INTRODUCTION

Low-molecular-mass (LMW) thiols play critical roles in cell physiology of all organisms. In eukaryotes and Gram-negative bacteria, glutathione (GSH) is the major LMW thiol (Fahey et al., 1978), while in Actinomycetes, mycothiol (MSH), consisting of \(N\)-mycothiol (Newton et al., 2008), is the major LMW thiol (Newton et al., 2008). We recently demonstrated that in low GC Gram-positive bacteria, such as Bacillus sp. and Staphylococcus, bacillithiol (BSH, CysGlcNMal, Fig. 1) is the major LMW thiol (Newton et al., 2009) along with coenzyme A (CoA) and cysteine (delCardayre & Davies, 1998). BSH is similar in structure to MSH with an \(L\)-cysteine linked to \(D\)-glucosamine, but differs from MSH in that \(L\)-malate replaces \(N\)-inositol (GlcNIns), is the major LMW thiol (Newton et al., 2008). We recently demonstrated that in low GC Gram-positive bacteria, such as Bacillus sp. and Staphylococcus, bacillithiol (BSH, CysGlcNMal, Fig. 1) is the major LMW thiol (Newton et al., 2009) along with coenzyme A (CoA) and cysteine (delCardayre & Davies, 1998). BSH is similar in structure to MSH with an \(L\)-cysteine linked to \(D\)-glucosamine, but differs from MSH in that \(L\)-malate replaces \(N\)-inositol and the cysteine is not \(N\)-acyetylated. The BSH biosynthetic pathway has been partially elucidated with the first step involving the formation of \(N\)-acyetylGlcNMal from UDP-\(N\)-acetylglucosamine and \(L\)-malate, a reaction catalysed by the glycosyltransferase BshA (Gaballa et al., 2010; Parsonage et al., 2010). The last step involves the ligation of cysteine to GlcNMal. The biochemical reaction has not been elucidated although Bacillus subtilis YLLA appears to be associated with this step (Gaballa et al., 2010).

Bacillithiol (BSH), an \(\alpha\)-anomeric glycoside of \(L\)-cysteynyl-\(D\)-glucosaminyll-\(L\)-malate, is a major low-molecular-mass thiol found in bacteria such as Bacillus sp., Staphylococcus aureus and Deinococcus radiodurans. Like other low-molecular-mass thiols such as glutathione and mycothiol, BSH is likely to be involved in protection against environmental toxins including thiol-reactive antibiotics. We report here a BSH-dependent detoxification mechanism in S. aureus. When S. aureus Newman strain was treated with monobromobimane and monochlorobimane, the cellular BSH was converted to the fluorescent \(S\)-conjugate BSH-bimane. A bacillithiol conjugate amidase activity acted upon the BS-bimane to produce Cys-bimane, which was then acetylated by an \(N\)-acetyltransferase to generate \(N\)-acetyl-Cys-bimane, a mercapturic acid. An S. aureus mutant lacking BSH did not produce mercapturic acid when treated with monobromobimane and monochlorobimane, confirming the involvement of bacillithiol. Furthermore, treatment of S. aureus Newman with rifampycin, the parent compound of the first-line anti-tuberculosis drug, rifampicin, indicated that this thiol-reactive antibiotic is also detoxified in a BSH-dependent manner, since mercapturic acids of rifamycin were observed in the culture medium. These data indicate that toxins and thiol-reactive antibiotics are detoxified to less potent mercapturic acids in a BSH-dependent manner and then exported out of the cell in S. aureus.

Detoxification of toxins by bacillithiol in Staphylococcus aureus

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Abbreviations: LMW, low molecular mass; GSH, glutathione; MSH, mycothiol; BSH, bacillithiol; GST, glutathione-S-transferase; MST, mycothiol-S-transferase; BST, bacillithiol-S-transferase; BSmB, bacillithiol-S-bimane; CySmB, cysteine-S-bimane.

