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Analysis of mutants disrupted in bacillithiol metabolism in *Staphylococcus aureus*

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ABSTRACT

Bacillithiol (BSH), an α-anomeric glycoside of L-cysteinyl-D-glucosaminyl-L-malate, is a major low molecular weight thiol found in low GC Gram-positive bacteria, such as *Staphylococcus aureus*. Like other low molecular weight thiols, BSH is likely involved in protection against a number of stresses. We examined *S. aureus* transposon mutants disrupted in each of the three genes associated with BSH biosynthesis. These mutants are sensitive to alkylating stress, oxidative stress, and metal stress indicating that BSH and BSH-dependent enzymes are involved in protection of *S. aureus*. We further demonstrate that BshB, a deacetylase involved in the second step of BSH biosynthesis, also acts as a BSH conjugate amidase and identify *S. aureus* USA 300 LAC 2626 as a BSH-*S*-transferase, which is able to conjugate chlorodinitrobenzene, cerulenin, and rifamycin to BSH.

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1. Introduction

Low molecular weight (LMW) thiols play critical roles in cell physiology. In most cells, the tripeptide, glutathione (GSH), is the major LMW thiol; however, Gram-positive bacteria lack GSH, but instead produce other LMW thiols. Mycothiol (MSH) is the dominant LMW thiol in Actinomycetes (e.g. *Mycobacterium tuberculosis*), serving analogous functions to GSH [1–3], and in Firmicutes, including *Staphylococcus*, bacillithiol (BSH) is the major thiol [4,5]. The BSH biosynthetic pathway consists of the formation of *N*-acetylglucosaminylmalate (GlcNAc-Mal) from UDP-*N*-acetylglucosamine (UDPGlcNAc) and L-malate, a reaction catalyzed by the glycosyltransferase, BshA [6–8]. This is followed by the deacetylation of GlcNAc-Mal by the deacetylase, BshB, to yield glucosaminylmalate (GlcN-Mal) [7,8]. The last step involves BshC and the ligation of cysteine to GlcN-Mal [7].

LMW thiols, like GSH, maintain an intracellular reducing environment in the cell via the reduction of toxic oxidants such as hydrogen peroxide or nitric oxide [9]. The oxidized thiols are reduced through the action of reductases, which use the electron equivalents from NADH/NADPH [10]. LMW thiols also act as nucleophiles to form *S*-conjugates, reacting rapidly to form adducts with alkylating agents, such as *N*-ethylmaleimide, iodoacetamide, and electrophiles, such as formaldehyde, methylglyoxal or *S*-nitrosocompounds. In the case of less chemically reactive xenobiotics, *S*-conjugate formation is catalyzed by *S*-transferases [11]. Recently, Nathan and colleagues [12] observed MSH-conjugates and *N*-acetyl cysteine conjugates (mercapturic acids) of an anti-inflammatory, mycobactericidal drug, oxyphenbutazone, in *M. tuberculosis*, indicating that this drug is detoxified in a MSH dependent manner. In *S. aureus*, it has been shown that FosB functions as a bacillithiol dependent *S*-transferase (Bst) responsible for the detoxification of fosfomycin [13,14]. Evidence has also recently emerged for a BSH-dependent detoxification system, similar to the GSH and MSH dependent pathways, which may be responsible for detoxification of rifamycin [5], and other thiol reactive antibiotics.

Herein, we undertook the analysis of *S. aureus* transposon mutants disrupted in genes involved in BSH biosynthesis and BSH dependent detoxification. We demonstrate that mutants disrupted in the three biosynthetic genes are sensitive to a range of stresses, including antibiotic stress, identify a second BSH-*S*-transferase (Bst), and demonstrate that BshB has a dual function as a BSH conjugate amidase (Bca).

2. Materials and methods

2.1. Culture conditions

Wild-type strains, *Bacillus subtilis* CU1065 and *S. aureus* USA300 LAC JE2, were grown in trypticase soy broth (TSB), and unless otherwise noted, liquid media were inoculated from an overnight pre-culture and incubated at 37 °C with shaking at 170 rpm.

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S. aureus USA300 LAC transposon mutants were obtained from the "Network on Antimicrobial Resistant in *Staphylococcus aureus*" (NARSA) Program and propagated on media containing erythromycin (10 μgml^{-1}). *B. subtilis* mutants disrupted in BSH biosynthesis were kindly provided by Dr. John Helmann (Cornell University) and propagated on appropriate antibiotics [7].

