1,120-bp region (GenBank accession no. AF121672).

Strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *S. aureus* 8325-4 (31) and SPW1 (43) were grown in a chemically defined medium (CDM) containing 0.1% glucose (22, 44) or in brain heart infusion (BHI) broth (Oxoid). Agar plates were prepared by the addition of 1% agar (wt/vol) to the above-described culture. Liquid cultures were grown with high aeration (10 ml of medium in a 250-ml flask, 250 rpm orbital shaking) or low aeration (100 ml of medium in a 250-ml flask, 125 rpm linear shaking). Amino acid- and glucose-limited starvation cultures were prepared as described in Watson et al. (44), and starved cells were recovered as described by Clements and Foster (10). Viable counts were determined by serial dilution of cultures with phosphate-buffered saline (PBS) and plating on CDM agar (1% wt/vol). Results shown are representative of at least three independent experiments showing less than 10% variation, unless otherwise stated.

Construction of reporter fusion strains. The *sodA*::Tn*917*-LTV1 locus was transduced from SPW1 into PC6911 (*agr*::*tet*), PC1839 (*sar*::*km*) and PC400 (*sigB*::*tet*) by phage transduction, by using Φ11 as carrier (32), selecting for transductants on erythromycin (5 μg ml^{-1}) containing BHI agar, giving rise to strains MC51, MC52, and MC53, respectively.

β-Galactosidase and luciferase assays. β-Galactosidase assays with cell lysates, with 4-methylumbelliferyl-β-D-galactoside as substrate, and luciferase assays were performed as previously described (6).

TABLE 1. Bacterial strains

Strain	Genotype or relevant characteristics	Origin (reference)
8325-4	Wild-type strain cured of known prophages	R. Novick (31)
SPW1	*sodA*::Tn*917*-LTV1 Ery^r	Laboratory stock (43)
PC6911	*agr*Δ::*tetM* Tc^r	Laboratory stock (6)
MC51	*sodA*::Tn*917*-LTV1 *agr*Δ::*tetM* Tc^r Ery^r	This study
PC1839	*sarA*::*km* Km^r	Laboratory stock (6)
MC52	*sodA*:Tn*917*-LTV1 *sarA*::*km* Km^r Ery^r	This study
PC400	*sigB*::*tet* Tc^r	Laboratory stock (6)
MC53	*sodA*::Tn*917*-LTV1 *sigB*::*tet* Tc^r Ery^r	This study

Pathogenicity study. A mouse abscess model of infection was used as described in Chan et al. (7). Results from six mice were recorded, and their significance was determined by the Mann-Whitney test.

Oxygen free-radical resistance assay. Cells during different phases of growth in CDM were pelleted by centrifugation (5,000 rpm, 10 min), resuspended to a cell density of 5×10^6 CFU ml^{-1}, and incubated at 37°C in either an equal volume of PBS containing 1 mM xanthine plus 0.1 U of xanthine oxidase (external free-radical generation [16]) or 10 mM methyl viologen (internal free-radical generation [20]). Viability was determined by CFU ml^{-1} on CDM agar.

SOD activity assay. Cell lysates were prepared by resuspension of cells in lysis buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA, 25 μg of lysostaphin [Sigma]/ml^{-1}), followed by repeated freeze thawing until cell lysis was observed by microscopic examination. One hundred micrograms of soluble protein was loaded on a 12.5% (wt/vol) nondenaturing polyacrylamide gel, and electrophoresis was carried out according to standard procedures (28) but without sodium dodecyl sulphate in the gel or in the electrophoresis buffer. Enzyme activity was visualized by negative staining by the nitroblue tetrazolium method (3). Quantification of SOD activity in cell lysates was determined by the inhibition of the auto-oxidation of pyrogallol as described by Marklund and Marklund (30).

Metal depletion and reconstitution of crude cell extracts. Metal depletion was performed by dialyzing cell extracts against metal depletion buffer (20 mM 8-hydroxyquinoline, 2.5 M guanidium chloride, 5 mM Tris-HCl [pH 3.8], 0.1 mM EDTA) as described by Kirby et al. (27). Reconstitution of metal-depleted cell extracts with either manganese chloride or ferric and ferrous ammonium sulphate was performed as described by Vasconcelos and Deneer (41).

