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Bacillithiol: a key protective thiol in *Staphylococcus aureus*

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Abstract

Bacillithiol is a low-molecular-weight thiol analogous to glutathione and is found in several Firmicutes, including *Staphylococcus aureus*. Since its discovery in 2009, bacillithiol has been a topic of interest because it has been found to contribute to resistance during oxidative stress and detoxification of electrophiles, such as the antibiotic fosfomycin, in *S. aureus*. The rapid increase in resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) to available therapeutic agents is a great health concern, and many research efforts are focused on identifying new drugs and targets to combat this organism. This review describes the discovery of bacillithiol, studies that have elucidated the physiological roles of this molecule in *S. aureus* and other Bacilli, and the contribution of bacillithiol to *S. aureus* fitness during pathogenesis. Additionally, the bacillithiol biosynthesis pathway is evaluated as a novel drug target that can be utilized in combination with existing therapies to treat *S. aureus* infections.

Keywords

bacillithiol; bacillithiol conjugate amidase; drug resistance; fosfomycin; oxidative stress; pathogenesis; *Staphylococcus aureus*

Thiols in aerobic bacteria

Aerobic bacteria live in an oxidizing environment and often require this environment for life, yet they maintain a reducing intracellular environment. Generating and maintaining this reducing environment requires not only energy but also specialized molecules in the cell that store this reducing potential and use it for various biochemical processes. These molecules include low-molecular-weight thiols such as glutathione (GSH), cysteine (Cys) and coenzyme A (CoA), each of which play different cellular roles [1]. Free cysteine levels are typically kept low because cysteine is prone to metal-catalyzed oxidation [2], which produces hydrogen peroxide, which can damage both DNA and proteins. CoA has a high thiol pK_a compared to cysteine and glutathione (see “Biophysical Properties” below, [3–5]), such that the availability of the thiolate anion form of CoA required for reaction with

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electrophiles and thiol-disulfide exchange reactions is very limited. CoA is not a storage form of cysteine and cannot resupply the cell with Cys for protein synthesis during times of shortage, which can occur during oxidative stress. GSH is oxidized more slowly than is Cys, and it can be broken down to release Cys when necessary, so it plays a key role in redox reactions and redox homeostasis. GSH is present in Gram-negative bacteria and also in eukaryotic cells and was at one time assumed to be present in all living aerobic cells. However, many Gram-positive bacteria were subsequently shown to lack GSH and contain instead other low-molecular-weight thiols [1·6·7]. For example, the Actinobacteria, which include human pathogens such as *Mycobacterium tuberculosis*, use mycothiol (MSH), whereas several Firmicutes, including the human pathogens *Bacillus anthracis* and *Staphylococcus aureus*, use bacillithiol (BSH). Since its discovery, BSH has become the focus of considerable biochemical and physiological attention. In this review, we summarize this work and we evaluate the BSH system in *Staphylococcus aureus* as a potential target for the development of chemotherapeutic agents.

The discovery of mycothiol & bacillithiol

Once it became clear that GSH was not ubiquitous, the search was underway for alternative low-molecular-weight thiols. To study these thiols, an assay was developed utilizing the thiol-labeling compound monobromobimane in combination with reverse phase separation of the resulting fluorescent thiolbimane derivatives (RSmB) [8·9]. The bimanes (such as the widely used 9,10-dioxa-*syn*-dimethylbimanes [10]) are among the smallest sulfhydryl-reactive fluorophores known and allow the thiol moiety to dominate the physical chemical properties of the conjugate and its chromatographic behavior. Another advantage of the bimane label is that it is highly conjugated and has a simple proton nuclear magnetic resonance spectrum, which is beneficial in solving novel biological thiol structures [11–14]. Application of the bimane assay to a variety of bacteria revealed novel thiols, but most were present at low levels relative to GSH or CoA. An exception was the *Actinomycetes* where a major novel thiol, U17, was identified in *Streptomyces clavuligerus* and in other actinobacteria [15]. The structure of U17 was solved by three laboratories and renamed mycothiol (Figure 1, [11·12·16]). Mycothiol would later guide the determination of the structure and discovery of the biosynthetic pathway for bacillithiol.

Although the Firmicutes were recognized to lack glutathione [7], the biologically relevant low-molecular-weight thiol in these bacteria remained unclear for several years because the bimane derivative of bacillithiol (BSmB) coeluted with the cysteine bimane derivative (CySmB) [17] in the sodium acetate buffer that was used in the HPLC separation method #1 [9]. The addition of an online mass detector in parallel with the HPLC proved extremely productive because bimane-thiol derivatives ionize easily in electrospray mass spectrometry. During a systematic thiol survey of *B. anthracis* in 2006, an unknown thiol was found to coelute with CySmB on the HPLC analysis and had a different mass. After the pH of the buffer was adjusted from 3.4 to 4.0, this novel thiol was fully resolved from CySmB. In retrospect, this was due to the ionization of the malate carboxylates of bacillithiol at the higher pH. U12 had a mass of 398, which did not match any of the biological thiols identified in previous surveys [18]. Subsequently, it was reported that treatment of *Bacillus subtilis* with cumene hydroperoxide led to the formation of mixed disulfides between the

organic hydroperoxide regulator OhrR and either cysteine, CoA or an unknown thiol of the same mass as U12 [19]. U12 was reported soon afterward in *Deinococcus radiodurans* [14]. Thus, the common occurrence of the novel thiol justified solving the structure.

The structure of U12-bimane from *D. radiodurans* was determined from a 2D NMR and analysis of acid hydrolysis products, the same strategy used to solve the bimane derivative of mycothiol [12]. U12 was found to be the α -anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid (Figure 1) and was named BSH because of its widespread occurrence within the Bacilli [14]. Bacillithiol resembles mycothiol in that it has the same central cysteine moiety amide bonded to glucosamine, but it has L-malate in an α -glycosidic bond to glucosamine in place of the inositol moiety in mycothiol, and the cysteine of MSH is *N*-acetylated (Figure 1). Bacillithiol was found in seven species of *Bacillus*, in *Geobacillus* and in some but not all species of *Staphylococcus* and *Streptococcus* [14]. A survey of Firmicutes growing exponentially in trypticase soy broth with shaking at 37°C, including *B. subtilis* and *S. aureus*, revealed BSH levels at 0.2–0.7 $\mu\text{mol/g}$ dry weight [0.1–0.2 mM], some 10-fold lower than the concentration of GSH in Gram-negative bacteria or of MSH in Actinomycetes, but similar to the levels of Cys and CoA in these organisms. Thus, the Firmicutes appear to have three major low-molecular-weight thiols (cysteine, coenzyme A and bacillithiol) that are maintained at similar, low levels during exponential growth.

Biophysical properties of bacillithiol

The solution structure of BSH is dominated by its zwitterionic character at physiological pH [20], which also contributes to its biological activities. The redox potential and the microscopic and macroscopic pK_a s for each ionizable group were determined by a combination of spectroscopic techniques. The redox potential for BSH is –221 mV, which is similar to that of most biological monothiols including Cys (–223 mV), CoA (–234 mV) and GSH (–240 mV). In contrast, all of the pK_a values for BSH are lower than the corresponding values for Cys or GSH. The first two pK_a values of BSH (3.1 and 4.4) correspond to those of the malate moiety, which are lower than the respective values for malic acid alone (3.4 and 5.1). This indicates that the malate moiety in BSH is a dianion at physiological pH (Figure 2). The most unusual aspect of BSH ionization is the microscopic amine pK_n of 7.63, which is just ~0.3 pH units below that of the thiol pK_s of 7.97. Hence when BSH is at the physiological pH of 7.7, there will be two major forms of BSH dianion, the $- + - -$ and the $00 - -$ forms ([20], Figure 2). The amount of BSH in the reactive thiolate form (RS^- , 21.9%) is greater than that of Cys (14.6%) and much greater than that of CoA, the two other major thiols found in the *B. subtilis* and in *S. aureus*. Thus, the major nucleophilic thiol available for reaction with electrophiles or disulfides in these organisms is BSH.

Many of the unique biophysical properties of BSH may be clarified by the solution structure of this thiol. A 3D structure for a major physiologically relevant form of BSH shows a close proximity of the carboxylate of malate to the amine of cysteine, which may contribute to the deprotonation of the amine and the low amine pK_a (Figure 3). When the major $0+ - -$ ionized form of bacillithiol (Figure 2) was analyzed in ChemDraw 3D, the energy minimized structure rapidly converged to that shown in (Figure 3) The proximity of the

ammonium, carboxylate and thiol groups in BSH have previously been postulated to contribute to metal chelation [14]. Importantly, in *B. subtilis* bacillithiol has recently been shown to chelate Zn^{2+} and to act as a Zn^{2+} buffer for metal homeostasis [21]. *B. subtilis* wildtype and a bacillithiol (*bshC*) mutant did not show a Zn^{2+} or Cd^{2+} stress phenotype in the wildtype background; however a double metal exporter mutant, *cadA czcD*, did show increased sensitivity to Zn^{2+} and Cd^{2+} in the absence of BSH [21]. Bacillithiol-dependent Zn^{2+} sensitivity has not been reported for *S. aureus*, but BSH mutants of *S. aureus* USA300 were reported to be sensitive to Cd^{2+} [22]. Gene expression studies revealed that metal-sensing regulators are more responsive to zinc perturbation in a mutant lacking bacillithiol, presumably because BSH buffers the zinc labile pool. This suggests that bacillithiol plays a role in zinc homeostasis by holding free zinc in a cytoplasmic pool, perhaps keeping the metal available for the estimated 5–10% cellular enzymes that require a Zn^{2+} cofactor [23]. It has been noted that during *S. aureus* infection, neutrophil proteins sequester and limit Zn^{2+} availability to the pathogen, reducing *S. aureus* growth and making it more vulnerable to oxidative stress [21,24].