The *S. aureus* transposon mutants were cultured in triplicate in 50 ml TSB until OD_{600} 0.5 and pelleted for thiol analysis. For stress treatments, *S. aureus* USA300 LAC JE2 wild-type and *B. subtilis* Cu1065 were cultured in triplicate in 100 ml TSB until OD_{600} 0.5, and treated with oxidants and metals for 45 min and 30 min, respectively. Cultures were pelleted for thiol analysis.

2.2. Synthesis of BSH, BSSB, and BSmB and HPLC analysis of LMW thiols

BSH, BSmB and BSSB were chemically synthesized as previously described [14]. LMW thiols were measured by HPLC analysis of fluorescent thiol adducts with monobromobimane (mBBr) as described previously [4].

2.3. Sensitivity assays

Disk assays were performed to assess sensitivity of the mutants to a wide range of oxidants, antibiotics, and other toxins. *S. aureus* and *B. subtilis* strains were grown to log phase (OD_{600} = 0.5) in TSB media and plated on trypticase soy agar (TSA).The diameter of the zone of clearance around the filter disks was measured after 24 h. These experiments were performed in quadruplicate three times [15].

2.4. Enzyme assays

Cell-free protein extracts were prepared by growing *S. aureus* strains in 100 ml TSB until OD_{600} was approximately 1.0. The cells were harvested and the cell pellet was resuspended in 1 ml of 25 mM HEPES pH 7.5. Glass beads (0.1 mm) were added and the cells were lysed three times in a Research Product International Ribolyzer for 30 s at speed 6.5, with cooling on ice between cycles. The cell lysate was centrifuged for 10 min at 14,000 rpm and the supernatant was loaded on either a Bio-Gel P-6 column (to remove molecules smaller than 6 kDa for the Bca and Bst assay) or a Bio-Gel P-30 column (to remove molecules smaller than 40 kDa for the BSSB reductase (Bdr) assay). Glycerol was added to the protein extract to a final concentration of 10%. Protein concentration was determined by a Bio-Rad protein assay or by measuring absorbance at 280 nm. All assays were performed in triplicate.

For all enzymatic reactions, control reactions in the absence of BSH and cell-free protein extract were performed for the different activities. The reactions were performed at room temperature (22 °C) in 100 μl reaction volume. Bca activity assay consisted of 30 μM of the model substrate, bacillithiol-*S*-bimane (BSmB), 2 mM β-mercaptoethanol, and 100 μg of cell-free protein extract in 25 mM HEPES (pH 7.5) buffer. Aliquots of 25 μl were taken at 0, 5, 15, and 30 min and the reaction was terminated with the addition of 25 μl acetonitrile on ice. The aliquots were centrifuged to pellet denatured proteins and other cell debris. Each time point was then diluted to 500 μl with 10 mM methanesulfonic acid and injected on the HPLC column and subjected to HPLC analysis.

The *S*-transferase activity was determined using cerulenin, rifamycin, chlorodinitrobenzene (CDNB), as substrates. For cerulenin, the Bst assay consisted of 200 μM cerulenin, 30 μM BSH, 200 μg of cell-free protein extract in 25 mM HEPES, 100 mM NaCl (pH 7.5) buffer. Aliquots of 25 μl were taken at 0, 5, 15, and 30 min and the reaction was terminated with the addition of 25 μl acetonitrile on ice followed by HPLC analysis. For rifamycin, the assay consisted of 200 μM rifamycin, 200 μM BSH and 200 μg of cell-free protein extract. Cerulenin levels were monitored by a decrease in absorbance at 220 nm [16] and the decrease in rifamycin levels and the increase in the level of rifamycin conjugates were followed at 315 nm, where rifamycin eluted at 24.5 min, RifS13 at 13 min, and RifS17 at 17 min [17]. For CDNB, the Bst assay consisted of 1 mM CDNB, 1 mM BSH and 200 μg of cell-free protein extract in phosphate buffered saline (pH 6.5) and the appearance of the product (BS-DNB), was monitored spectophotometrically at 340 nm after addition of 200 μM CDNB [18]. Finally, the *N*-acetyltransferase activity was assayed as previously described [5].