Acid tolerance and the adaptive response. Acid tolerance and the acid-adaptive response were determined as described in Chan et al. (7). Briefly, for acid tolerance, cells were resuspended in CDM acidified to pH 2, and viability was determined by CFU ml^{-1}. For the acid-adaptive response, cells were resuspended in CDM (pH 4) for 1 h, prior to determination of acid tolerance (7).

RESULTS AND DISCUSSION

The transposon insertion of SPW1 is within a *sodA* homologue. The Tn*917*-LTV1 insertion mutant, SPW1, was isolated in a previous study, screening for starvation survival mutants of *S. aureus* (43). Chromosomal DNA flanking the transposon insertion was cloned and sequenced as described in Materials and Methods. Sequence analysis of this region (1,120 bp) by using the Staden and GCG packages (SEQNET; Daresbury Laboratory, Warrington, United Kingdom), revealed that the Tn*917* had inserted into an ORF of 226 amino acids which encodes a putative protein of 23 kDa. The Tn*917* insertion occurred after residue 54. Database searches revealed that the ORF has sequence similarity to the SOD family of proteins (Fig. 1). Comparison of the amino acid sequence to other bacterial SOD of the Mn^{2+} type (*sodA*; *E. coli* [P00448], *Salmonella typhimurium* [P43019], *Listeria monocytogenes* [P28764], *Bacillus subtilis* [P54375]) and the Fe^{2+} type (*sodB*; *E. coli* [P09157], *Pseudomonas putida* [P77928], *Legionella pneumophila* [P31108]) showed that the sequence had greater

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E.col (Fe) .SFELPALPYAKDALAPHISAETIEYHYGKHHQTYVTNLNNLIKG.TAFEGKSLEEIIRS.......SEGGVFNNAAQVWNHTFYWNCLAPNAGGEPTGK 100
P.put (Fe) .AFELPPLPYAHDALQPHISKETLEFHHDKHHNTYVVNLNNLVPG.TEFEGKTLEEIVKT.......SSGGIFNNAAQVWNHTFYWNCLAPNAGGQPTGA 100
L.pne (Fe) MTFTLPQLPYALDALAPHVSKETLEYHYGKHHNTYVTNLNKLIPG.TEFESMTLEEIIMK.......AKGGIFNNAAQVWNHTFYWHSMSPNGGGEPKGR 100
E.col (Mn) .SYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALESLPEFANLPVEELITKLDQLPADKKTVLRNNAGGHANHSLFWKGL..KKGTTLQGD 100
S.typ (Mn) .SYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALENLPEFASLPVEELITKLDQVPADKKTVLRNNAGGHANHSLFWKGL..KTGTTLQGD 100
S.aur (Mn) MAFELPKLPYAFDALEPHFDKETMEIHHDRHHNTYVTKLNAAVEG.TDLESKSIEEIVANLDSVPANIQTAVRNNGGGHLNHSLFWELLSPN..SEEKGT 100
S.aur (?)  .................YIDQRTMEFHHDKHHNTYVTKLNATVEG.TELEHQSLADMIANLDKVPEAMRMSVRNNGGGHFNHSLFWEILSPN..SEEKGG 100
L.mon (Mn) MTYELPKLPYTYDALEPNFDKETMEIHYTKHHNTYVTKLNEAVAGHPELASKSAEELVTNLDSVPEDIRGAVRNHGGGHANHTLFWSILSPNGGGAPTGN 100
B.sub (Mn) .AYELPELPYAYDALEPHIDKETMTIHHTKHHNTYVTNLNKAVEGNTALANKSVEELVADLDSVPENIRTAVRNNGGGHANHKLFWTLLSPNGGGEPTGA 100
                                                  *******