The dianion character of BSH drives the substrate specificity of the BSH biosynthesis enzymes BshB1 and BshB2 and of the fosfomycin detoxification enzyme FosB (see below) [25,26]. The catalytic efficiency (k_{cat}/K_m) of BshB1 and BshB2 was diminished by $\sim 10^4$ – 10^5 when GlcNAc-Mal was replaced with the *des*-malate analog GlcNAc. This specificity is likely determined by opposing arginines (Arg53 and Arg109) present in the active site near the malate moiety when GlcNAc-Mal is computationally docked in the crystal structure of *B. cereus* BshB (BC1534, [25]). Similarly, the catalytic efficiency of FosB was diminished by 10^3 when BSH (Cys-GlcN-Mal) was replaced with the *des*-malate analogs Cys-GlcN-O-methyl or Cys-GlcN-O-benzyl [26]. In the catalytic sites of the crystallized FosB proteins, Arg35 and Lys36 (*B. cereus* and *B. anthracis*), or Lys35 and Lys36 (*S. aureus*), are positioned in the active site ~ 10 Å apart in an orientation to bind the malate moiety of BSH [26]. It may be possible to find BSH-binding enzymes by identifying opposing positively charged residues with the appropriate geometry for malate binding near their active sites.

Bacillithiol biosynthesis enzymes

BshA

The structural similarity of MSH and BSH made identification of the first two BSH biosynthetic genes relatively straightforward since they encoded homologs of the previously identified MSH biosynthetic enzymes [27]. For example, the first step in mycothiol biosynthesis is the formation of the glycosidic linkage between 1-L-inositol-1-phosphate (Ins-P) and UDP-*N*-acetylglucosamine (UDP-GlcNAc), which is catalyzed by the glycosyltransferase (MshA) [28]. *B. subtilis* has five glycosyltransferases with low sequence homology to MshA, but the prime *bshA* candidate with $\sim 25\%$ identity over 40% of the sequence was *ypjH*, which is located immediately downstream from the *mshB* homolog *ypjG*. *ypjH* was shown to be essential for BSH biosynthesis and the purified YpjH protein catalyzed the production of *N*-acetylglucosamine-malate (GlcNAc-Mal). The BshA glycosyltransferase reaction was able to utilize UDP-GlcNAc and L-malate as substrates (Figure 4), but not D-malate. The *B. anthracis* homolog of *B. subtilis* BshA (BA_1558, 65%

identity) had been crystallized without ligands prior to the discovery of BSH and was observed to have an active site that was similar to MshA (PDB entry 2JJM, [29]). This structure aided in the solution of a ternary complex of *B. anthracis* BshA, UDP and L-malate (PDB entry 3MBO, [30]). Kinetic studies of the *B. anthracis* BshA [30] and the *S. aureus* BshA [31], which share ~56% sequence homology, indicated that the proteins perform similar catalytic functions. Allosteric inhibition of *B. subtilis* BshA by BSH (IC₅₀ ~ 0.7 mM) suggests that the enzyme is subject to feedback regulation to modulate the intracellular level of BSH by inhibiting the first biosynthetic enzyme in the pathway, as is the case for many pathways, including those involved in GSH and MSH biosynthesis [31]. Significantly, the heterologous expression of *bshA* in non-BSH-containing bacteria led to the production of significant quantities of GlcNAc-Mal, providing a source for this non-commercially available substrate for BshB.

BshB

The second bacillithiol biosynthesis gene *bshB* is analogous to the corresponding MSH biosynthesis gene *mshB*, which encodes a deacetylase. *bshB* (*ypjG*) is immediately upstream of the *bshA* gene in some BSH-producing organisms, such as *B. subtilis*, and its product was proposed to catalyze the deacetylation of GlcNAc-Mal. The BSH levels in a *B. subtilis* *bshB1* deletion strain were at about 40% of wildtype levels. A second unlinked homolog of *bshB1*, *bshB2* (*yojG*), was then discovered in the *B. subtilis* genome, and deletion of both *bshB1* and *bshB2* yielded a mutant that failed to produce bacillithiol [27]. Thus, the *B. subtilis* BshB1 and BshB2 enzymes have redundant functions, but BshB1 can provide all of the necessary deacetylase activity since the *bshB2* mutant has wildtype levels of bacillithiol [27]. The genome of *B. anthracis* appears to have one *bshB1* and two *bshB2* homologs. The *B. anthracis* *bshB1* gene (BA_1557) was cloned into *E. coli*, and the purified protein showed high deacetylation activity with GlcNAc-Mal [27:30]. Unlike *B. subtilis*, the genome sequence of *S. aureus* USA300 indicated that there was only one deacetylase gene closely related to *bshB2*, leading to the prediction that a mutation in this gene would produce no BSH. Analysis of a *S. aureus* USA300 strain containing a transposon insertion in *bshB* demonstrated that this is the case [22].

A *Bacillus cereus* BC1534 (BshB1) crystal structure, published before the discovery of BSH, revealed a metalloprotein with structural similarity to the metalloproteins LpxC and MshB from *M. tuberculosis* [32]. Although the native substrate of the BC1534 was not known, the protein was reported to deacetylate GlcNAc and chitobiose [33]. However, a detailed analysis of the substrate specificity of BshB1 and BshB2 type proteins from *B. cereus* and *B. anthracis* failed to confirm this activity [25], perhaps because the malate moiety of the native substrate GlcNAc-Mal is a major determinant for BshB substrate specificity. Deacetylation of GlcNAc by BshB proteins is found to be at least 10⁵ fold lower ($k_{\text{cat}}/K_{\text{m}}$) than that of the native substrate GlcNAc-Mal [25:30]. Interestingly, the BshB enzymes have a second function in addition to their deacetylation activity, as they can also act as a bacillithiol conjugate amidase (BCA) and cleave bacillithiol *S*-conjugates during detoxification reactions (Figure 4). This activity is described below.

BshC

The enzyme utilized in the third step in mycothiol biosynthesis is the L-Cys, GlcN-Ins ligase MshC. This enzyme was purified from crude extracts of *M. smegmatis* by following activity, and the protein was identified by aminoterminal sequencing [34]. The corresponding open reading frame (ORF) in the *M. tuberculosis* genome, ORF Rv2130c, had been designated *cysS2* and annotated as a second Cys tRNA synthase. However, neither the *B. subtilis* nor *S. aureus* genome encoded a MshC homolog or a second gene annotated as a putative Cys tRNA synthase, and no BshC activity was associated with the one Cys tRNA synthase in *S. aureus* [27]. Therefore, to identify candidate BshC proteins, EMBL STRING was queried with a BshA input sequence to identify genes that co-occur with *bshA* in the genomes of bacteria that produce bacillithiol. This phylogenetic profiling identified *ylla* as a *bshC* candidate in *B. subtilis*. The product of this gene is a member of DUF2317, but it has no recognizable Pfam domains. The null mutant lacked BSH and produced excess GlcN-Mal, which would be the expected substrate for BshC [27]. Thus, the *bshC* gene is essential for BSH production, and in both *B. subtilis* and *S. aureus* *bshC* mutants are completely devoid of BSH [22-27]. However, when the *Bacillus subtilis* *bshC* gene was cloned and expressed in *E. coli*, activity was not detected when purified BshC was assayed under conditions that had been used to assay MshC activity [27]. The lack of activity may have been due to the assay conditions or to misfolding of the 539 amino acid BshC protein. Recently, *B. subtilis* BshC was crystallized as a dimer with an overall architecture of the Rossmann fold active site similar to MshC and Cys t-RNA synthase except for the absence of the canonical adenylate and zinc-binding residues in MshC [35]. However, because BshC was enzymatically inactive, it is difficult to evaluate the biological significance of this structure. It remains possible that additional cofactors or protein partners are necessary for BshC enzymatic activity.

Bacillithiol as a redox buffer

Any thiol that serves as a redox buffer in aerobic organisms must have the ability to regenerate the reduced thiol after it is oxidized to the disulfide form. Glutathione disulfide reductase (Gor) was originally isolated from *E. coli* and was thought to be necessary for reduction of GSH during redox stress [36]. The first complete genome of *Mycobacterium tuberculosis* H37Rv [37] contained a gene that was annotated as glutathione disulfide reductase (*gor*). It seemed likely that the mycobacterial *gor* was instead a gene for a mycothiol disulfide reductase because *M. tuberculosis* contains millimolar levels of mycothiol but no glutathione. This was demonstrated when the *M. tuberculosis* gene product Mtr was found to reduce only disulfides of mycothiol and truncated versions containing at least cysteinyl-glucosamine, but not oxidized GSH (GSSG) or cystine [38].

Identifying a redox thiol in the Firmicutes was more difficult than in the Actinomycetes given the variety of low-molecular-weight thiols present at low levels in the cell and the absence of likely candidates for proteins involved in redox recycling of these molecules. CoA disulfide reductase (CoADR) was identified in *Staphylococcus aureus* and some Bacillaceae including *Bacillus anthracis* [39-41]. A paradox arose in the model organism *Bacillus subtilis* when a CoA disulfide reductase (CoADR) homolog was not identified in

the complete genome sequence. Additionally, no cystine disulfide reductase from Firmicutes had been reported, so the search began for a bacillithiol disulfide reductase in *Bacillus subtilis*. Although candidate proteins for the bacillithiol disulfide reductase have been proposed, no flavin-containing, NADH- or NADPH-utilizing reductase has been identified in either *B. subtilis* or *S. aureus* [14·22]. It is possible that the highly reduced state of bacillithiol [14·20] is maintained by a thioredoxin-thioredoxin reductase type system as found for glutathione in *gor* mutants of *E. coli* K12 [42].

Interestingly, a fluorescent reporter system has recently been used to image real-time changes in the redox status of MSH during *M. tuberculosis* infection of macrophages [43]. This technology could in principle be applied in *S. aureus* to evaluate the BSH redox status during various stages of growth and mammalian cell infection.

The role of bacillithiol in Firmicutes: phenotypes of biosynthesis defective mutants

The first BSH mutants were produced in the genetically tractable *B. subtilis* [27]. Deletion of *bshA*, or of both *bshB1* and *bshB2*, or of *bshC* gives rise to completely bacillithiol-deficient *B. subtilis*. Each of these deletion strains was readily constructed and appeared to grow normally, demonstrating that BSH is not essential for growth in nutrient-rich media. However, *B. subtilis* BSH mutants were reported to be sensitive to salt stress and to acid stress, and mildly sensitive to oxidants and electrophiles such as *N*-ethylmaleimide, iodoacetamide, monobromobimane, diamide and methylglyoxal [27].