One of the major functions of LMW thiols is to protect the cell against toxins. The thiols function as nucleophiles that readily react with electrophiles such as formaldehyde, methylglyoxal and alkyl halides. The formation of a thiol-S-toxin conjugate may occur spontaneously or, if the electrophile is less chemically reactive, the formation of the conjugate may be catalysed by an S-transferase. The thiol-S-toxin conjugate is then either excreted from the cell, presumably through a transporter, or further processed before excretion. This system has been well described in eukaryotes where GSH-dependent detoxification of drugs is a major cause of concern (Townsend & Tew, 2003). Indeed, screening lead compounds for reactivity with GSH has become a mainstay in drug discovery programmes and lead compounds that react with GSH are rapidly removed from the drug pipeline or are subjected to further medicinal chemical refinement. In higher organisms, GSH reacts with K. Lo, D. Holden, R. C. Fahey & M. Rawat, unpublished data. This is followed by the deacetylation of \(N\)-acyetylGlcNMal by the deacetylase BshB to yield GlcNMal (Gaballa et al., 2010; Parsonage et al., 2010). The last step involves the ligation of cysteine to GlcNMal. The biochemical reaction has not been elucidated although Bacillus subtilis YLLA appears to be associated with this step (Gaballa et al., 2010).
the toxin in the liver, a reaction that can occur chemically or is catalysed by GSH-S-transferase (GST). Peptidases then cleave the glutamic acid and glycine residues on the GSH-S-conjugate resulting in a cysteine-S-toxin conjugate, which is acetylated by an acetyl-CoA-dependent N-acetyltransferase in the kidney to form a mercapturic acid that is excreted (Hinchman & Ballatori, 1994) (Fig. 2a). In mycobacteria, a similar detoxification mechanism occurs (Newton et al., 2000; Steffek et al., 2003; Rawat et al., 2004), with the formation of a MSH-S-toxin conjugate either spontaneously or by catalysis with a mycothiol-S-transferase (MST). A mycothiol conjugate amidase (Mca) then cleaves the amide bond in the MSH-S-toxin to yield an N-acetylcysteinyl-S-toxin, the mercapturic acid of the toxin, which is excreted from the cell, and the retained GlcNIns is used for resynthesis of MSH (Fig. 2b) (Steffek et al., 2003).

Recently, we reported that B. subtilis mutants lacking BSH are sensitive to fosfomycin and moderately sensitive to methylglyoxal, suggesting that, like GSH and MSH, BSH plays a role in the detoxification of electrophiles and antibiotics (Gaballa et al., 2010). We have also shown that FosB functions as a BSH-dependent S-transferase (BST) that is responsible for the detoxification of fosfomycin (Sharma et al., 2011). In this report, we demonstrate the presence of a BSH-dependent detoxification system analogous to the MSH- and GSH-dependent detoxification systems.

**METHODS**

**Bacterial strains and culture conditions.** Staphylococcus aureus Newman and RN4220 strains were kind gifts from Dr V. Nizet (University of California, San Diego). S. aureus SH1000 and NCTC 8325 were generously provided by Dr A. Horswill (University of Iowa). All strains were grown in trypticase soy broth (TSB, BBL) or trypticase soy agar (TSA) at 37 °C unless otherwise indicated.

**Determination of thiol and glucosaminyl malate levels.** Derivatization of cell extracts with monobromobimane and HPLC analysis of the derivatized samples to determine thiol content were performed as described by Anderberg et al. (1998). Control samples treated with N-ethylmaleimide followed by monobromobimane labelling were also analysed. GlcNMal was assayed by pre-column derivatization with the fluorescent amine reagent AccQ Tag (Waters) by a minor modification of the method of Anderberg et al. (1998), as described by Gaballa et al. (2010).

**Determination of amidase activity.** N-Acetylbacillithiol-S-bimane (AcBSmB) was synthesized from bacillithiol-S-bimane (BSmB) and a 10-fold excess of acetic anhydride as described for GlcNAcIns (Newton et al., 2000). Bacillithiol conjugate amidase (Bca) activity was analysed in a reaction volume of 100 μl with 100 μg cell-free protein extract in 25 mM HEPES, pH 7.5, 3 mM β-mercaptoethanol containing either 78 μM BSmB or 44 μM AcBSmB (Newton et al., 2000). The samples were incubated at 30 °C and 25 μl aliquots were taken at 10, 20 and 40 min. The reaction was stopped by adding 25 μl 40 mM methanesulfonic acid and subjected to HPLC analysis.

**Analysis of S. aureus strains treated with monobromobimane and monoclorobimane in culture.** S. aureus Newman culture

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**Fig. 1.** Structure of bacillithiol.