E.col (Fe) VAEAIAASFGSFADFKAQFTDAAIKNFGSGWTWLVKNSDGKLAIVSTSNAGTPLTTDAT......PLLTVDVWEHAYYIDYRNARPGYLEHFWALVNWEF 200
P.put (Fe) LADAINAAFGSFDKFKEEFTKTSVGTFGSGWGWLVKKADGSLALASTIGAGCPLTSGDT......PLLTCDVWEHAYYIDYRNLRPKYVEAFWNLVNWAF 200
L.pne (Fe) LAEAINKSFGSFAAFKEQFSQTAATTFGSGWAWLVQDQSGALKIINTSNAGTPMTEGLN......ALLTCDVWEHAYYIDYRNRRPDYIEAFWSLVNWDF 200
E.col (Mn) LKAAIERDFGSVDNFKAEFEKAAASRFGSGWAWLVLK.GDKLAVVSTANQDSPLMGEAISGASGFPIMGLDVWEHAYYLKFQNRRPDYIKEFWNVVNWDE 200
S.typ (Mn) LKAAIERDFGSVDNFKAEFEKAAATRFGSGWAWLVLK.GDKLAVVSTANQDSPLMGEAISGASGFPILGLDVWEHAYYLKFQNRRPDYIKEFWNVVNWDE 200
S.aur (Mn) VVEKIKEQWGSLEEFKKEFADKAAARFGSGWAWLVVN.NGQLEIVTTPNQDNPLTEGKT......PILGLDVWEHAYYLKYQNKRPDYIGAFWNVVNWEK 200
S.aur (?)  VIDDIKAQWGTLDEFKNEFANKATTLFGSGWTWLVVN.DGKLEIVTTPNQDNPLTEGKT......PILLF.............................. 200
L.mon (Mn) LKAAIESEFGTFDEFKEKFNAAAAARFGSGWAWLVVN.DGKLEIVSTANQDSPLSDGKT......PVLGLDVWEHAYYLKFQNRRPEYIETFWNVINWDE 200
B.sub (Mn) LAEEINSVFGSFDKFKEQFAAAAAGRFGSGWAWLVVN.NGKLEITSTPNQDSPLSEGKT......PILGLDVWEHAYYLNYQNRRPDYISAFWNVVNWDE 200
                                                  *

E.col (Fe) VAKNLAA..........................  233
P.put (Fe) VAEQFEGKTFKA.....................  233
L.pne (Fe) ASSNLK...........................  233
E.col (Mn) AAARFAAKK.........................  233
S.typ (Mn) AAARFALK..........................  233
S.aur (Mn) VDELYNATK.........................  233
S.aur (?)  ...................................  233
L.mon (Mn) ANKRFDAAK.........................  233
B.sub (Mn) VARLYSDENNGTNKVLIMGPCFFMSSFYISRFE 233
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FIG. 1. Alignment of the *S. aureus* SodA amino acid sequence with those of *E. coli* [P00448, P09157], *S. typhimurium* [P43019], *L. pneumophila* [P31108], *B. subtilis* [P54375], *P. putida* [P77928], *L. monocytogenes* [P28764], and the putative *S. aureus* enzyme [S54793] (only partially sequenced). Numbers represent the position of the amino acid sequence. Fe and Mn denote metal iron cofactor of enzyme. Residues in bold are predicted to bind metal iron cofactor. Boxed residues have been predicted to bind Mn^{2+}. Asterisks pinpoint deletions, which discriminate Mn- from Fe-SOD.

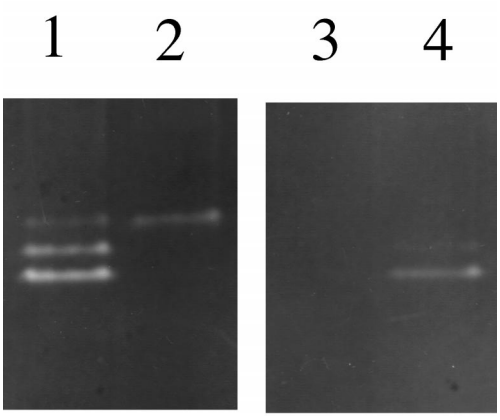


FIG. 2. SOD activity of whole-cell extracts of *S. aureus* 8325-4 and SPW1. Extracts were separated on a 12.5% (wt/vol) nondenaturing polyacrylamide gel, and stained for SOD activity. Lane 1, 8325-4; lane 2, SPW1; lane 3, 8325-4 metal ion depleted; lane 4, 8325-4 metal ion depleted, reconstituted with Mn^{2+}.