Bacillithiol also participates in the detoxification of fosfomycin by serving as the cosubstrate for the FosB enzyme [22·26·27·44]. FosB is a fosfomycin resistance protein found in Firmicutes that was initially thought to catalyze the reaction of cysteine with the epoxide moiety of fosfomycin, a cell wall inhibitor [45]. However, after the discovery of bacillithiol, several biochemical studies found that bacillithiol is the preferred FosB thiol cosubstrate for fosfomycin detoxification, making FosB the first bacillithiol transferase to be described (see “Bacillithiol Transferases” section below) [26·46·47]. The importance of bacillithiol in fosfomycin detoxification was confirmed in *B. subtilis* [22·27·44] and *B. anthracis* Sterne [30] as mutants lacking bacillithiol were as sensitive to fosfomycin as the *fosB* mutant. This has also been observed in *S. aureus* [22·26] where mutants lacking *bshA* are 15- to 60-fold more sensitive to fosfomycin than their corresponding wildtype methicillin-sensitive and methicillin-resistant (MRSA) *S. aureus* strains [44].

One sequenced *S. aureus* strain, NCTC 8325 (www.phe-culturecollections.org.uk), harbors an 8 basepair frameshift mutation in the *bshC* gene [44·48·49]. This naturally occurring mutation is also found in lineages derived from this strain, such as RN4220 and SH1000, and was the first BSH mutant of *S. aureus* to be studied. NCTC 8325 was found to lack BSH and contains elevated levels of GlcNAc-L-Mal and GlcN-L-Mal, the biosynthetic precursors of BSH [48]. NCTC 8325 and other *S. aureus* bacillithiol mutants do not exhibit growth defects in nutrient-rich media, but are severely hindered when challenged with fosfomycin [22·26·27].

Staphylococcus aureus bshA mutants are also sensitized to exogenous H₂O₂ and diamide [44]. As expected, a *S. aureus* strain with the *bshC* frameshift (SH1000) treated with monochlorobimane or the antibiotic rifamycin S did not show *N*-acetylcysteinyl *S*-conjugates (mercapturic acid products) of the toxins in the medium. The production of mercapturic acid products (see Figure 4 and bacillithiol *S*-conjugate amidase below) was dependent on the presence of bacillithiol even though SH1000 cells contained normal levels of cysteine, demonstrating that cysteine does not directly react with target substrates. Interestingly, *S. aureus* SH1000 converted all of the monochlorobimane to dimethylbimane [48], indicating the presence of a source of electrons not normally utilized in the wildtype strain. Dimethylbimane was also observed during the labeling of spinach chloroplasts and was thought to be produced by electron transfer from photosystem II [50].

In a recent metabolomics study, a *S. aureus* NCTC 8325 derived natural *bshC* mutant strain (HG001) was treated with fosfomycin and UDP-GlcNAc, GlcNAc-Mal, GlcN-Mal levels were observed by LCMS [51]. Interestingly, fosfomycin treatment significantly increased levels of UDP-GlcNAc (3.4-fold) and GlcNAc-Mal (the product of BshA, 2.2-fold), but levels of GlcN-Mal (product of BshB) were similar to treatment by the other drugs tested (ciprofloxacin, erythromycin, vancomycin and ampicillin). UDP-GlcNAc is a substrate for the first enzyme in BSH biosynthesis BshA, and the fosfomycin targets MurA and MurZ, which UDP-N-acetylglucosamine enolpyruvyl transferase activity. Thus, it is possible that the inhibition of MurA and MurZ by fosfomycin can increase flux through the BSH biosynthesis pathway, leading to an increase in the thiol cofactor utilized in FosB-dependent resistance to fosfomycin. This could limit the utility of BshA as a drug target during fosfomycin treatment of *S. aureus* (see “Expert commentary & five-year view”).

Identification of bacillithiol-dependent & bacillithiol-related enzymes

Bacillithiol transferases

Thiol transferases are enzymes that catalyze the addition of a low-molecular-weight thiol to target substrates forming a new thioether bond. The native substrates for two mycothiol-dependent enzymes from the *S*-transferase like (STL) superfamily [52], formerly known as the DinB/YfiT-like superfamily, have been characterized from Actinomycetes.

Corynebacterium glutamicum was found to grow on aromatic hydrocarbons like gentisate as a sole carbon source in a mycothiol-dependent manner. The key step in the degradation of gentisate in *C. glutamicum* is the isomerization of malylpyruvate to fumarylpyruvate in which mycothiol serves as a cofactor for the STL enzyme Ncgl2918 [53]. In *Streptomyces lincolnensis* LmbE, a mycothiol conjugate amidase (MCA), and LmbV, a STL mycothiol transferase, have been shown to be involved in the biosynthesis of lincomycin A [54]. LmbV installs the sulfur moiety derived from mycothiol in a lincomycin A precursor. This is the first example of mycothiol, a MCA, and a STL mycothiol transferase being used in the biosynthesis rather than the detoxification of secondary metabolites.

The two bacillithiol transferases identified in the Firmicutes thus far are members of two distinct superfamilies. The first bacillithiol transferase to be identified in *S. aureus* is FosB (Figure 5), which confers resistance to the antibiotic fosfomycin. FosB is a member of the vicinal oxygen chelate (VOC) superfamily. VOCs are metalloenzymes that catalyze a diverse

set of reactions, but all coordinate a divalent metal cation through vicinal oxygen atoms of a substrate, intermediate or transition state in the reaction [55]. FosB was identified before the discovery of bacillithiol, and cysteine was believed to be the thiol cofactor of FosB [45]. Early studies with the *B. subtilis* FosB reported a K_m value for cysteine of 35 ± 3 mM, which is well above the calculated cysteine concentration for this organism (~ 230 μ M, [14]). Since the discovery of bacillithiol, the thiol cofactor of both the *B. subtilis* and *S. aureus* FosB enzymes was reevaluated and determined to be bacillithiol [26,46,47]. The *S. aureus* FosB K_m for bacillithiol was determined to be 4.2 ± 0.7 mM [26]. This value is also above the intracellular concentration of bacillithiol. However, due to the low thiol p*K*_a of bacillithiol (see “Biophysical Properties” above), there is more of the reactive thiolate form of bacillithiol present than that of cysteine in *S. aureus*. Genetic data also support these findings: *fosB* and bacillithiol null mutants show similar sensitivity to fosfomycin [22,27,44]. Thus, the thiol cofactor of FosB is bacillithiol.

The first non-FosB bacillithiol transferase identified was *B. subtilis* YfiT (*B. subtilis* BstA), a member of the STL superfamily [56]. The single predicted *S. aureus* Newman STL enzyme (ORF ID NWMN_2591) was identified in a Superfamily search (<http://supfam.org/SUPERFAMILY/>) using *B. subtilis* BstA as a query sequence against the *S. aureus* Newman genome. The *S. aureus* enzyme was confirmed to be a bacillithiol transferase on the basis of biochemical studies of cell free extracts [22] and the purified protein and was named *S. aureus* BstA [52]. Kinetic studies with *S. aureus* BstA demonstrated that the K_m for BSH is 16 ± 4 μ M, which is ~ 10 -fold lower than the intracellular concentration of bacillithiol in *S. aureus*, indicating that the enzyme is saturated with bacillithiol *in vivo* [52]. *S. aureus* BstA was found to catalyze the addition of bacillithiol to cerulenin [22] at a low rate *in vitro* [52]. This newly identified STL superfamily is a diverse protein family that contains $\sim 30,000$ proteins with structural similarity but very little sequence identity. Phylogenetic studies have demonstrated that *B. subtilis* BstA and *S. aureus* BstA are distantly related, but are more related to each other than they are to the non-STL bacillithiol transferase *S. aureus* FosB (Figure 6). The number of predicted STL transferases varies between species, with only one predicted structural homolog of YfiT in *S. aureus* Newman (BstA) and eight in *B. subtilis* [56].

Although additional substrates of *S. aureus* BstA have not been identified, the genomic context of *bstA* may give insight into its function (Figure 7). Five genes downstream and in the same orientation as *bstA* is a gene encoding an *N*-acetyltransferase, an enzyme that could *N*-acetylate the Cys-adduct product of the bacillithiol conjugate amidase, and three genes downstream is a gene encoding RarD, an efflux transporter that might export acetylated Cysadducts (mercapturic acids, Figure 4). Products of these types of enzymatic reactions have been previously observed in the detoxification of electrophiles by *S. aureus* Newman [48]. A 2-oxoglutarate/malate translocator is adjacent to the *bstA* gene, which is significant because L-malate is a BshA substrate. Upstream of *S. aureus* *bstA* is a gene encoding PadR, a component of the phenolic acid stress response in *B. subtilis* that functions as a negative regulator of *padC*, a gene encoding a phenolic acid decarboxylase that converts toxic phenolic acids to their vinyl phenol derivatives [57]. The ortholog of *padC*ⁱⁿ *Lactobacillus plantarum* *padA*, is transcribed divergently from its negative regulator *padR* [58], just as *S. aureus* *padR* is transcribed divergently from *bstA*. This organization of genes

can indicate a negative regulation relationship. In *Lactococcus lactis*, the *padR* analog *ImrR* encodes a negative regulator of *ImrCD*, which encodes an ATP-binding-cassette transporter, and is in the operon with these genes [59]. The gene upstream of *padR*, Newman_2589, encodes a hypothetical protein that shares structural motifs with efflux pumps. If PadR regulates expression of *bstA*, then the natural BstA substrate(s) might bind PadR and relieve transcriptional repression of *bstA* and could provide a method to identify the BstA natural substrate(s).

BshB has bacillithiol conjugate amidase activity in addition to GlcNAc-Mal deacetylase activity

The first mycothiol-dependent enzyme identified was mycothiol conjugate amidase (MCA) from *M. smegmatis* (MSMEG_5261) and *M. tuberculosis* (Rv1082) [60]. This enzyme cleaves a mycothiol *S*-adduct at the amide linkage between the cysteinyl and glucosamine moieties to generate a *N*-acetylcysteinyl *S*-adduct. The adduct is excreted and the residual glucosaminyl-inositol pseudo-disaccharide is retained in the cell and cycles back to MSH biosynthesis [60-61]. This is a commonly observed detoxification reaction among mycothiol-containing actinomycetes. Mycothiol conjugate amidase is a zinc-containing hydrolase and a close homolog of the mycothiol biosynthetic amidase, MshB [62]. These enzymes have both mycothiol conjugate amide hydrolase and GlcNAc-Ins deacetylase activities [63].