**Fig. 2.** Thiol-dependent detoxification. (a) Glutathione-dependent detoxification system in humans, (b) mycothiol-dependent detoxification in mycobacteria and (c) proposed bacillithiol-dependent detoxification in S. aureus.
(1 l) was grown to exponential phase (OD₆₀₀ 2.0), harvested and then brought up in ice-cold TSB medium. Triplicate (25 ml) samples of culture were assayed for initial thiol and GlcNMal content as described above. This thiol and GlcNMal assay was designated the zero time point (Figs 3 and 4). The culture was then split into four 100 ml cultures; one set of two cultures was incubated on ice without shaking and another set was incubated at 37 °C with shaking. In each set, 0.5 mM monobromobimane was added to one culture and 0.5 mM monochlorobimane was added to the other culture. At 30 and 160 min, triplicate 25 ml samples were harvested and the cell pellets and medium were both analysed for thiol-bimane conjugates. In the case of the cell pellet, the thiol-bimane conjugates were extracted by washing the cell pellet with fresh media followed by extraction with 1 ml 50 % acetonitrile in 25 mM HEPES, pH 7.5, the cell pellet. After incubation at 60 °C for 15 min, the reaction was acidified with 2 μM methanesulfonic acid and centrifuged for 5 min at maximum speed to precipitate the proteins. The resulting supernatant along with the medium were subjected to HPLC analysis.

Similarly, S. aureus strains SH1000 and Newman were grown to exponential phase (OD₆₀₀ 1.5), harvested, brought up in fresh ice-cold TSB media, and triplicate samples of cultures were assayed for initial thiol and GlcNMal content as described above. Rifamycin S (100 μg ml⁻¹) was added and the culture was incubated on ice for 1 h without shaking. The culture was harvested, washed twice with 250 ml ice-cold TSB, resuspended in 250 ml prewarmed TSB medium, and incubated at 37 °C. At 1, 10 and 60 min and 18 h, 45 ml samples were harvested and the cell pellet and medium were analysed for rifamycin S conjugates, as described by Steffek et al. (2003). Rifamycin S conjugates were quantified by HPLC at 315 nm, an isosbestic wavelength for the rifamycin S (RifS) and rifamycin SV (RifSV) forms, using an approximate extinction coefficient of 24 000 M⁻¹ cm⁻¹ and RifS and RifSV standards. Standards of cysteine-rifamycin (CyS-Rif) and N-acetylcysteine-rifamycin (AcCyS-Rif) were synthesized by chemically reacting rifamycin with cysteine and acetylcysteine, respectively (Steffek et al., 2003). Sample peaks with identical elution times as the standards, CyS-Rif and AcCyS-Rif, were collected and the mass was checked by mass spectroscopy to confirm the identity of the peaks. The AcCyS-Rif represents the sum of AcCyS-RifS and AcCyS-RifSV forms and similarly CyS-Rif represents the sum of CyS-RifS and CyS-RifSV forms.

**Determination of acetyltransferase activity.** Acetyltransferase activity was determined on cell-free protein extracts of S. aureus Newman under the following conditions: the reaction volume of 100 μl consisted of 100 μg cell-free extract protein sample in 25 mM HEPES, pH 7.5, 3 mM β-mercaptoethanol, 30 μM BSMB and 1 mM acetylCoA. The samples were incubated at 30 °C and 25 μl aliquots were taken at 10, 20 and 40 min. The reaction was stopped by adding 25 μl 40 mM methanesulfonic acid and subjected to HPLC analysis.

**Analysis of S. aureus strains treated with rifamycin S in culture.** A S. aureus culture (4 l) was grown to exponential phase (OD₆₀₀ 1.0) and the cells were collected by centrifugation; the sample was brought up in 250 ml ice-cold TSB media, and triplicate 1 ml samples of culture were assayed for initial thiol content as described above. Rifamycin S (100 μg ml⁻¹) was added and the culture was incubated on ice for 1 h without shaking. The culture was harvested, washed twice with 250 ml ice-cold TSB, resuspended in 250 ml prewarmed TSB medium, and incubated at 37 °C. At 1, 10 and 60 min and 18 h, 45 ml samples were harvested and the cell pellet and medium were analysed for rifamycin S conjugates, as described by Newman under the following conditions: the reaction volume of 1 l consisted of 100 μg cell-free extract protein sample in 25 mM HEPES, pH 7.5, 3 mM β-mercaptoethanol, 30 μM BSMB and 1 mM acetylCoA. The samples were incubated at 30 °C and 25 μl aliquots were taken at 10, 20 and 40 min. The reaction was stopped by adding 25 μl 40 mM methanesulfonic acid and subjected to HPLC analysis.