The Bdr assay consisted of 100 μM BSSB, 200 μM NADH or NADPH (made fresh and quantified by absorbance at 340 nm), 300 μg cell-free protein extract in 25 mM HEPES (pH 7.5) buffer. Aliquots of 25 μl were taken at 0, 5, 15, and 30 min and the reaction was terminated with the addition of 25 μl acetonitrile on ice. After pelleting denatured protein and cell debris, 2 mM mBBr was added and the aliquot was incubated at 60 °C for 10 min to derivatize the resulting BSH from the disulfide reductase reaction, which was then analyzed by HPLC. Control reactions in the absence of BSSB and cell-free protein extract were performed.

3. Results

3.1. BSH levels in S. aureus wild-type and BSH biosynthetic pathway mutants

In *S. aureus*, like *B. subtilis* and *Bacillus anthracis*, BSH is one of the major LMW thiols. The three *S. aureus* USA 300 LAC transposon mutants, NE1728 disrupted in ORF 1349 ($bshA^-$), NE1596 disrupted in 552 ($bshB^-$), and NE230 disrupted in 1071 ($bshC^-/yllA^-$), do not contain BSH (<0.01 μmol g^{-1} dry weight), as compared to the JE2 wild-type strain (0.32 ± 0.02 μmol g^{-1} dry weight) but do contain normal levels of cysteine (JE2, 0.26 ± 0.11; NE1728, 0.26 ± 0.05; NE1596, 0.46 ± 0.09; NE230, 0.57 ± 0.09 μmol g^{-1} dry weight). The growth of these mutants is not significantly different from wild-type in liquid media (data not shown).

3.2. Mutants disrupted in BSH biosynthesis are sensitive to a variety of stresses

B. subtilis mutants disrupted in BSH biosynthesis are known to be sensitive to osmotic and acidic stress, alkylating agents, and toxins, such as methylglyoxal [7]. To determine if *S. aureus* transposon mutants disrupted in BSH biosynthesis are susceptible to the same stresses, disk assays were performed (Table 1A). Like *B. subtilis*, *S. aureus* mutants lacking BSH are more susceptible to the alkylating agents, iodoacetamide and CDNB (a model substrate for glutathione *S*-transferase) and the toxin, methylglyoxal, which is detoxified in glutathione containing organisms by glutathione dependent glyoxalases [19]. In addition, like *B. subtilis* and *B. anthracis*, *S. aureus* BSH mutants are sensitive to epoxide containing antibiotics fosfomycin [7,8], and cerulenin, as well as to rifamycin, the parent compound of the drug, rifampin (Table 1A). As the structure of BSH contains a number of potential metal coordinating ligands (carboxylate, amine, thiol), which might serve to bind metals more tightly than cysteine, sensitivity to metals was also assessed for both *S. aureus* and *B. subtilis*. BSH mutants of both *B. subtilis* and *S. aureus* demonstrated sensitivity to metal stress induced by cadmium, copper, and dichromate ions. In contrast to *B. subtilis* mutants, *S. aureus* mutants lacking BSH were more susceptible to oxidative stress in the form of hydrogen peroxide, plumbagin, cumene hydroperoxide, and diamide (Table 1A) and did not differ in sensitivity to acid and osmotic stress (data not shown).

Table 1A
Susceptibility of *S. aureus* and *B. subtilis* wild-type and BSH mutants to toxins, oxidants, and metals as determined by disk assays on TSA. Values shown are averages and SD ($n \geqslant 3$). $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.005$ using student's *t*-test.