It appears there are specialized enzymes that control the degradation of the major low-molecular-weight thiols such as GSH, MSH and BSH, which can ultimately supply the cell with cysteine. The unique γ -glutamyl peptide bond in GSH (γ -Glu-Cys-Gly) prevents the unwanted breakdown of GSH by peptidases. For example, γ -glutamyltranspeptidase is required to hydrolyze the γ -glutamyl-cysteine peptide bond, which releases Cys-Gly, a substrate for carboxypeptidase. γ -glutamyltranspeptidase is found in eukaryotes and GSH-producing bacteria. The obligate human pathogen *M. tuberculosis*, which does not produce GSH, contains a glutathione ABC transport protein and γ -glutamyltranspeptidase that can supply Cys to *M. tuberculosis* from host GSH [64]. MSH-containing organisms possess MCA, which has been shown to hydrolyze the unusual Cys-GlcN amide bond of MSH to provide AcCys and ultimately Cys for protein synthesis [60-61-65]. It was predicted that the same holds in true in BSH-containing organisms: hydrolysis of the Cys-GlcN amide bond of BSH by bacillithiol conjugate amidase should release of Cys in the cell.

A similar pathway was observed in the BSH containing organisms *B. cereus* and *B. anthracis*, which encode two BshB enzymes (BshB1 and BshB2) that have dual GlcNAc-Mal deacetylase and bacillithiol *S*-conjugate amidase activities [25]. A sensitive assay that monitors hydrolysis of the fluorescent bimane derivative of bacillithiol (BSmB) to the corresponding derivative of cysteine (CySmB) was developed to measure bacillithiol conjugate amidase activity. Assays of purified *B. anthracis* BshB1 (BA_1557) showed low amidase activity, about 10^{-5} in comparison with the deacetylation rate with GlcNAc-Mal [25-30]. *B. anthracis* BshB2 (BA_3888) showed considerably more BSmB deacetylase activity, only 100-fold less than the GlcNAc-Mal deacetylase activity. Both *B. anthracis* enzymes show low amidase activity toward unmodified BSH that is about 10^{-3} – 10^{-4} of the

GlcNAc-Mal deacetylase rate. The authors indicated that this low level of BSH amide hydrolysis activity was unlikely to be sufficient to supply cysteine to the cell. However, the MCA activity in *M. smegmatis* was also about 10^3 below that of the MSmB amidase reaction and was shown to be responsible for MSH degradation [60-65]. It remains unclear if this low level of BCA activity with BSH is capable of supplying Cys in a rapidly growing organism such as *S. aureus*.

S. aureus contains only one BshB2-type enzyme and the *bshB* NARSA transposon mutant (SAUSA300_0552) was determined by thiol analysis to be a complete BSH knockout [22,52]. The same *bshB* transposon mutant also lacks 90% of the wildtype bacillithiol *S*-conjugate amidase activity, indicating that this protein is responsible for all of the BshB activity and the majority of bacillithiol conjugate amidase activity in *S. aureus* [52]. An inhibitor of *S. aureus* BshB in combination with fosfomycin might provide a novel cotherapy method against *S. aureus*. Such a method would eliminate both BSH synthesis and BCA detoxification and would prevent fosfomycin detoxification.

Glyoxylases GlxA & GlxB

Methylglyoxal is a toxic, electrophilic byproduct formed during many metabolic pathways such as glycolysis (e.g., the conversion of dihydroxyacetone phosphate (DHAP) to methylglyoxal) [66] and can also diffuse into the cell from the environment (Figure 8) [67]. Methylglyoxal detoxification in *E. coli* and can be separated into glutathione-dependent and -independent pathways. In the glutathione-dependent pathway, methylglyoxal reacts spontaneously with glutathione to form GS-hemithioacetal. The glyoxylase enzyme GlxI then converts GS-hemithioacetal to *S*-lactoyl-glutathione, which GlxII then converts to D-lactate and glutathione [68]. Production of *S*-lactoyl-glutathione activates the KefGB and KefKC potassium efflux pumps, leading to cytoplasmic acidification and protection from methylglyoxal presumably by antagonizing its reaction with cellular targets [69].

Recently bacillithiol-independent and dependent pathways for methylglyoxal detoxification in *B. subtilis* have been characterized [67]. Unlike *E. coli*, the primary resistance pathway in *B. subtilis* is thiol-independent. The BSH-dependent pathway is similar to glutathione-dependent methylglyoxal detoxification. Methylglyoxal reacts spontaneously with bacillithiol to form BS-hemithioacetal. The glyoxylase GlxA then converts BS-hemithioacetal to *S*-lactoyl-bacillithiol, which GlxB converts to D-lactate and bacillithiol. This pathway involves the acidification of the cytoplasm, which is both necessary and sufficient for bacillithiol-dependent methylglyoxal resistance. Acidification depends on both bacillithiol and a K^+ efflux pump KhtU, which is in the same family of transporters as the *E. coli* KefGB and KefKC, and the ancillary proteins KhtS and KhtT (collectively called KhtSTU). The authors propose that the *S*-lactoyl-BSH conjugate gates the KhtU efflux pump in the manner similar to the gating of the *E. coli* efflux pump by *S*-lactoyl-glutathione, although direct evidence is still lacking. Thus, bacillithiol is proposed to mediate methylglyoxal resistance through cytoplasmic acidification rather than methylglyoxal detoxification (Figure 8).

The closest *B. subtilis* KhtU analog in *S. aureus* Newman is NWMN_0880, a Na^+/H^+ transporter (23% sequence identity with 69% coverage) in the same CPA2 family as the *E.*

coli transporter. Interestingly, *glxA* shares 25–30% sequence identity with *S. aureus* FosB, depending on the subspecies. Structural and biochemical studies could elucidate whether these enzymes also bind bacillithiol.

Bacilliredoxins debacillithiolate proteins

Upon exposure of cells to oxidants such as cumene hydroperoxide or sodium hypochlorite (NaOCl), bacillithiol has been shown to form mixed disulfides with the cysteine residues of certain proteins. This process, termed *S*-bacillithiolation, protects protein cysteine residues from overoxidation and irreversible damage of the protein [19,70,71]. Transcriptomic and proteomic studies revealed that several proteins are bacillithiolated, including the redox-sensing regulator OhrR, the methionine synthase MetE and two proteins of unknown function YtxJ and YphP. Inactivation of OhrR results in the upregulation of *ohrA*, which encodes a peroxiredoxin that contributes to NaOCl resistance. Bacillithiol biosynthetic gene mutants show the same sensitivity to NaOCl as do *ohrA* knockouts, suggesting that bacillithiolation of OhrR is essential for *ohrA* expression [70]. *S*-bacillithiolation of MetE decreased the expression of methionine biosynthesis genes, resulting in transient methionine autotrophy [71], which also occurs during *S*-glutathionylation of *E. coli* MetE [72].

Debacillithiolation of protein mixed disulfides was hypothesized to be analogous to deglutathionylation, which is a process catalyzed by glutaredoxins. The first evidence that Firmicutes contained glutaredoxin-like proteins emerged from a bioinformatics search for the bacillithiol biosynthesis gene BshC. An EMBL STRING analysis remarkably identified four putative thiol-disulfide oxidoreductase proteins that had yet to be characterized, including YtxJ, a putative monothiol (TCPIS) active site glutaredoxin-like protein, and YpdA, a thioredoxin reductase-like protein and putative bacilliredoxin reductase. The other two proteins, YphP and YqiW, are paralogs of the DUF1094 family (53% identity) and contain a CxC motif that is typical of glutaredoxins [27]. Both proteins were recently demonstrated to have bacilliredoxin activity and were renamed BrxA (YphP) and BrxB (YqiW) [73]. These proteins were found to debacillithiolate active site cysteine residues of OhrR and MetE, two bacillithiolated redox-sensing proteins [19,70]. The missing part of the bacilliredoxin story is the disulfide reductase needed to recycle the bacillithiolated forms of BrxA and BrxB. One candidate for this enzyme is the thioredoxin reductase like protein YpdA found in early bioinformatics studies of BSH [27], which was observed to be bacillithiolated in its putative active site [70,71].

The *B. subtilis* BrxA and BrxB proteins share a high level of sequence identity with their *S. aureus* Newman homologs NWMN_1339 (57% with 97% sequence coverage) and NWMN_1420 (69% with 99% sequence coverage), respectively. Thus, bacilliredoxins are likely to participate in the oxidative stress response in *S. aureus*.

Response of Firmicutes to electrophilic & oxidative stress

Data on cellular responses to electrophiles and oxidants predates the discovery of bacillithiol, and reexamination of these transcriptomic and proteomic studies provides significant evidence for bacillithiol-dependent stress responses. Catabolism of phenolic acids like salicylic acid occurs during the decay of plant material in the soil and the response of

Bacillus subtilis to salicylic acid indicates a role for bacillithiol transferases in this process. One of the eight putative STL enzymes from *B. subtilis*, *yuaE*, is upregulated in response to salicylic acid along with *bshB2*, which encodes an enzyme with both BshB deacetylase and bacillithiol conjugate amidase activity (Tables 1 & 2, [25-74]). Exposure of *B. subtilis* to the fungal-related antimicrobial compounds methylhydroquinone and 6-brom-2-vinyl-chroman-4-on elicited a response similar to that of salicylic acid: two bacillithiol transferases (*yjoA*, *yuaE*), the *bshB2* gene *yjoG* and the bacillithiol biosynthesis gene *bshC* were upregulated (Tables 1 & 2, [75]). Bacilliredoxin (*brxB*) was also upregulated, indicating that excessive protein thiolation might be occurring.

Firmicutes are exposed to hydrogen peroxide during infection, where bacillithiol-dependent defenses function as an adjunct to catalase activity. In *Bacillus anthracis* exposed to H₂O₂, all three bacillithiol biosynthesis genes as well as *bshB2* and the bacilliredoxin reductase candidate *ypdA* are upregulated (Tables 1 & 2, [76]). Exposure of *S. aureus* MW2 to H₂O₂ resulted in a twofold downregulation of *bshA*, but upregulation of *bstA*, the glyoxylase genes *glxA* and *glxB*, and the two putative bacilliredoxins *brxA* and *brxB* (Tables 1 & 2).