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**Fig. 3.** Detoxification of monohalobimanes in S. aureus Newman. After addition of 0.5 mM monohalobimane, levels of intracellular thiol-bimanes, cysteiny-l-bimane (CySmB) (□), N-acetylcysteiny-l-bimane (AcCySmB) (△), bacillithiol-bimane (BSmB) (○), extra-cellular thiol-bimanes in the medium, CySmB (■), AcCySmB (▲) (left y-axis), and intracellular glucosaminylmalate, GlcNMal (half-shaded square) (right y-axis), were determined after incubation for 0, 30 and 165 min. Cultures were incubated at 0 °C without shaking after addition of 0.5 mM monobromobimane (a) or at 37 °C with shaking after addition of 0.5 mM monobromobimane (b) and at 37 °C with shaking after addition of 0.5 mM monochlorobimane (c). Error bars, SD.
RESULTS

NCTC 8325 and its derivatives are yllA mutants lacking bacillithiol

_S. aureus_ NCTC 8325, a strain with an 11 bp deletion in the _rsbU_ gene, which encodes a regulator of sigma factor B (Kullik et al., 1998), was cured of all prophages to obtain strain NCTC 8325-4, which was then chemically mutagenized to obtain RN4220 (Novick, 1990). RN4220 is used as an intermediate cloning host for generating allelic replacement _S. aureus_ mutants as it lacks the restriction modification system, which prevents foreign DNA from incorporating into the host genome in other _S. aureus_ strains. In the process of making _S. aureus_ knockouts, we noticed that RN4220 lacks BSH. When checked, the parent strain, NCTC 8325-4 also lacked BSH (Table 1), suggesting that the mutation in _rsbU_ is responsible for the lack of BSH in these strains. To confirm this hypothesis, we analysed the thiol content of SH1000, the _rsbU_-repaired strain of NCTC 8325 (Horsburgh et al., 2002), and discovered that this strain also lacks BSH. The levels of the BSH biosynthetic intermediate, GlcNACMal, and to a lesser extent the level of GlcNMal, are elevated in _S. aureus_ NCTC 8325-4 and SH1000 compared with _S. aureus_ Newman strains. These results are similar to the results obtained with _Mycobacterium smegmatis_ Tn1 and Tn2 mutants, which lack MSH and MSH ligase activity, but have elevated levels of the MSH biosynthesis intermediate GlcNIns (Rawat et al., 2002). These mutants were originally thought to be disrupted in MSH ligase activity (MshC) but the presence of an intact _mshC_ gene suggests another mutation is responsible for the lack of MSH ligase activity.

When the sequences of genes associated with BSH biosynthesis were examined in the _S. aureus_ strains, _S. aureus_ NCTC 8325 _bshA_ and _bshB_ did not appear to be significantly different in sequence from other _S. aureus_ strains, including the Newman strain; however, _yllA_, associated with BSH ligase activity (Gaballa et al., 2010), was disrupted in NCTC 8325. In _S. aureus_ NCTC 8325, SAOUHSC_01139 (1 090 331–1 090 558) codes for the first 71 aa of YllA, followed by SAOUHSC_01140 (10 905 000–1 090 661 complement), whose sequence overlaps with the last 58 aa at the C terminus of SAOUHSC_01139. SAOUHSC_01140 is coded by the complementary strand and does not match any protein sequence in the genus _Staphylococcus_. The rest of the _yllA_ protein is coded by SAOUHSC_01141 (1 090 750–1 091 952), which codes for aa 138–537 of the C terminal portion of _yllA_. Thus, it appears that NCTC 8325 and all strains derived from it are disrupted in the _yllA_ gene.

**BSH detoxifies monobromobimane by mercapturic acid formation**

Thiol-dependent detoxification of environmental toxins and antibiotics has been described for both GSH and MSH (Fig. 2) (Allocati et al., 2009; Newton et al., 2000). We have already described the MSH-dependent detoxification of monobromobimane, a thiol-reactive, fluorescent, alkylating agent in mycobacteria (Newton et al., 2000). We thus

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**Table 1. Levels of thiols and intermediates in _S. aureus_ strains NCTC 8325-4, SH1000 and Newman**

<table>
<thead>
<tr>
<th>Thiol [nmol (mg dry weight)⁻¹]</th>
<th><em>S. aureus</em> strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCTC 8325-4</td>
</tr>
<tr>
<td>BSH</td>
<td>≤0.01</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>CoA</td>
<td>6.3±0.2</td>
</tr>
<tr>
<td>Acetylglucosaminylmalate</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>Glucosaminylmalate</td>
<td>5.5±0.4</td>
</tr>
</tbody>
</table>

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_**Fig. 4.** Detoxification of bimanes in _S. aureus_ SH1000 lacking yllA and BSH. The thiol-bimanes, cellular CySmB ( ●), extracellular CySmB ( □), and intracellular syn-dimethylbimane ( ○) and extracellular syn-dimethylbimane ( ●) were assayed at 0, 30, 160 and 1200 min after incubation at 37 °C with shaking after addition of (a) 0.5 mM monobromobimane and (b) 0.5 mM monochlorobimane. Error bars, SD._
compared the detoxification of the monobromobimane by *S. aureus* Newman at physiological temperature (37 °C) and on ice in order to slow the reactions so the intermediates could be monitored.