similarity to the *sodA* family of Mn-SOD. Thus, the *S. aureus* gene characterized in this study was designated *sodA*. The four Mn^{2+} metal ion binding residues characteristic of Mn-SOD were conserved (34). The greatest identities are to SodA from *B. subtilis* and *L. monocytogenes*, with which the *S. aureus* SodA shows 80% identity over 226 and 203 amino acids, respectively. Additionally the alignment revealed the lack of a 7-amino-acid deletion (position 59 to 66; Fig. 1) conserved amongst the Fe-SOD and the presence of a 1-amino-acid deletion conserved amongst the Mn-SOD. Poyart et al. (36) identified a putative SOD gene from *S. aureus* RN4220 (a derivative of 8325), using PCR with degenerate primers based on the sequence of several gram-positive Mn-SODs. The alignment of this sequence (Z49245) with the *sodA* identified in this study demonstrates only 71.3% identity, and therefore they most probably represent different enzymes. Both sequences have residues which are conserved among the Mn-SOD family.

The *sodA* gene is likely to be monocistronic, as putative Rho-independent terminators are present up- and downstream of the gene ($\Delta G -6.2$ and -14.4 kJ mol^{-1}, respectively, calculated according to Tinoco et al. [39]).

***S. aureus* SodA is a manganese-requiring enzyme.** The SOD activity of *S. aureus* 8325-4 was analyzed by nondenaturing polyacrylamide gel electrophoresis staining for SOD activity (Fig. 2, lane 1). Three bands of SOD activity were identified, with the upper band demonstrating the least activity. Comparison of whole-cell lysates of SPW1 with *S. aureus* 8325-4 showed that the lower two bands of activity were absent in SPW1 (Fig. 2, lanes 1 and 2). To determine the metal ion requirement of the superoxide dismutases, whole-cell lysates of *S. aureus* 8325-4 were treated to remove all metal ions. As can be seen in Fig. 2, lane 3, this abolished all SOD activity from the lysate. Replacement of the metal ions with Mn^{2+} restored activity of the lower two bands, but not the upper band of activity, establishing that they require Mn^{2+} for activity (Fig. 2, lane 4). The activity of the upper band was sensitive to H_2O_2 (data not shown), which is typical of Fe-SODs, although the addition of Fe^{2+} did not restore this activity to metal ion-depleted cell lysates of *S. aureus* 8325-4.

Since SODs are dimeric proteins, it may be that the two activities associated with SodA are due to a homodimer and a heterodimer with the proposed low-level, constitutive, Fe-SOD. This type of activity profile has been demonstrated in *E. coli* (9) and would explain why inactivation of *sodA* results in the loss of two bands of activity.

SOD activity and *sodA* expression during growth of *S. aureus* 8325-4. SOD activity of bacterial cell lysates during different phases of growth (Fig. 3A) was determined by the inhibition of the auto-oxidation of pyrogallol (30). Activity during the exponential phase of growth was low (2 U) but increased 18-fold (36 U) upon entry into postexponential phase (5 h), remaining high throughout stationary phase (13 h). This increase was observed only when cultures were incubated with high aeration; low aeration resulted in a 10-fold increase in SOD activity after 4 h, which rapidly fell to 8 U after 6 h incubation. The stationary-phase increase in SOD activity was dependent on *sodA*, since SPW1 demonstrated only a constitutive basal level of SOD activity throughout growth (Fig. 3A). The basal level of SOD observed in the *sodA* mutant is probably due to the activity of the putative Fe-dependent superoxide dismutase, which would protect the cell from low-level $O_2^{\cdot}$.