Furthermore, mammalian peptidoglycan recognition proteins (PGRP) are a component of the host response and have been shown to kill *B. subtilis* by a combination of oxidative stress, thiol depletion and metal stress from release of Zn²⁺ and Cu²⁺ ions [77]. Treatment with one isoform of PGRP (PGLYRP4) increased expression of the bacillithiol transferase *yuaE* ~8 fold [77]. Diamide leads to disulfide stress by oxidizing cellular thiols to disulfides. *S. aureus* COL responds with upregulation of the bacillithiol biosynthesis genes *bshA* and *bshC* and downregulation of *bstA* [78]. The putative bacilliredoxin reductase gene *ypdA* is also upregulated, which is consistent with the observation that diamide increases protein thiolation [79]. Oxidizing agents also induce bacillithiol-related genes in *S. aureus*. Nitrite upregulates all three bacillithiol biosynthesis genes, *glxA* and *ypdA* in *S. aureus* N315 [80]. Nitric oxide leads to the upregulation of genes encoding the BSH detoxification proteins *bshB2* and *ypdA* [81]. Oxidizing agents produced in neutrophils, hypochlorous acid, azurophilic granule proteins and H₂O₂ downregulate *bshA* and *brxA* ~2-fold, but induce *bstA*, *glxA* and *brxB* ~2-fold [78].

From these studies, we see that electrophilic compounds tend to induce specific bacillithiol transferases in the Firmicutes, whereas the major bacillithiol conjugate amidase protein BshB2 is upregulated in response to both electrophiles and oxidants. The bacillithiol biosynthesis genes are most often upregulated in response to oxidants such as H₂O₂, diamide and nitrite. Thus, bacillithiol is likely to play a key role in responding to these toxic molecules for both *B. subtilis* in soil and *S. aureus* infections.

Spontaneous enzyme-independent reactions with bacillithiol

Thiols can react spontaneously with electrophilic substrates such as monobromobimane and methylglyoxal. Preliminary evidence indicates that bacillithiol participates in a wider range of spontaneous reactions than other major low-molecular-weight thiols, presumably because its thiol group is more acidic and thus more reactive at cytoplasmic pH (Figures 2 and 3). This property would help ensure rapid detoxification of certain harmful substances. The

uncatalyzed reaction of H₂O₂ with bacillithiol was the first such reaction to be described, and it was found to proceed at a rate of ~80 nmol min⁻¹ mg⁻¹, 40-fold higher than the rate with glutathione or mycothiol [56]. Bacillithiol has been found to react in an enzyme-independent manner with a number of substrates, such as the antibiotic rifamycin S and the thiol-reactive molecules etacrynic acid, 4-hydroxynonenal and sulforaphane [52]. The antibiotic cerulenin also reacts with bacillithiol spontaneously [52], but the *S. aureus* bacillithiol transferase BstA enhances this rate of reaction 2 fold [20:52:56]. These results suggest that bacillithiol may be more reactive than glutathione or mycothiol in uncatalyzed reactions with certain electrophiles.

Contribution of bacillithiol to pathogenesis

The model for bacillithiol-dependent detoxification

The models for GSH- and MSH-dependent detoxification pathways involve similar enzymatic components and both produce a mercapturic acid, an *N*-acetylated cysteine-toxin conjugate that is excreted from the organism and is usually non-toxic [60:61:82:83]. In *S. aureus*, bacillithiol conjugate amidase and *N*-acetyltransferase activity were both detected using cell-free extracts [48]. Studies with whole cells demonstrated that BSH was added to the toxins prior to the appearance of the cysteine-toxin conjugate, demonstrating that the cysteine-toxin conjugate was likely the product of cleavage of the bacillithiol-conjugate. The cysteine-toxin conjugate is then *N*-acetylated and excreted from the cells. In support of this interpretation, the levels of GlcN-L-malate increased with the appearance of the cysteine-toxin conjugate [48].

Bacillithiol protects *S. aureus* against mammalian cells involved in immune responses

Bacteria that have been engulfed by macrophages and neutrophils are exposed to a number of reactive oxygen and nitrogen species, and bacillithiol likely serves as a cellular redox buffer that can neutralize these highly damaging molecules. Indeed, the BSH-deficient *Staphylococcus aureus* strain NCTC 8325 showed diminished survival in murine macrophages in comparison to strains that produce BSH [49]. A similar deficiency was observed in whole blood with the BSH deficient *S. aureus* strain SH1000 as compared with the BSH containing methicillin-resistant *S. aureus* strain COL [44]. Taken together with the increased sensitivity of bacillithiol-deficient mutants to H₂O₂ [44] and with the rapid rate of reaction of bacillithiol with H₂O₂ [56], the decreased survival of the bacillithiol mutants in macrophages might be explained by their increased susceptibility to oxidative stress.

Bacillithiol levels have been reported to be relatively low in exponentially growing *S. aureus* [14:26:44] compared with the glutathione levels in Gram-negative bacteria, but comparable to the bacillithiol levels in *B. subtilis*. Bacillithiol levels in *B. subtilis* continue to increase to ~5.2 mM during late stationary phase, but it is unclear whether the same occurs in *S. aureus*. Cysteine and bacillithiol levels did rise rapidly in *S. aureus* in response to treatment with rifamycin S, however [48].

Interestingly, *S. aureus* has been found to import glutathione from the growth medium during stationary phase [49]. Although *S. aureus* does not encode any known enzymes that

utilize this thiol as a cofactor, glutathione can react spontaneously with thiol-reactive molecules and may still serve as a protective redox buffer for the bacterium. Other Gram-positive bacteria such as *Streptococcus mutans* [84] and *Streptococcus pneumoniae* have also been reported to import glutathione, which conferred oxidative stress resistance and increased survival in the mouse model of infection [85]. Interestingly, the *S. aureus* genome encodes a protein (NWMN_0147) that shares 27% sequence identity with 91% coverage to the *M. tuberculosis* γ -glutamyltranspeptidase, which is required to hydrolyze the γ -glutamyl-cysteine peptide bond of GSH to release cysteine in glutathione producing organisms [86]. *M. tuberculosis* γ -glutamyltranspeptidase provides this intracellular pathogen the critical activity to utilize macrophage GSH as a source of cysteine for protein synthesis during infection [64]. The *S. aureus* γ -glutamyltranspeptidase analog has not been studied to the same extent as the *M. tuberculosis* γ -glutamyltranspeptidase, and it is unclear whether this enzyme is expressed during uptake of GSH. It remains an open question whether glutathione is imported by *S. aureus* during eukaryotic infection and whether it contributes to the fitness of the pathogen.

Expert commentary & five-year view

The rapid rate of the development of resistance of methicillin-resistant *S. aureus* to the current therapeutic options is a great health concern, and many research efforts are focused on identifying new antibiotics and new antibiotic targets to combat this organism [87-88]. The cell wall synthesis inhibitor fosfomycin is currently used in the treatment of urinary tract and gastrointestinal *S. aureus* infections. Fosfomycin can enter the cell via the L- α -glycerophosphate transport system and/or the hexose-monophosphate transport system (UhpT) [89]. Mutations in these transporters contribute to fosfomycin resistance and are presumably a major mechanism of fosfomycin resistance [90]. The plasmid borne *fosB* gene has been identified in 34% of fosfomycin-resistant clinical *Staphylococci* isolates [91]. Recently, deletion of the chromosomally encoded *fosB*^{OR} *bshA* in clinically relevant MRSA isolates was determined to be the major source of inherent fosfomycin susceptibility [44]. The use of fosfomycin as a monotherapy has resulted in rapid development of resistance [92]. The FDA recently recommended that one antimicrobial therapy that should be considered for streamlined development are those incorporating an agent that inhibits drug resistance. We predict that the bacillithiol biosynthetic enzymes would be good targets for neutralizing resistance, because mutants lacking bacillithiol show increased sensitivity both to oxidative stress and to drugs such as fosfomycin that are detoxified by a FosB/bacillithiol-dependent process [22:27:44:49]. Thus, inhibition of BSH biosynthesis should sensitize *S. aureus* to the innate immune response and to antibiotics such as fosfomycin.

The glycosyltransferase BshA may be a problematic drug target when used in combination therapy with fosfomycin due to increases in UDP-GlcNAc levels. Treatment of *S. aureus* with fosfomycin, which targets peptidoglycan biosynthesis by inhibiting MurA and MurZ, has been shown to increase levels of UDP-GlcNAc, the substrate of BshA [51]. This accumulation of a BshA substrate could hinder the ability of a potential competitive inhibitor to effectively block BshA activity and decrease bacillithiol levels. Thus, bacillithiol would still be synthesized at some level and would confer resistance to fosfomycin. BshC,

the final enzyme in the BSH biosynthesis pathway, has yet to be characterized, so significant development would be required before a BshC inhibitor screen could be devised.

S. aureus BshB may be a good candidate drug target, as this enzyme is involved in bacillithiol biosynthesis and also provides most of the bacillithiol conjugate amidase detoxification activity [22,25,52]. Unlike the *Bacillus* species discussed in this review, there are no compensating activities for *S. aureus* BshB, so the appropriate drug could completely inhibit BSH biosynthesis. *S. aureus* BshB is ~25% identical with *M. tuberculosis* MshB (Rv1170) and MCA (Rv1082), and is easily expressed in *E. coli*. A screen for natural product inhibitors yielded μM inhibitors of *M. tuberculosis* MCA and MshB (Figure 9, [93–95]), and these would constitute a logical starting point for *S. aureus* BshB2 inhibition studies. Inhibition of *S. aureus* BshB2 activity would diminish the production of bacillithiol and thereby render the organism more susceptible to being killed by the host defenses such as the oxidizing environment of macrophages, neutrophils and blood [44,49]. Depletion of bacillithiol would also diminish the BCA detoxification activity of BshB2 and inhibit excretion of mercapturic acids. A decrease in bacillithiol levels could result in a 15- 60-fold decrease in resistance to fosfomycin [44], suggesting a combination therapy of fosfomycin and a BshB/BCA inhibitor could be effective against multidrug resistant *S. aureus* infections. Identifying an inhibitor of this enzyme should be feasible within the next 5 years, especially with the numerous inhibitor chemotypes identified for the homologous *M. tuberculosis* MCA (Figure 9).