When *S. aureus* Newman cultures were treated with 0.5 mM monobromobimane, the cellular BSH and cysteine were converted to their bimane derivatives (BSmB and CySmB, respectively). Both BSmB and CySmB were virtually undetectable inside the cell by 30 min at 37 °C (Fig. 3b) while for cells incubated on ice, the levels of BSmB dropped intracellularly but levels of CySmB did not (Fig. 3a). Extracellularly, CySmB was evident in the culture medium in both treatments by 30 min. By 30 min, the extracellular level of CySmB in cultures incubated on ice was 0.65 nmol (mg dry weight)⁻¹, approximately twofold higher than the initial level of intracellular CySmB, and remained at this level at 160 min. At 37 °C, the levels of CySmB remained low within the cells but the extracellular levels increased 12-fold more than the initial level of intracellular CySmB within the cell. In the cultures incubated on ice, the mercapturic acid of the bimane (AcCySmB) was detected in the cell and in the medium, and the amount of mercapturic acid of the bimane was higher within the cells. In contrast, in 37 °C- incubated cultures, AcCySmB was not evident within the cells but could be detected extracellularly, and these extracellular levels had increased to 2.5 nmol (mg dry weight)⁻¹ by 160 min.

Since a mercapturic acid derivative of the bimane was detected within the cells, we reasoned that if AcCySmB was derived from BSH then glucosaminylmalate (GlcNMal) levels should also be elevated in these cells. Indeed, the intracellular levels of GlcNMal increased by 50 % by 160 min in the 0 °C cells and threefold by 160 min in 37 °C cells. These data indicate that GlcNMal is being newly synthesized within the *S. aureus* cell upon depletion of BSH and cysteine by monobromobimane. It should be noted that the thiol and GlcNMal levels at 0 time for the experiments in Figs 3, 4 and 5 are 5- to 15-fold less than those reported in Table 1 for freshly harvested *S. aureus* cells. The extensive cold pretreatments and washes (cold shock) prior to the zero time point appear to depress BSH and intermediate levels.

**BSH also detoxifies the less reactive monochlorobimane**

While monobromobimane is able to react spontaneously with LMW thiols, the chemical reaction of monochlorobimane with LMW thiols is slow under physiological conditions indicating that S-conjugate formation is enzyme-catalysed (Cook et al., 1989). When *S. aureus* Newman cultures were treated with 0.5 mM monochlorobimane at 37 °C, both BSmB and CySmB were virtually undetectable inside the cell after 30 min (Fig. 3c) similar to treatment with monobromobimane (Fig. 3b). At 37 °C, the levels of intracellular and extracellular thiol-bimanes and mercapturic acid were generally lower in the monochlorobimane-treated cells compared with the monobromobimane-treated cells. The amount of CySmB in the medium was more than threefold less in monochlorobimane-treated cultures at 160 min compared with monobromobimane-treated cells. In addition, AcCySmB was present intracellularly; after 160 min, 0.6 nmol mg⁻¹ was detected in contrast with the monobromobimane-treated cells, where there was no evidence of intracellular mercapturic acid. The amount of extracellular AcCySmB was 2.0 nmol mg⁻¹ in monochlorobimane-treated cells, while there was 2.7 nmol mg⁻¹ at 37 °C in monobromobimane-treated cells. However, the total amount of intracellular and extracellular AcCySmB detected in monobromobimane-treated cells (2.7 nmol mg⁻¹) is equivalent to the total amount in monochlorobimane-treated cells (2.0 and 0.6 nmol mg⁻¹). The chemical conjugation of monochlorobimane to BSH is too slow to account for the formation of BSmB, the precursor of the
mercapturic acid of the bimane. Since our data indicate that the treatment with either monohalobimane results in the production of similar amounts of mercapturic acid, this is strong evidence for the presence of a BST that mediates the conjugation of BSH to monochlorobimane. GSTs preferentially use GSH over cysteine and these data indicate that this is likely to be the case with BST since the levels of CySmB in the monochlorobimane-treated cells were less than in the monobromobimane-treated cells. In monochlorobimane-treated cells, the intracellular levels of GlcNMal increased by ~25%.