	S. aureus USA 300 Lac ORF zone of clearing (mm)			
	JE2 wild-type	NE1728 *bshA*⁻	NE1596 *bshB*⁻	NE230 *bshC*⁻
Toxins (μmol)				
Iodoacetamide (0.5)	22 ± 1	27 ± 1**	28 ± 2**	26 ± 1**
Chlorodinitrobenene (1.5)	15 ± 1	18 ± 1*	19 ± 1*	19 ± 1*
Methylglyoxal (1.0)	9 ± 0	11 ± 1*	12 ± 1*	11 ± 1*
Oxidants (μmol)				
H_2O_2 (6.0)	26 ± 0	29 ± 1**	31 ± 1**	29 ± 1**
Plumbagin (0.2)	13 ± 1	18 ± 2**	16 ± 1*	20 ± 1**
Cumenehydroperoxide (0.1)	14 ± 1	16 ± 1*	20 ± 2**	16 ± 0*
Diamide (10)	16 ± 0	18 ± 1*	20 ± 0**	19 ± 0**
Antibiotics (μg)				
Cerulenin (100)	15 ± 1	25 ± 1**	22 ± 3**	19 ± 1**
Fosfomycin (350)	36 ± 1	39 ± 1*	40 ± 1**	39 ± 1*
Rifamycin (10)	33 ± 1	NA	NA	37 ± 1**
Metals (μmol)				
Cd^{2+} (0.7)	11 ± 1	22 ± 0**	22 ± 0**	18 ± 2**
Cu^{2+} (12.5)	14 ± 0	16 ± 1*	18 ± 2**	17 ± 0**
$Cr_2O_7^{2-}$ (5)	11 ± 1	18 ± 3**	13 ± 1*	25 ± 1**
	Bacillus subtilis Wild-type	*bshA*⁻	*BshB1*⁻	*bshC*⁻
Metal (μmol)				
Cd^{2+} (0.7)	11 ± 1	22 ± 0**	22 ± 0**	18 ± 2**
Cu^{2+} (10)	18 ± 4	29 ± 4*	31 ± 1**	37 ± 2**
$Cr_2O_7^{2-}$ (5)	22 ± 5	37 ± 3**	36 ± 1**	37 ± 1**

To complement the studies on mutant sensitivity to oxidants and metals, BSH levels after treatment with various stresses were also analyzed. Upon treatment with diamide, BSH levels dropped dramatically in *B. subtilis* but remained the same in *S. aureus*. The levels also decreased, albeit to a lesser extent, when *B. subtilis* was treated with hydrogen peroxide and CHP. In contrast, *S. aureus* BSH levels did not decrease with these treatments and in fact increased slightly upon exposure to hydrogen peroxide (Table 1C). Treatment with plumbagin and menadione resulted in decreased BSH levels in both species, presumably due to conjugation of these compounds with BSH, similar to the data reported for MSH [15]. In response to metal treatment, there was a decrease in BSH levels during copper and cadmium treatment in both species, perhaps as a result of autooxidation of BSH to BSSB. In contrast, exposure to dichromate did not affect BSH levels in either species, although mutants lacking BSH were more susceptible to dichromate as compared to wild-type (Fig. 1A).

3.3. Enzymes involved in BSH dependent detoxification in S. aureus

The first step in BSH dependent detoxification is the formation of a conjugate of the toxin with BSH, which is catalyzed by an *S*-transferase [16]. A novel class of thiol-*S*-transferases of the DinB family was recently identified, of which *B. subtilis* YfiT was shown to have Bst activity with a wide range of substrates, including CDNB, cerulenin, cumene hydroperoxide (CHP) and monochlorobimane [16]. Cell-free protein extracts from wild-type and the *S. aureus* USA300 LAC transposon mutant NE248 disrupted in the structural homolog of *B. subtilis* YfiT (ORF 2626), were tested for *S*-transferase activity. An increase in BS-DNB, the product of CDNB conjugation with BSH, was observed only in the wild-type. Although only 25% of CDNB was converted to BS-DNB in the wild-type cell free extract, no BS-DNB was detected in the mutant cell-free extract or the control reactions (Fig. 1A), suggesting the involvement of BSH and Bst. Similarly, a decrease in the levels of the substrate cerulenin was observed in the wild-type, while in the mutant extract and control reactions the levels of cerulenin remained constant (Fig. 1B). In the case of rifamycin, a decrease in rifamycin and an increase in RifS13 and RifS17 (BS-conjugates of rifamycin [17]) were observed in the wild-type. There was also decrease in rifamycin in the mutant with the appearance of RifS13 and RifS17, but these changes were similar to those observed in the no protein control suggesting a chemical reaction (Fig. 1C). NE248 was more susceptible to CDNB, cerulenin, and rifamycin than wild-type (Table 1B).

The second step in BSH and MSH dependent detoxification involves an *S*-conjugate amidase, Bca and Mca, which releases the cysteine-toxin and the acetylcysteine conjugate, respectively [5]. A BLAST search with *M. tuberculosis* Mca and the paralog MshB, catalyzing the deacetylation of glucosaminylinositol, the third step in MSH biosynthesis, yielded a single gene in *S. aureus* (ORF 552), which codes for BshB. To test whether this gene serves a detoxification function (Fig. 2), the *bshB* transposon mutant and the wild-type were assayed for Bca amidase activity using the model substrate BSmB. In Fig. 2, it can be seen that the wild type, but not the bshB mutant, contains Bca activity converting the substrate BSmB to CysmB. A decrease in BSmB levels was observed in the mutant, perhaps due to chemical degradation of BSmB, although no thiol breakdown products were observed during HPLC chromatography.