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Key issues

- Bacillithiol is a recently discovered low-molecular-weight thiol found in the Firmicutes, which include the pathogens *Staphylococcus aureus* and *Bacillus anthracis*.
- The low-molecular-weight thiols bacillithiol and mycothiol are related by their Cys-glucosamine core moiety.
- In *S. aureus*, three unlinked genes encode the bacillithiol biosynthesis enzymes, which are each essential for bacillithiol biosynthesis.
- The BshB enzyme has dual activities: it is a deacetylase utilized in the second step of bacillithiol biosynthesis and a bacillithiol conjugate amidase that can cleave *S*-conjugates.
- Compared to other low-molecular-weight thiols found in bacteria, bacillithiol has structural differences that make it more reactive with hydrogen peroxide, xenobiotics and drugs, particularly fosfomicin.
- Bacillithiol is the thiol cofactor of the enzyme FosB. Collectively, bacillithiol and FosB-derived fosfomicin resistance is the major detoxification mechanism of multi-drug resistant *Staphylococcus aureus* to fosfomicin.
- *S. aureus* mutants deficient in bacillithiol are more susceptible to killing by macrophages, neutrophils and whole blood.
- A possible new strategy for treatment of multidrug resistant *Staphylococcus aureus* is the combination therapy of a bacillithiol biosynthesis inhibitor with fosfomicin.

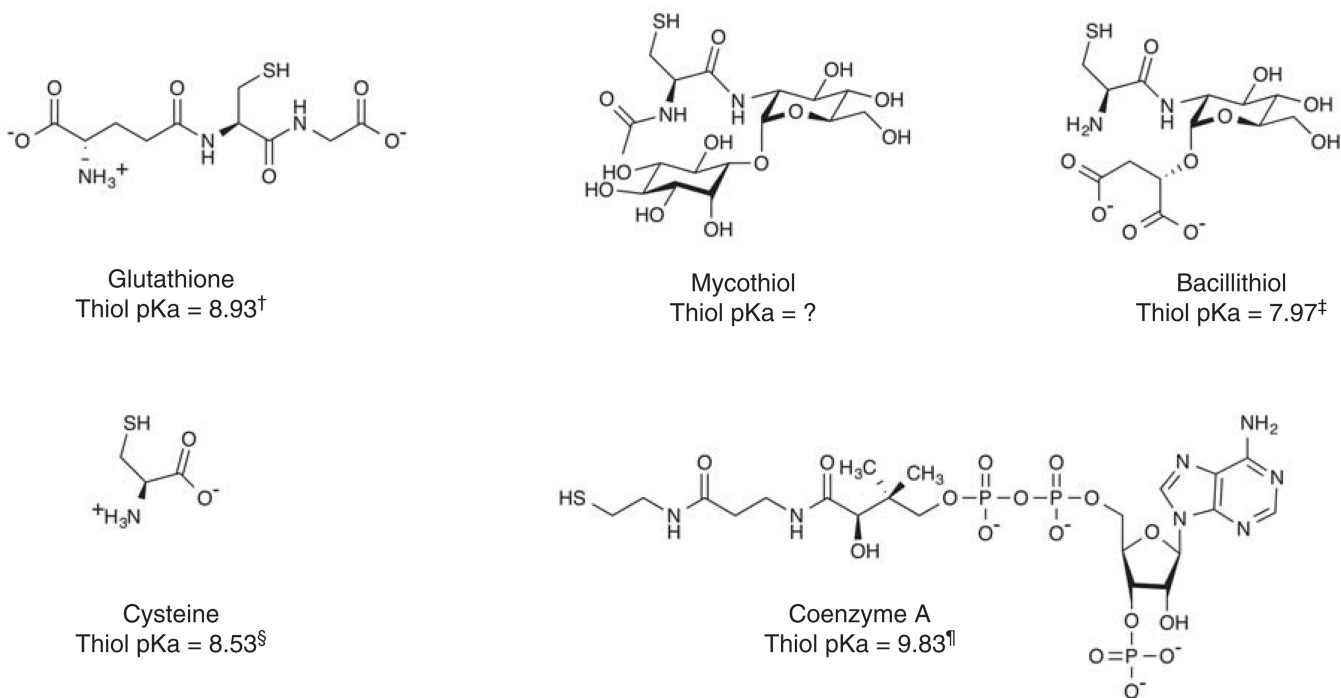
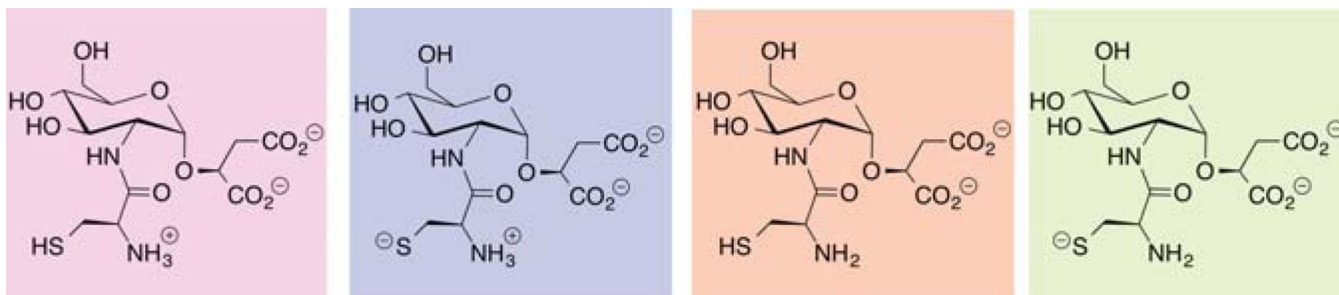


Figure 1. Structures and pKa values of the major low-molecular-weight thiols found in bacteria
 Thiols shown as the major ionized form at pH = 7.7 and represent microscopic thiol pKa values.

[†]Rabenstein 1973 [5]; [‡]Sharma *et al.* 2013 [20]; [§]Benesch and Benesch 1955 [4]; [¶]Keire *et al.* 1992 [3].



Thiol	Ionizable group				Net charge	% Total	Total RS ⁻
	SH	NH2	COOH-1	COOH-2			
Bacillithiol	0	+	-	-	-1	36.5	21.9%
	-	+	-	-	-2	20.9	
	0	0	-	-	-2	41.6	
	-	0	-	-	-3	1.0	
Cysteine	0	+	-	-	0	79.1	14.6%
	-	+	-	-	-1	14.4	
	0	0	-	-	-1	6.3	
	-	0	-	-	-2	0.2	
CoA	-	-	-	-	-5	0.7%	0.7%
	0	-	-	-	-4	93.3%	

Figure 2. Comparison of the major forms of bacillithiol, cysteine and CoA at pH 7.7
 Proportions of each form are based on microscopic pKa values. The four major forms of bacillithiol are depicted. The ionizable phosphate groups of CoA are not listed.
 Figure from Sharma *et al.* 2013 [20].

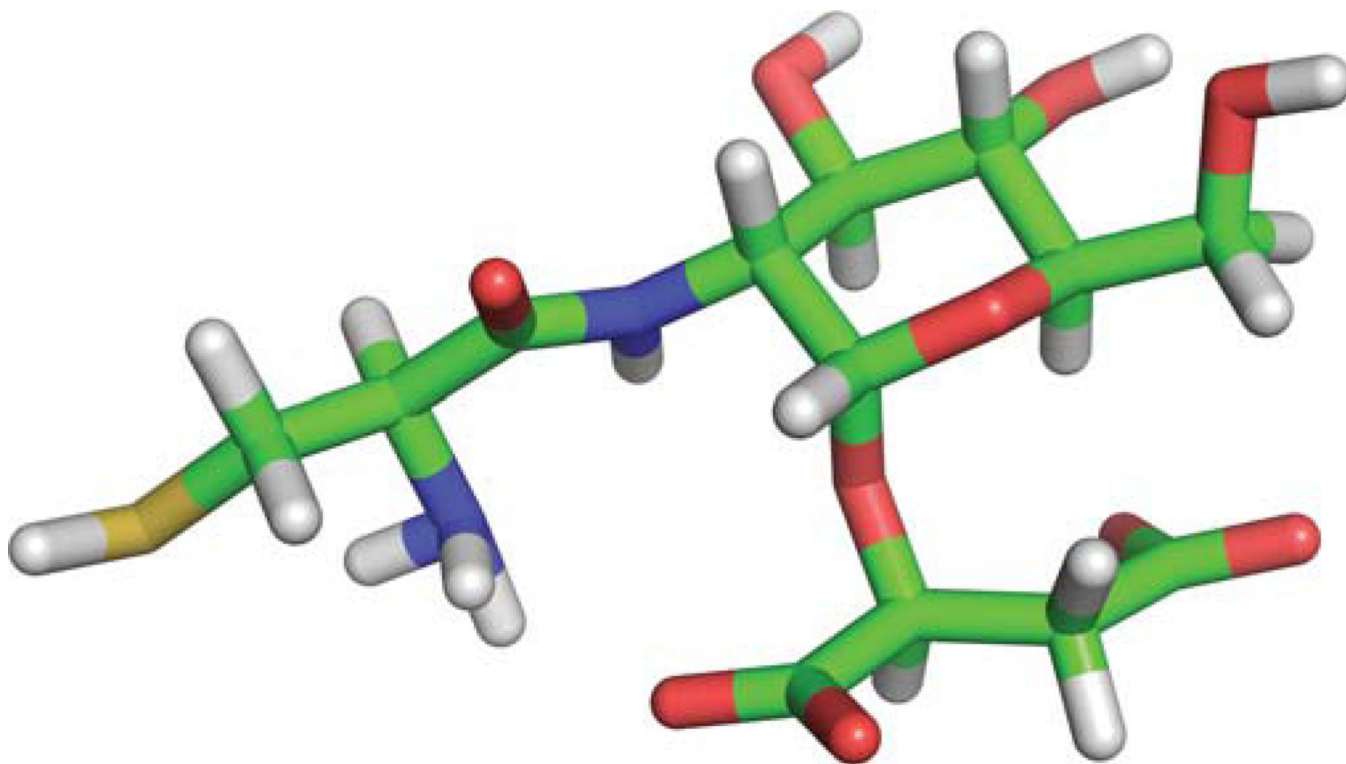


Figure 3. 3-dimensional structure of bacillithiol in the ionized 0 + - - form (Figure 2)
The proximal location of thiol, amine and carboxylate moieties in the energy-minimized structure may support divalent metal ion chelation. Atom labels: C, green; N, blue; O, red; H, white, and S, yellow.