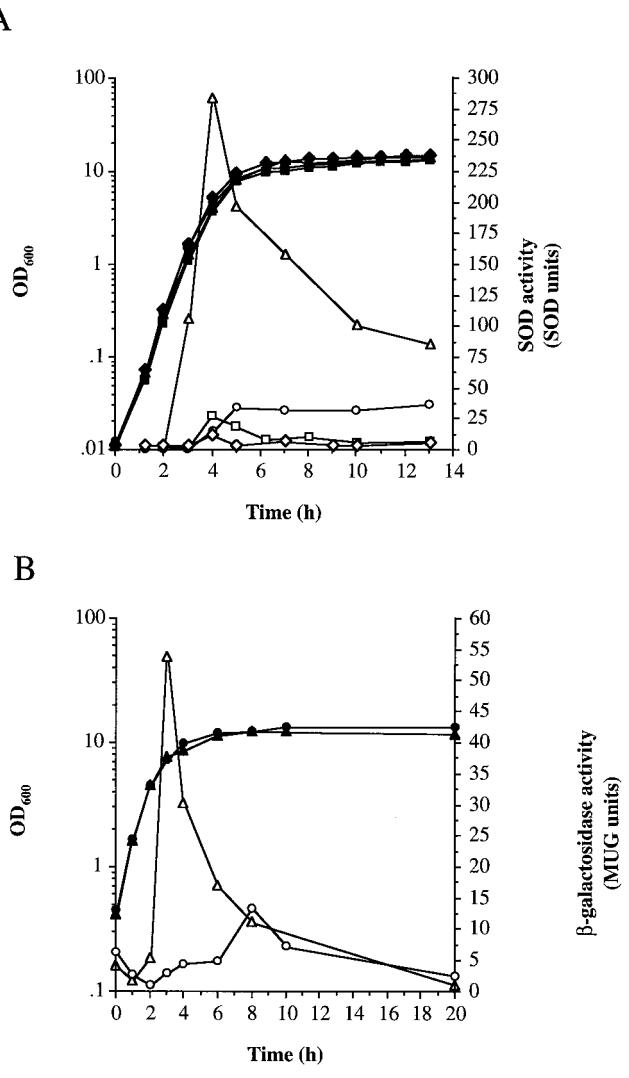
The SPW1 Tn*917*-LTV1 insertion generated a *lacZ* transcriptional fusion with *sodA*, enabling its expression to be monitored throughout growth, by measuring the levels of β-galactosidase activity. Expression of *sodA* was low during exponential-phase growth (1 U at 2 h) but increased 13-fold (13 U at 8 h) during the stationary phase, when cultures were incubated with high aeration, correlating with the SOD activity results. The fact that SPW1 is a *sodA* mutant may affect the finite levels of expression. Culture under conditions of high aeration may result in increased internal superoxide levels, which leads to oxidative stress for the cell. Expression of *sodA* is induced to deal with this potentially lethal assault.

Increased levels of SOD during stationary-phase growth have been observed in many bacteria, including *E. coli*, *B. subtilis*, *L. monocytogenes*, and *Legionella* (5, 25, 38, 41). The increase in SOD in cells during stationary-phase growth may not result from an increase in $O_2^{\cdot}$ generation but may be a mechanism to prevent the accumulation of $O_2^{\cdot}$ damage to proteins, since damaged proteins will accumulate due to the low rate of de novo protein synthesis and, hence, low protein turnover (33). Since SPW1 (*sodA*) is less able to survive in the stationary phase of growth, as are SOD-deficient mutants of *E. coli* (5) and *Legionella* (38), the protective role of SOD during starvation survival is supported.

Induction of SOD activity and *sodA* expression by methyl viologen. Methyl viologen, when accumulated within bacterial cells, is a potent generator of $O_2^{\cdot}$ via redox cycling (20). When methyl viologen was incubated with *S. aureus* 8325-4, an approximately ninefold maximal increase in SOD activity over the untreated control was observed (Fig. 3A). This correlated with an approximately fourfold increase in *sodA* expression compared to the untreated culture (Fig. 3B). Interestingly, the methyl viologen induction of SOD activity and *sodA* expression was growth-phase dependent, occurring specifically during the post-exponential phase, irrespective of the time of addition of methyl viologen to the culture (Fig. 3C and data not shown). If, however, methyl viologen was added after stationary phase was reached, there was no induction of *sodA* expression.

In *E. coli*, addition of methyl viologen leads to an immediate induction of *sodA* expression (40), whereas in *B. subtilis* the addition of methyl viologen does not induce *sodA* at all (25). The range of methyl viologen effects could be due to differences in permeability.

Role of *sodA* in oxidative stress resistance. Oxidative stress can be induced internally, within bacterial cells, by the addition of methyl viologen, while external free radicals can be generated by the incubation of cells with xanthine and xanthine oxidase (16). Surprisingly, the addition of methyl viologen to