The third step involves the acetylation of CysmB catalyzed by a putative *N*-acetyltransferase to form a mercapturic acid [5]. A transposon mutant (NE1951) disrupted in ORF 2460, annotated as an *N*-acetyltransferase, contained similar levels of *N*-acetyltransferase activity as the wild-type suggesting that other ORF(s) must encode this enzyme (data not shown).

3.4. Search for bacillithioldisulfide reductase (Bdr)

The hallmark of an intracellular thiol buffer is a specific disulfide reductase generating a substantially reduced thiol redox status, defined as the thiol/disulfide ratio. In order to find Bdr, the *M. tuberculosis* mycothiol disulfidereductase, Mtr [20], sequence was used to BLAST search the genome of *S. aureus* USA 300 LAC.

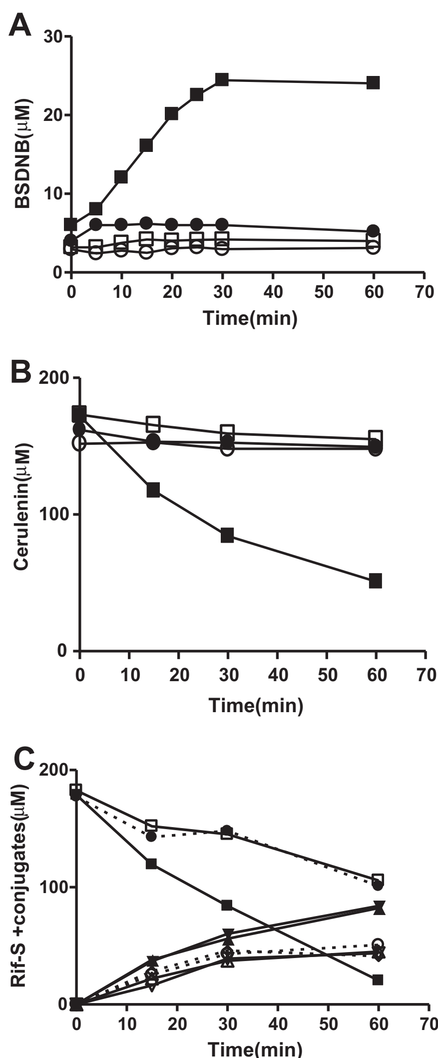

Fig. 1. *S*-transferase activity in *S. aureus* USA 300 LAC JE2 (wild-type) andNE248 (*bst* transposon mutant) cell-free extracts; with substrate (a) 200 μM chlorodinitrobenzene, resulting in BS-DNB (■) with wild-type but not with NE 248 cell-free extract (●), no BSH control (□); and no protein control (○); (b) 200 μM cerulenin resulting in a decrease in cerulenin with wild-type (■) but not with NE 248 cell-free extract (●), no BSH control (□) and no protein control (○); and (c) 200 μM rifamycin (■) resulting in RifS13 (▲) and RifS17 (▼), conjugates of BS-rifamycin, in wild-type; rifamycin (□), RifS13 (Δ), and RifS17 (▽) in NE248; rifamycin (●), RifS13 (○), and RifS17 (◇) in no protein added control (dotted line).Values shown are averages and SD ($n = 3$).

Table 1B
Sensitivity of *bst* transposon mutant to toxins and oxidants as determined by disk assays on TSA. Values shown are averages and SD ($n \geqslant 3$). $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.005$ using student's *t*-test.

S. aureus USA 300 Lac ORF	Zone of clearing (mm)		
	CDNB (1.5 μmol)	Cerulenin (100 μg)	Rifamycin (10 μg)
JE2	17 ± 1	22 ± 1	33 ± 1
NE248 *bst*⁻	38 ± 1**	25 ± 1**	37 ± 1**

ORFs 576,996, and 1467 showed the most sequence similarity. The BSH/BSSB ratio during exponential phase growth for the wild-type and transposon mutants disrupted in ORFs 576 (NE785), 996 (NE1610), 1467 (NE1896) was determined to be 14, 3, 6, and 28,respectively.The NE1896 BSH/BSSB ratio is twice that of the wild-type, while the other two mutants have a lower ratio than in the wild-type.