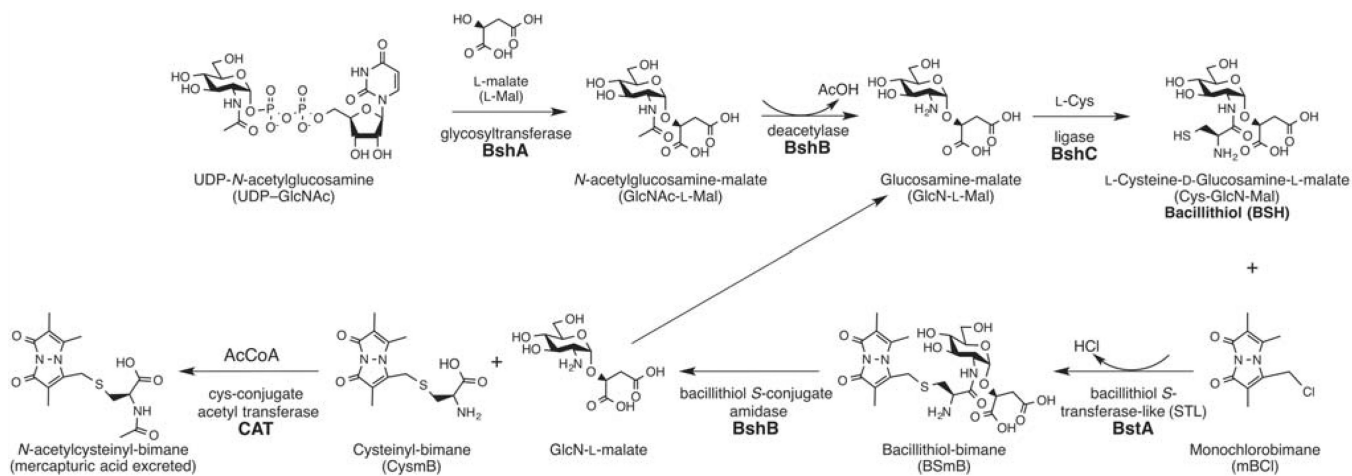


Figure 4. Bacillithiol biosynthesis (top) and bacillithiol-dependent detoxification (bottom) in *Staphylococcus aureus*

Data from Gaballa *et al.* 2010 [27], Newton *et al.* 2011 [56], Newton *et al.* 2012 [48], Fang *et al.* 2013 [25], and Perera *et al.* 2014 [52].

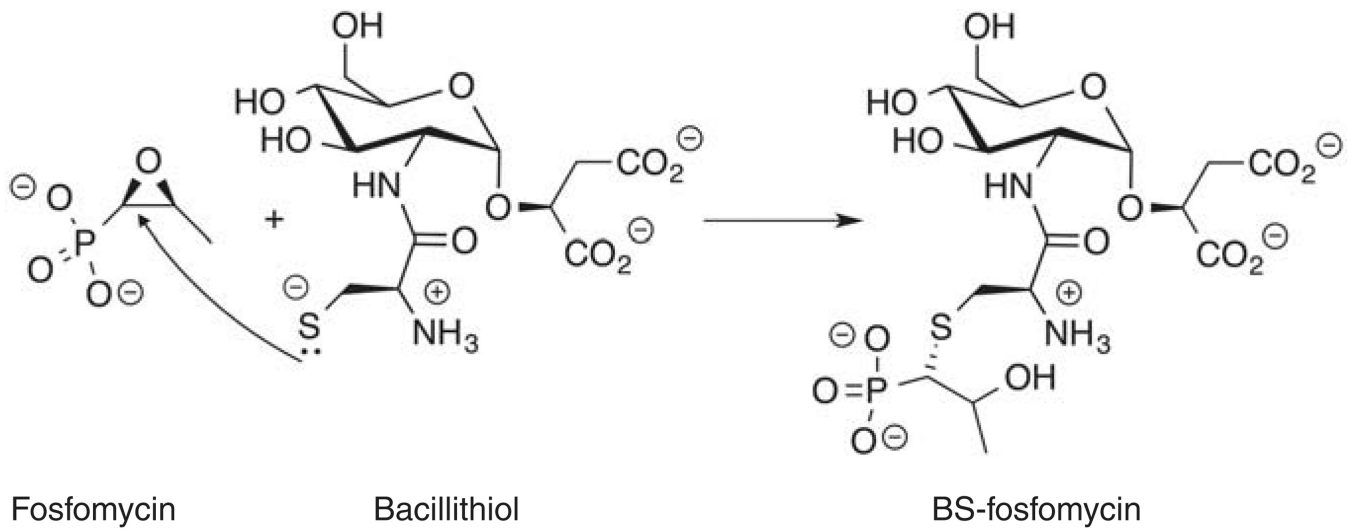


Figure 5. Bacillithiol-dependent detoxification of fosfomicin is catalyzed by *S. aureus* FosB
 Figure from Roberts *et al.* 2013 [26].

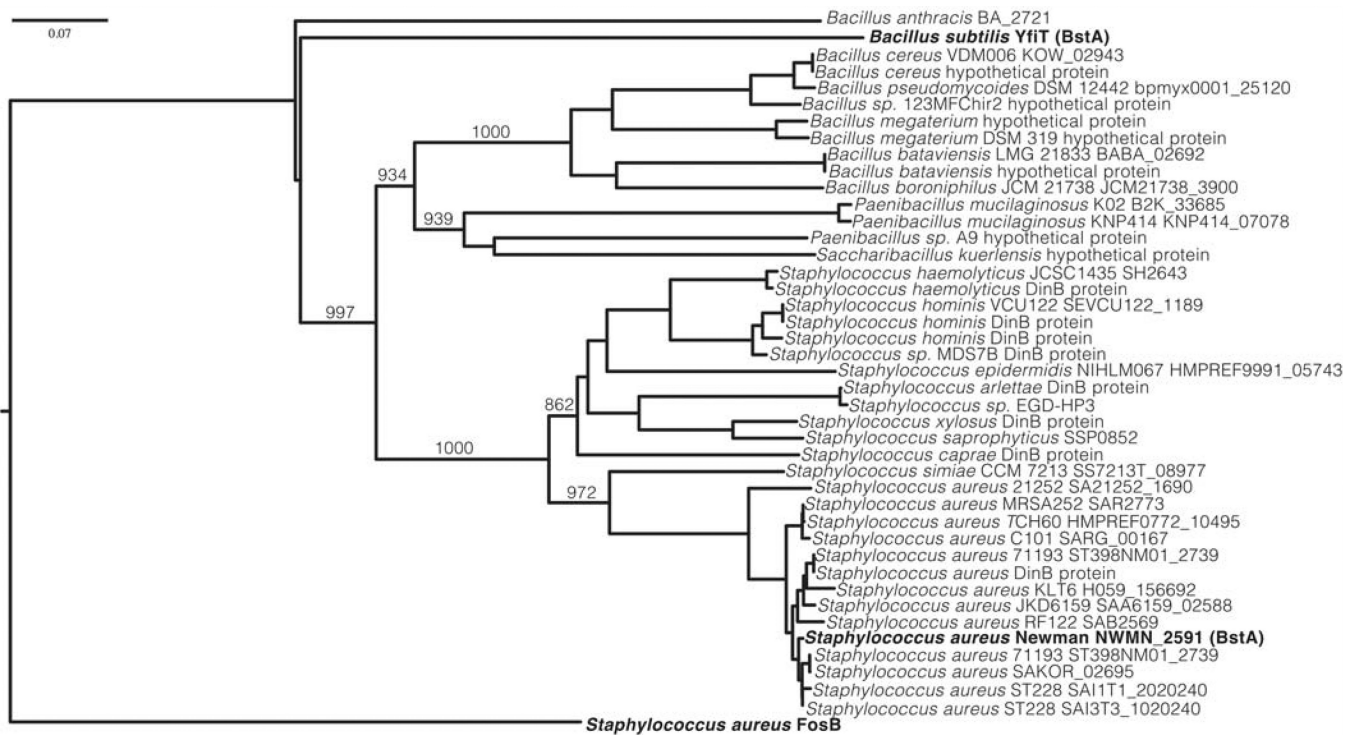


Figure 6. Close relatives of *S. aureus* Newman BstA in the S-transferase like (STL, formerly DinB/YfiT-like) superfamily
Staphylococcus aureus BstA is distantly related to *B. subtilis* YfiT (BstA) and a *B. anthracis* STL superfamily member (BA_2721), and unrelated to the bacillithiol transferase protein FosB. Bootstrap values are shown for the major branches.
 Figure from Perera *et al.* 2014 [52].

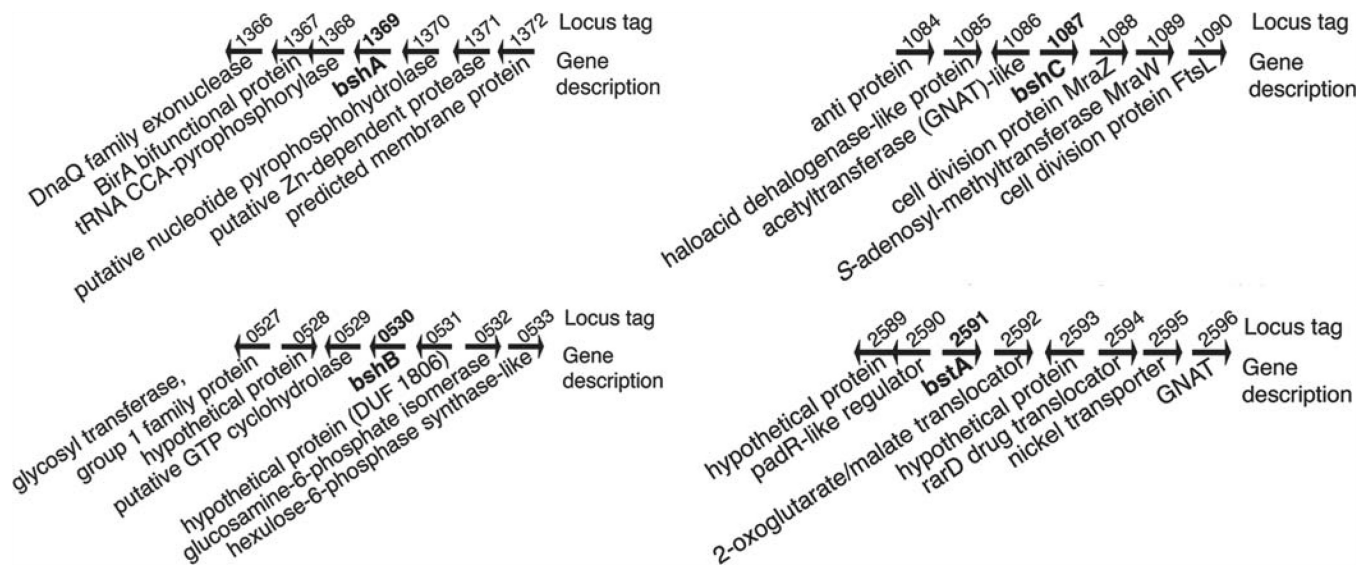


Figure 7. Genomic context of *S. aureus* *bshA*, *bshB*, *bshC* and *bstA* genes in *S. aureus* Newman
 The bacillithiol biosynthesis genes *bshA*, *bshB* and *bshC* and the *bstA* gene are unlinked (in bold). Overlapping arrows represent overlapping ORFs.