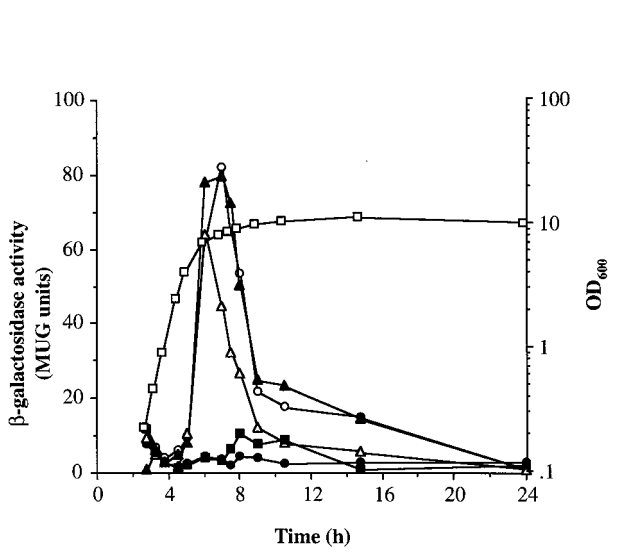


FIG. 3. SOD activity and expression of *sodA* during growth and the effect of methyl viologen. (A) Total SOD activity. 8325-4 (○,●,△,▲,□,■) and SPW1 (◆,◇) were grown under high (△,▲,○,●,◆,◇) or low (□,■) aeration in BHI or in BHI with 10 μM methyl viologen (△,▲). Closed symbols represent OD_{600}, and open symbols represent SOD activity as described in Materials and Methods. (B) Expression of *sodA*. SPW1 was grown in BHI with high aeration with (△,▲) or without (○,●) 10 μM methyl viologen. Growth was measured by OD_{600} (closed symbols) and β-galactosidase activity (open symbols) as described in Materials and Methods. (C) Effect of growth phase on methyl viologen induction of *sodA*. Expression of *sodA* was measured during growth of SPW1 in BHI (●) and in BHI with 10 μM methyl viologen added 0 h (○), 2.75 h (▲), 4.5 h (△), and 7 h (■) after inoculation. Cells were incubated with high aeration, and expression was measured as described in Materials and Methods. Growth as measured by OD_{600} is shown for one representative culture (□).

exponential-phase cells had no significant effect on the growth rate but did lead to a slight reduction in yield upon entry to stationary phase of *S. aureus* 8325-4 and SPW1 (data not shown). The addition of methyl viologen to stationary-phase cells (8 h postexponential phase; glucose limited) of 8325-4 had no effect on the rate of loss of viability (data not shown) but caused a 100-fold reduction in CFU of SPW1 after 24 h compared to the untreated control (Fig. 4). Untreated SPW1 showed the same death kinetics as 8325-4 over the duration of the experiment. Incubation of *S. aureus* 8325-4 or SPW1 in PBS containing xanthine and xanthine oxidase had no effect on the rate of growth, yield, or loss of cell viability irrespective of the growth phase of the cells (data not shown). Thus, lack of SodA results in increased sensitivity to internally generated oxidative stress, but this is apparent only in the stationary phase, which coincides with maximal *sodA* expression.

Role of *sodA* in starvation survival and recovery from starvation. SPW1 was initially isolated from a screen of mutants that demonstrated a decreased ability to survive amino acid starvation (43). Further analysis of the starvation defect demonstrated that the increased rate of loss of viability was dependent on the aeration of the culture during starvation. SPW1 demonstrated identical starvation kinetics to 8325-4 in amino acid-limiting CDM when the cultures were incubated statically, whereas SPW1 lost viability at a faster rate when the cultures were incubated with shaking (43; data not shown). The inactivation of *sodA* in SPW1 had no effect on the ability of the

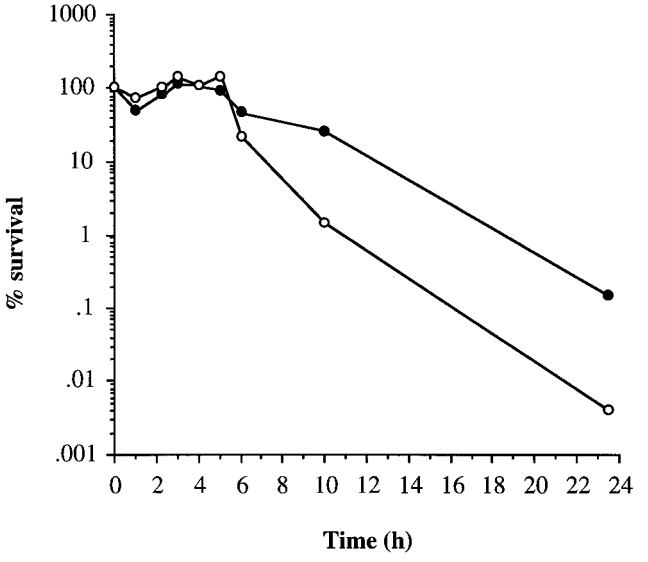


FIG. 4. Effect of methyl viologen on cell survival. Cells (8 h postexponential phase) of SPW1 were resuspended in PBS (●) or in PBS containing 10 μM methyl viologen (○). Cell viability was determined by plating on CDM agar.