**Mercapturic acid production requires BSH**

To determine the origin of the mercapturic acids of the bimane observed in *S. aureus* Newman upon treatment with monobromobimane and monochlorobimane, *S. aureus* SH1000, which does not have BSH, was also treated with these monohalobimanes. In Fig. 4, it can be seen that intracellular levels of CySmB declined in the first 30 min and then remained low during the course of the experiment, and little CySmB or AcCySmB was detected in the culture medium. However, large amounts of syn-dimethylbimane were detected within the cell and outside in the medium within 30 min of the addition of monobromobimane or monochlorobimane (Fig. 4). syn-Dimethylbimane is the product formed by displacement of the halide in monobromobimane or monochlorobimane by hydride. An authentic sample of dimethylbimane was generously supplied by Edward Kosower (Tel Aviv University) for use in quantification. By 160 min, monobromobimane had been converted to syn-dimethylbimane with 15 nmol syn-dimethylbimane g⁻¹ present extracellularly and 17 nmol g⁻¹ present intracellularly; these levels remained constant to 20 h (Fig. 4a). Similarly, when SH1000 was treated with 0.5 mM monochlorobimane, no CySmB or AcCySmB was detected in the medium and the monochlorobimane was substantially converted to dimethylbimane (Fig. 4b). The rate of conversion of monochlorobimane and monobromobimane to dimethylbimane in *S. aureus* SH1000 is very fast and was not observed in *S. aureus* Newman. The enzymic source of the hydride donor in *S. aureus* SH1000 is unknown. Since SH1000 cells were viable even after 20 h, the production of syn-dimethylbimane does not appear to be detrimental to SH1000 and may represent a detoxified form of the monohalobimanes. As SH1000 lacks BSH and also does not produce CySmB or AcCySmB when treated with monohalobimanes, this is clear evidence that production and excretion of CySmB and AcCySmB are linked to intracellular BSH and not cysteine in *S. aureus*.

**BSH participates in rifamycin detoxification**

To determine if *S. aureus* is able to detoxify rifamycin in a BSH-dependent manner, *S. aureus* Newman was exposed to RifS (Fig. 5). After just 1 min treatment, AcCyS-Rif was detected within the *S. aureus* Newman cell and also in the medium. By 10 min, the intracellular amount of AcCyS-Rif dropped to 0.10 nmol mg⁻¹ and kept on dropping until it was below 0.05 nmol mg⁻¹ by 15 h. The drop in the intracellular amount was accompanied by a concomitant rise in the extracellular amount of AcCyS-Rif (Fig. 5b). These results suggest that the mercapturic acid of rifamycin was excreted out of the cell into the medium. No CyS-Rif conjugate was detected in the medium although a small amount of CyS-Rif was present intracellularly. As with monohalobimane treatments, addition of RifS to *S. aureus* Newman cells resulted in the induction of cysteine and BSH biosynthesis (Fig. 5a). The increase in cysteine levels occurred prior to the increase in BSH levels indicating that the cysteine synthesis is needed for BSH biosynthesis (Fig. 5a).

**A bacilli thiol S-conjugate amidase is involved in mercapturic acid formation**

The *S. aureus* Newman cell extract was assayed for Bca activity with the bacilli thiol bimane derivative, BSmB, and the *N*-acetylated form of the BSmB, AcBSmB, as substrates. The two activities are essentially equivalent indicating that Bca is able to cleave the cysteine-glucosamine amide bond in either substrate (Fig. 6a). Furthermore, the Bca activity in the whole-cell extract is 0.7 nmol min⁻¹ mg⁻¹, which is within a factor of two of the McA activity (with 30 µM MSmB as substrate) from *M. smegmatis* whole-cell extracts (Newton et al., 2000). The action of Bca on AcBSmB and BSmB produces the mercapturic acid (AcCySmB) and CySmB, respectively. Although AcCySmB has been detected in cells treated with monohalobimanes (Fig. 3) AcBSmB has not been detected in cells. The gene encoding Bca remains to be identified.

**N-Acetyltransferase activity produces mercapturic acid from CySmB**

As a parallel to GSH and MSH biochemistry, a mercapturic acid of the toxin may be the product of the BSH detoxification mechanism. Since the amino group on the cysteine moiety in BSH is not acetylated, acetylation of the cysteine must occur for a mercapturic acid to form. The substrate for the acetylase can be the B- toxin conjugate forming AcBSmB, which can then be cleaved by Bca to generate AcCyS-toxin and GlcNMal. Alternatively, the substrate can be a cysteine-toxin adduct, such as CySmB, that results when *S. aureus* Bca cleaves the amide bond in the B- toxin conjugate (Fig. 6b). To determine the *in vivo* sequence of events, the levels of thiols were followed over time after the addition of BSmB to cell-free extracts of *S. aureus* Newman. The drop in BSmB level was accompanied by a sharp rise in CySmB level and a slower rise in AcCySmB level; AcBSmB was not detected. This suggests that CySmB is produced first and is then acetylated to form the mercapturic acid (Fig. 6b).