Table 1C
Bacillithiol content of *S. aureus* and *B. subtilis* after treatment with (A) oxidants for 45 min and (B) metal ions for 30 min. Cultures were grown in 10 ml TSB in triplicates and values shown are averages and SD ($n \geqslant 3$).

	Bacillithiol (μmolg^{-1} dry weight)			
	Staphylococcus aureus		*Bacillus subtilis*	
	Control	Treated	Control	Treated
Diamide (5 mM)	0.59 ± 0.01	0.53 ± 0.02	0.79 ± 0.02	0.09 ± 0.01
Plumbagin (0.01 mM)	0.67 ± 0.05	0.24 ± 0.01	0.69 ± 0.03	0.32 ± 0.01
Menadione (0.01 mM)	0.67 ± 0.05	0.22 ± 0.01	0.69 ± 0.03	0.15 ± 0.01
H_2O_2 (1 mM)	0.67 ± 0.05	0.77 ± 0.0	0.69 ± 0.03	0.46 ± 0.02
CHP (0.1 mM)	0.59 ± 0.01	0.56 ± 0.01	0.74 ± 0.03	0.64 ± 0.02
Cu^{2+} (100 μM)	0.62 ± 0.01	0.38 ± 0.01	0.75 ± 0.01	0.53 ± 0.02
Cd^{+} (100 μM)	0.52 ± 0.09	0.34 ± 0.01	0.60 ± 0.12	0.25 ± 0.06
$Cr_2O_7^{2-}$ (100 μM)	0.62 ± 0.01	0.62 ± 0.01	0.60 ± 0.12	0.15

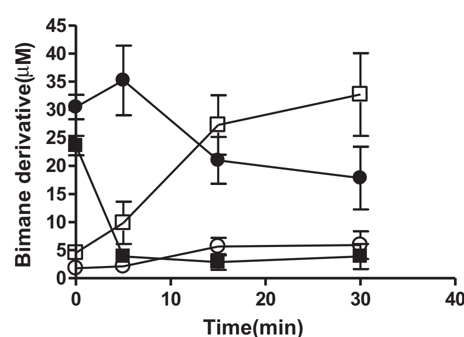

Fig. 2. Bacillithiol conjugate amidase activity in *S. aureus* USA 300 LAC JE2 (wild-type) and NE1596 (*bshB* transposon mutant) with BSmB (■) resulting in CySmB (□) in the wild-type cell-free extract. Addition of BSmB (●) to the NE1596 mutant cell-free extract does not result in an increase in CySmB (○). Values shown are averages and SD ($n = 3$).

Bdr activity assays were also performed on the cell-free protein extracts of the transposon mutants. The wild-type strain demonstrated the reduction of BSSB with NADPH as an electron donor and to a lesser extent with NADH (Fig. 3B), as measured by the derivatization of the resulting BSH with mBBr. As expected, in a control reaction where DTT replaced the cell-free protein extract, BSSB was reduced to BSH (Fig. 3C). Surprisingly, the three mutants demonstrated varying levels of BSSB reductase activity (Fig. 3A), with NE785 having the lowest level of Bdr activity and NE1896 having activity levels similar to the wild-type. These data suggest that the gene products of both ORF 576 and 996 have Bdr activity, which may each compensate for a mutation in the other gene.

4. Discussion

Methicillin resistant *S. aureus* (MRSA) is a serious source of life-threating nosocomial and community acquired infections. As our current treatments for MRSA infections are losing efficacy, a thorough understanding of mechanisms of drug resistance is needed. In addition, novel pathways, present in *S. aureus* but absent in the human host must be investigated for potential drug targets. We have demonstrated that *S. aureus* mutants disrupted in the BSH biosynthesis are susceptible to a range of toxins, including oxidants, alkylating agents, and metals (Table 1A). We have further identified two of the genes, involved in BSH dependent detoxification, *bst* and *bshB*/*bca*. Bst likely conjugates thiol reactive antibiotics, cerulenin and rifamycin, to BSH (Fig. 1) and is thus the second