starved cells to recover and resume growth after starvation (data not shown), by standard recovery protocols (10).

SodA is involved in acid tolerance and the acid-adaptive response and is induced by acid. We have previously shown that *S. aureus* develops increased resistance to acid stress upon starvation or by incubation of cells at a lower but nonlethal pH as part of the stress response (7). Exponential-phase cells of *S. aureus* 8325-4 are sensitive to CDM acidified to pH 2 but become tolerant when adapted for 1 h at pH 4 prior to challenge at pH 2 (Fig. 5A; 7). SodA is involved in acid resistance, since exponential-phase cells of SPW1 were less tolerant to CDM (pH 2) than *S. aureus* 8325-4 ($>10^3$-log loss of viability after 10 min compared to 1-log loss, respectively; Fig. 5A). SodA also has a role in the acid-adaptive response, as *sodA* showed a >2-log drop in viability compared to a 0.6-log drop by 8325-4 (30 min, pH 2 treatment after pH 4 adaptation [Fig. 5A]).

Exponential-phase cells (optical density at 600 nm [OD_{600}] = 0.8) of *S. aureus* 8325-4 develop increased acid resistance when incubated at pH 4 prior to challenge at pH 2 (Fig. 5A). The expression of *sodA* was compared between cells resuspended at pH 4 (adaptive) and pH 7 (nonadaptive) and is shown in Fig. 5B. The level of *sodA* expression remained relatively constant at pH 7 (0.2 to 0.8 MUG U), while at pH 4, the level increased almost 10-fold to 7.6 MUG U after 60 min. When total levels of SOD activity were measured in *S. aureus* 8325-4, a similar rise in SOD activity was observed (Fig. 5B).

How *sodA* is involved in acid resistance is unknown, but acid stress may lead to uncoupling of the respiratory chain, resulting in the increased internal synthesis of O_2^-. Alternatively, or additionally, inactivation of *sodA* may result in increased cellular damage from the effects of O_2^-, rendering the cell more sensitive to acid stress.

Regulation of *sodA* expression. In order to identify potential regulators of *sodA* in *S. aureus*, we examined *sodA* expression in strains defective in several global gene regulators. SigB is an alternative sigma factor (46), which is preferentially expressed in the stationary phase and has a role in acid and oxidative stress resistance (7). The global regulators of toxin production, *agr* and *sarA* (8, 35), are also preferentially expressed during stationary phase. We have recently shown that *sigB* expression is partially controlled by SarA (7). The *sodA*::*lacZ* fusion was introduced into a set of strains bearing defined mutations in *sigB*, *agr*, or *sarA*. No alteration in the pattern of *sodA*::*lacZ* expression was observed in any of the backgrounds (data not shown), during growth or induction by methyl viologen. In *E. coli*, superoxide stress induces the *soxRS* regulon, resulting in the synthesis of SodA and DNA repair mechanisms (13). To date, there is no evidence for a comparable mechanism in *S. aureus*.

Role of *sodA* in pathogenicity. Cu/Zn-SODs have been shown to be an important virulence factors in several pathogens, including *E. coli*, *Neisseria meningitidis*, and *Salmonella typhimurium* (1, 14, 45). It has been suggested that SOD is involved in protecting *S. aureus* from neutrophils, since it was shown that the attachment of SOD to the surface of *S. aureus* decreased the rate of killing (19). A mouse abscess model of *S. aureus* infection (7) was therefore used to determine the contribution of *sodA* to pathogenicity. No significant difference between *S. aureus* 8325-4 and SPW1 was observed in regard to the size of the lesion formed or the number of bacteria that were recovered from the lesion (data not shown).