**DISCUSSION**

Herein, we present data concerning a BSH detoxification system analogous to the MSH detoxification mechanism...
present in mycobacteria (Fig. 7). In this model, a toxin reacts with the thiol group of BSH to form a BS-toxin conjugate. An amidase, Bca, with similar activity to Mca, then cleaves the amide bond linking GlcNMal to the cysteine residue, resulting in a cysteine-toxin conjugate and GlcNMal. The latter can be recycled to BSH and the cysteine-toxin conjugate is acetylated to form a mercapturic acid, which is exported out of the cell.

First, we demonstrated that detoxification proceeds with the conjugation of the toxin with BSH, followed by the appearance of mercapturic acids in the culture medium. Using monobromobimane, a fluorescent alkylating agent that reacts rapidly with thiols, as a model substrate, we show the formation of BSmB conjugate, which is converted to CySmB and AccySmB, the mercapturic acid of the toxin. Both of these are pumped out of the cell (Fig. 3a and b). This detoxification mechanism occurs rapidly at physiological temperatures (37 °C) with the thiol-reactive monobromobimane such that the mercapturic acid of this alkylating agent is only found in the medium. By incubating the cells on ice, the reactions are slowed enough to see the mercapturic acid within the cell (Fig. 3a). Large amounts of CySmB are also detected in the medium when cells are incubated at 37 °C (Fig. 3b) and to a lesser extent when cells are incubated on ice (Fig. 3a). There are two explanations for this result: (1) CySmB is simply the product of the chemical reaction between cysteine and monobromobimane and is excreted out of the cell into the medium or (2) an acetyltransferase that adds an acetyl group to CySmB generated by the action of the amidase cannot keep up with the large amount of CySmB that is produced. Both explanations may be true; however, when SH1000, a strain that lacks BSH due to a disruption in yllA, is treated with monobromobimane, there is very little CySmB in the medium indicating that the CySmB seen in S. aureus Newman medium originates from BSmB (Fig. 4a). Second, the levels of intracellular GlcNMal also increased within the cells indicating a turnover of BSH, especially in the 37 °C cells (Fig. 3b).

Treatment of SH1000, lacking BSH, with monohalobimanes resulted in the production of large amounts of syn-dimethylbimane that was excreted into the medium whereas CySmB and AccySmB were not excreted (Fig. 4). This indicates that in the absence of BSH, S. aureus is able to detoxify and rid itself of toxins like monobromobimane using an unidentified reactive source of hydride (electrons). This system may be induced in the absence of BSH and may have broader detoxification activity. It clearly merits further investigation.

In S. aureus, the BSH detoxification mechanism is also able to detoxify the antibiotic rifamycin S, the parent compound of the anti-tubercular drug rifampicin (Fig. 5). Examination of the S. aureus Newman strain treated with RifS indicated that a substantial quantity of the cellular BSH reacted to produce the BS-Rif conjugate that was rapidly converted to the mercapturic acid of the rifamycin, Accys-Rif. The intracellular levels dropped as the mercapturic acid was exported out of the cell with the concomitant rise of the mercapturic acid in the medium (Fig. 5b). No Cys-Rif conjugate was detected in the medium indicating that rifamycin is detoxified principally through BSH detoxification pathway. Similarly to M. smegmatis, a significant fraction of the cellular BS-Rif conjugate produced in the cell subsequently disappeared and could not be accounted for in terms of cellular Accys-Rif or identified secreted forms. The HPLC analysis indicated that significant levels of additional rifamycin derivatives are produced in the cells and released into the medium (data not shown).

The presence of large amounts of CySmB and AccySmB inside and outside the cell (Fig. 3b), compared with initial levels of BSH and cysteine in the S. aureus cell, suggests that treatment with a thiol-reactive toxin, such as monobromobimane, may induce genes involved in cysteine and BSH biosynthesis. As with monohalobimanes, exposure to

Fig. 6. Amidase and N-acetyltransferase activity in S. aureus Newman cell extract. (a) Amidase activity with substrate 78 μM BSmB (▼) resulting in CySmB (■) and substrate 44 μM AcBSmB (▲) resulting in AccySmB (▲); and (b) N-acetyltransferase activity with substrate BSmB (■) resulting in CySmB (■) followed by AccySmB (▲).
rifamycin S results in an increase in the induction of cysteine and BSH biosynthesis (Fig. 6a).