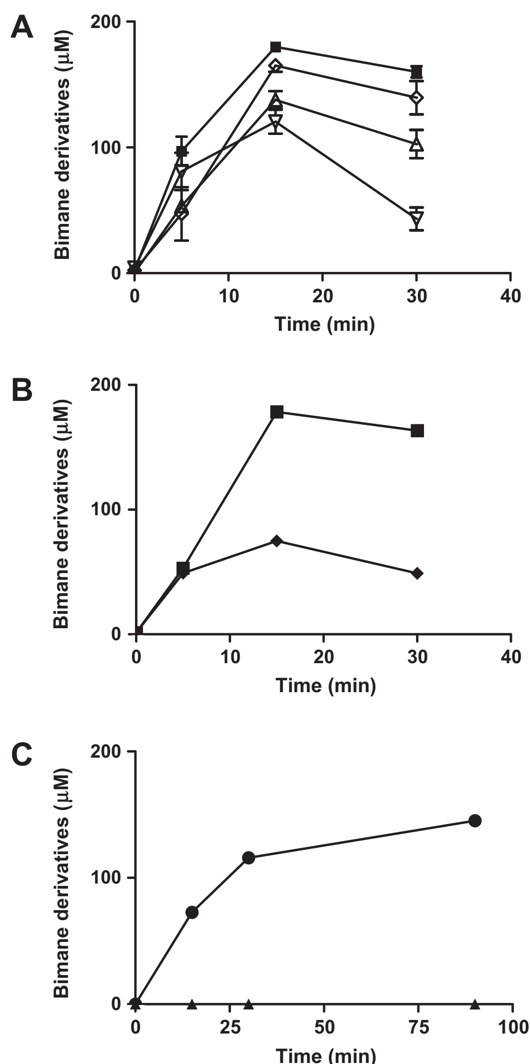

Fig. 3. BSH disulfide reductase assays measuring BSH released (A) with cell-free protein extract (300 µg) from *S. aureus* JE2 wild-type (■), and transposon mutants NE1896 (◇),NE1610 (Δ), and NE785 (▽) and NADPH; (B) with NADPH (■) and NADH (♦) as electron donor; and, (C) with DTT instead of protein extract (■). Values shown are averages and SD ($n = 3$).

Bst after FosB to be identified [13,14]. The other enzyme, BshB/Bca, participates in both the biosynthesis of BSH and detoxification of BS-toxin conjugates (Fig. 2).This dual activity of BshB/Bca is of great interest since bromotyrosine alkaloids (such as Exeg1706) which inhibit Mca in micromolar quantities [21], are bactericidal not only for *M. smegmatis* but also *S. aureus* [22].

Another important enzyme involved in LMW thiol metabolism and regarded as a good drug target is the disulfide reductase. The use of mutants to identify Bdr proved to be less successful (Fig. 3) than the identification of BshB/Bca and Bst. Mutants disrupted in the three genes identified to potentially code for reductases had Bdr activity, albeit the amount of Bdr activity differed in the mutants. Double or triple mutants disrupted in more than one of these genes may clarify the contribution of these genes. It is equally plausible that other genes not identified in the BLAST search may code for Bdr. One possible candidate is the thioredoxin reductase. In *Saccharomyces cerevisae*, glutathione is reduced by the thioredoxin/thioredoxinreductase system [23].

Interestingly, there appears to be significant differences in BSH metabolism in BSH producing bacteria with regards to oxidative stress. In *B. anthracis*, the BSH biosynthesis genes are induced by hydrogen peroxide but not by paraquat, a redox cycling agent [24]. *B. subtilis* BSH mutants are not sensitive to oxidative stress, although *B. subtilis* treated with cumene hydroperoxide (CHP) accumulates mixed disulfide forms of the Ohr repressor, OhrR, with cysteine, CoA and BSH [25]. *S. aureus* BSH mutants, on the other hand, are sensitive to oxidative stress, implying that BSH may play a greater role in protection against oxidative stress in *S. aureus*. In addition, *S. aureus* maintains constant BSH levels in response to disulfide stress and oxidative stress from hydrogen peroxide and cumene hydroperoxide. In contrast, BSH levels decrease with the same treatments in *B. subtilis*. The BSH levels in *S. aureus* are presumably maintained by increased biosynthesis of BSH, as observed when *S. aureus* was treated with the alkylating agent monobromobimane [5].

In conclusion, we have established that BSH and BSH dependent enzymes play an important protective role in *S. aureus*. Validation of the genes identified as Bst, BshB/Bca, and Bdr(s), via inhibition studies and enzyme kinetics, awaits successful cloning and expression of the recombinant proteins.

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