Interestingly, SodA of *Haemophilus influenzae* has been shown to play a role in nasopharyngeal colonization (12), which is also a preferred site of *S. aureus* colonization. It is therefore possible that SodA may be important in the successful colonization of specific niches.

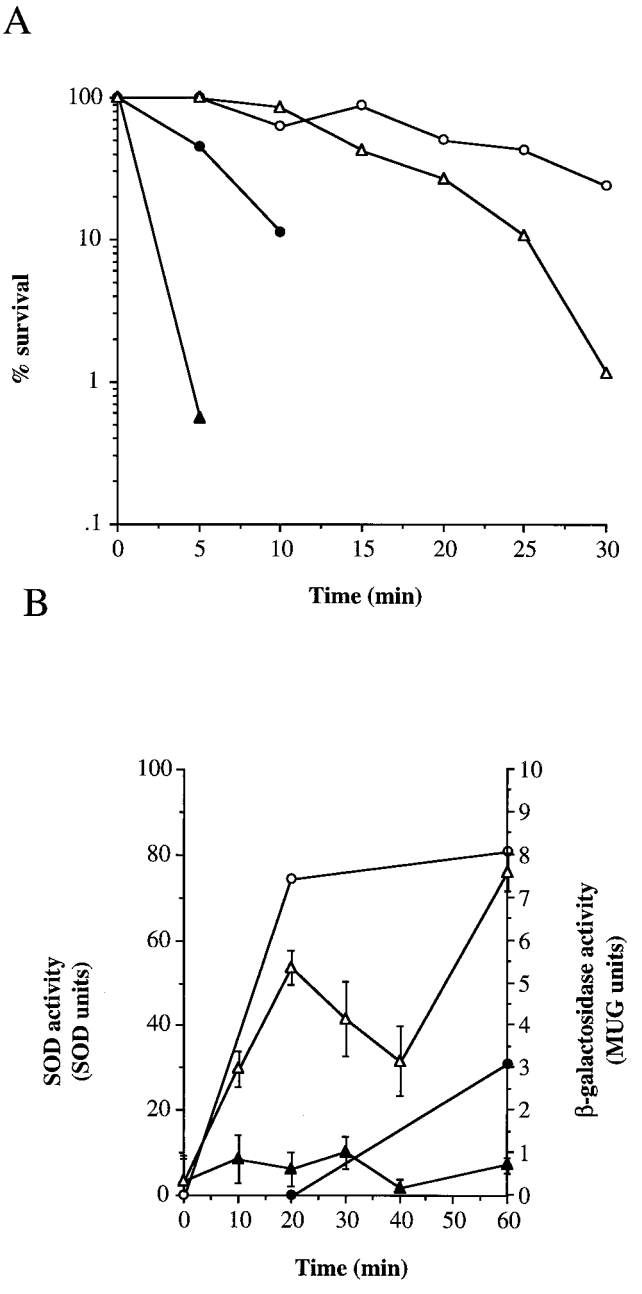


FIG. 5. Role of SodA in acid tolerance and adaptation. (A) *S. aureus* 8325-4 (●,○) and SPW1 (▲,△) were grown and treated with acid as described in Materials and Methods. Exponential-phase cells were either incubated at pH 4 (37°C) for 1 h (○,△) or untreated (●,▲) prior to pH 2 assault. Viability was determined by plating on CDM agar. Results are representative of three independent experiments showing less than fivefold variability. (B) Induction of *sodA* expression and SOD activity during acid adaptation. Exponential-phase cells (OD_{600} = 0.8) were resuspended in CDM pH 7 (●,▲) or pH 4 (○,△). Expression of *sodA* (SPW1, ▲,△) and total SOD activity (8325-4, ●,○) were determined as described in Materials and Methods. Error bars represent the standard deviations of three independent samples.

The resistance to oxidative stress involves the interplay between several resistance mechanisms and components. In order to understand how *S. aureus* responds and adapts to oxidative stress inside and outside the host, it is important to

identify all the components of the resistance mechanism and to determine how they are regulated in response to potentially lethal stresses. It is by such exquisite mechanisms of adaptation that *S. aureus* has become such a versatile and successful pathogen.

ACKNOWLEDGMENTS

This work was supported by the Royal Society (S.J.F.), BBSRC (S.P.W.), and the BBSRC/Celsis Connect Program (M.O.C.).

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