GSTs are a large protein superfamily, which are highly specific for the thiol substrate, GSH, and less specific for the second substrate. However, all catalyse the nucleophilic attack by the thiol on the electrophilic groups of a wide range of compounds. The Mn$^{2+}$-dependent GST, FosA, confers resistance to fosfomycin since the conjugation of fosfomycin to GSH opens the epoxide ring and inactivates fosfomycin. We have demonstrated that BSH is the actual substrate for the homologue, FosB, in S. aureus (Sharma et al., 2011). When we followed the fate of the fluorescent alkylating agent monochlorobimane, which is less chemically reactive and thus would require a BST, analogous to a GST, to catalyse its conjugation to BSH, the mercapturic acid of the bimane was detected intracellularly and extracellularly, indicating the presence of a BST, which catalyses the conjugation of monochlorobimane with BSH to produce BSmB. Recently, we have identified and characterized a B. subtilis BST which is able to catalyse the reaction of monochlorobimane with BSH; whether the S. aureus homologue does the same remains to be seen (Newton et al., 2011).

A key enzyme postulated in our model is the Bca. The homologue in mycobacteria, Mca, a zinc metalloenzyme, is able to cleave an amide bond in the MSH molecule of the mycothiol S-conjugates of monobromobimane, rifamycin S, cerulenin, etc. (Steffek et al., 2003), to release the mercapturic acid and GlcNIns. A paralogue of Mca, MshB, is responsible for the deacetylase activity needed in the third step of the biosynthesis of MSH. A prospective candidate for Bca in S. aureus Newman is NWMN_0530, containing an lmbE signature and having significant sequence similarity to Mca, and its parologue, MshB deacetylase. In Bacillus cereus, B. anthracis and B. subtilis, multiple paralogues with the lmbE signature exist but only BA1557 has been shown to have the BshB deacetylase activity responsible for the second step of BSH biosynthesis and very low amidase activity with bacillithiol S-bimane (Gaballa et al., 2010; Parsonage et al., 2010). Attempts to clone NWMN_0530 have been met with technical problems; the protein either precipitates as it is purified or does not express well. Bca-dependent cleavage of a BSH-conjugate of thiol-reactive antibiotics and other electrophiles may be as important in the protection against thiol-reactive toxins in BSH-containing micro-organisms as it is in the actinomycetes (Newton & Fahey, 2002). It remains to be seen if mercapturic acids of these toxins can be

Fig. 7. Detoxification of model toxins, monobromobimane or monochlorobimane by S. aureus Newman. Enzyme activities observed were: BST, bacillithiol S-transferase; Bca, bacillithiol S-conjugate amidase; BshC, bacillithiol synthase; CAT, cysteiny1 S-conjugate acetyltransferase.
detected in broths similar to the mercapturic acids found in fermentation broths of actinomycetes (Newton & Fahey, 2002).

An enzyme unique to the BSH detoxification pathway is the cysteine N-acetyltransferase (CAT; Fig. 7). The acetylated cysteine moiety in MSH obviates the need for a CAT in the MSH detoxification mechanism but in the BSH detoxification pathway, a CAT is required for the production of mercapturic acid. In Fig 6(b), it can be seen that the CyS toxin conjugate is the substrate for the CAT since CySMB appears first followed by AcCySMB instead of AcBSMB followed by AcCySMB, when BSmB is added with acetyl-CoA to \textit{S. aureus} Newman cell extract. There are 23 genes annotated GNAT \textit{N}-acetyltransferases present in the \textit{S. aureus} Newman genome. Analysis of the gene region and known and predicted associations of the annotated \textit{N}-acetyltransferase genes via the STRINGS database (http://string.embl.de/) indicated that a likely candidate for CAT is acetyltransferase genes via the STRINGS database (http://mic.sgmjournals.org 1125 Montelione, G. T. & Kennedy, M. A. (2008). Cort, J. R., Ramelot, T. A., Murray, D., Acton, T. B., Ma, L. C., Xiao, R., Monteilone, G. T. & Kennedy, M. A. (2008). Structure of an acetyl-CoA binding protein from \textit{Staphylococcus aureus} representing a novel subfamily of GCN5-related \textit{N}-acetyltransferase-like proteins. \textit{J Struct Funct Genomics} 9, 7–20. 


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