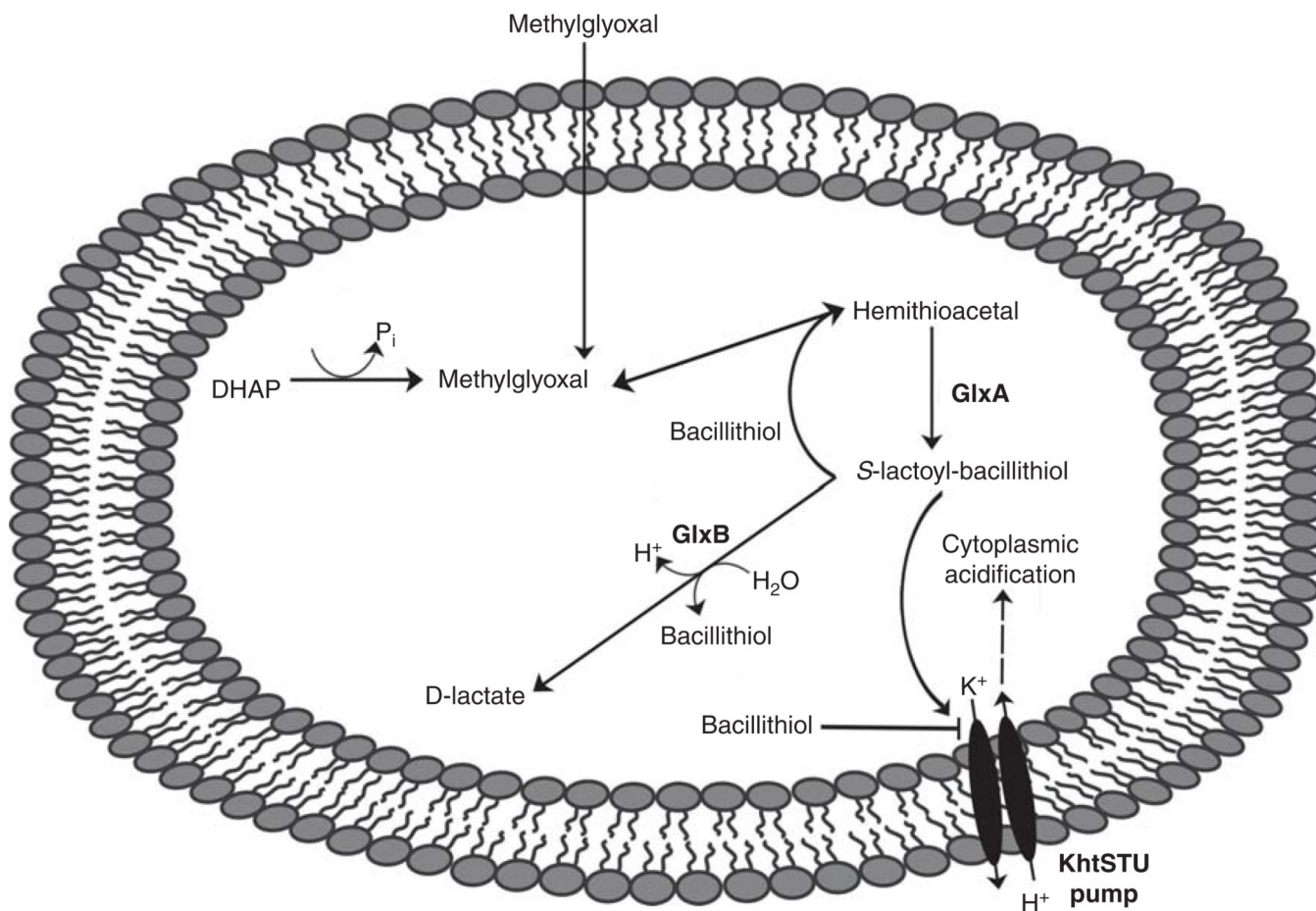
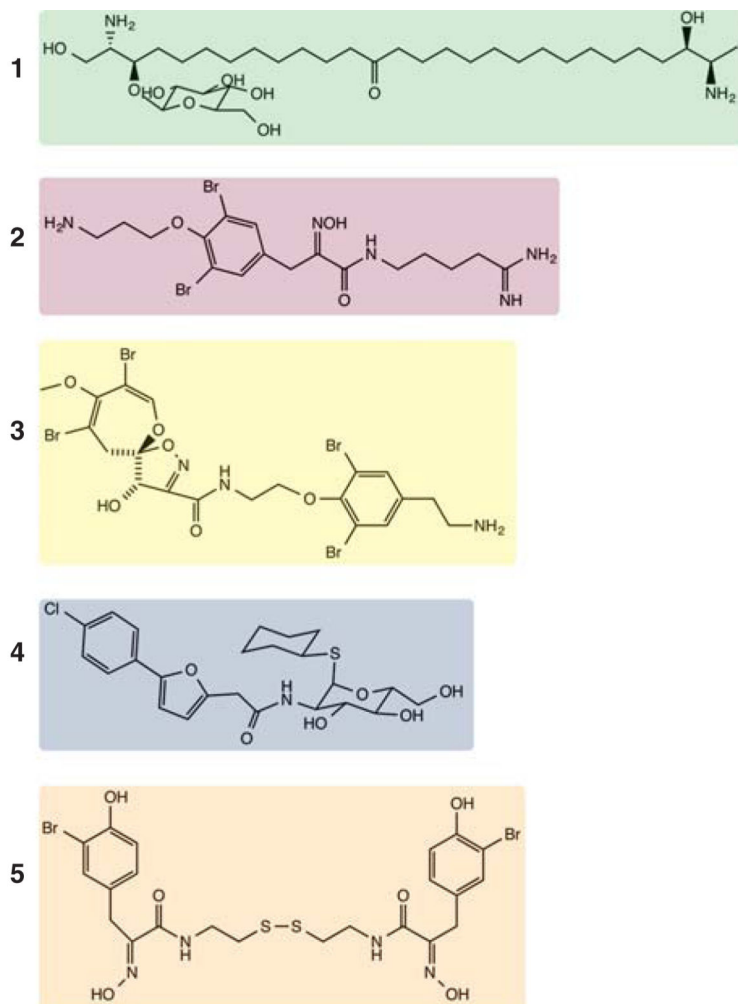


Figure 8. Bacillithiol-dependent methylglyoxal detoxification pathway in *Bacillus subtilis*
 Methylglyoxal can enter the cell via diffusion or can be formed intracellularly (i.e., from dihydroxyacetone phosphate, abbreviated DHAP). Bacillithiol and both glyoxalase A (GlxA) and glyoxylase B (GlxB) are utilized to convert methylglyoxal to D-lactate. Cytoplasmic acidification is mediated by the interplay of the KhtSTU pump with bacillithiol and *S*-lactoyl-bacillithiol.
 Model from Chandrangsou *et al.* 2013 [67].



Compound No.	Compound name	IC ₅₀ (μM) [†]	
		MshB	MCA
1	Oceanapiside [94]		10
2	Nicholas <i>et al.</i> , 8 [93]		2.8 ± 0.5
3	Psammplysin A [93]		20 ± 11
4	Metaferia <i>et al.</i> , 43 [95]	7 ± 1	33 ± 4
5	Psammaplin A [93]		2.8 ± 0.5

[†]*Mycobacterium tuberculosis* proteins MshB (Rv1170) and MCA (Rv1082)

Figure 9. Natural product and synthetic inhibitors of *M. tuberculosis* metalloproteins MshB and MCA

Oceanapiside (compound **1**) is a non-competitive inhibitor of *M. tuberculosis* MCA; a brominated derivative of tyrosine (compound **2**) and psammplysin A (compound **3**) are competitive inhibitors of MCA. Psammaplin A (compound **5**) and (compound **2**) contain oxime moieties, known to chelate metal ions. Compound **4** is a synthetic substrate analog of GlcNAc-Ins (substrate of MshB), and one of the few compounds that inhibits both *M. tuberculosis* MshB and MCA.

Table 1Bacillithiol-related genes in *S. aureus* and *Bacillus* strains.

Bacillithiol-related gene	<i>S. aureus</i> Newman	<i>S. aureus</i> USA300_ FPR3757	<i>B. anthracis</i> Ames	<i>B. subtilis</i> 168
<i>bshA</i>	NWMN_1369	SAUSA300_1349	BA_1558	BSU22460 (<i>ypjH</i>)
<i>bshB1</i>	–	–	BA_1557	BSU22470 (<i>ypjG</i>)
<i>bshB2</i>	NWMN_0530	SAUSA300_0552	BA_3524 BA_3888	BSU19460 (<i>yojG</i>)
<i>bshC</i>	NWMN_1087	SAUSA300_1071	BA_4058	BSU15120 (<i>yjIA</i>)
<i>brxA</i>	NWMN_1339	SAUSA300_1321	BA_2173	BSU21860 (<i>yphP</i>)
<i>brxB</i>	NWMN_1420	SAUSA300_1463	BA_4378	BSU23990 (<i>yqiW</i>)
Thioredoxin-reductase like enzyme	NWMN_1388	SAUSA300_1369	BA_1515	BSU22950 (<i>ypdA</i>)
Thioredoxin family enzyme	NWMN_0710	SAUSA300_0725	BA_4931	BSU29760 (<i>ytxJ</i>)
<i>glxA</i>	NWMN_2420	SAUSA300_2461	BA_3208	BSU38370 (<i>ywbC</i>)
<i>glxB</i>	NWMN_1416	SAUSA300_1458	BA_5452 BA_2111	BSU32660 (<i>yurT</i>)
<i>fosB</i>	NWMN_2234	SAUSA300_2280	BA_4109	BSU17840 (<i>yndN</i>)
S-transferase-like (STL) superfamily genes	NWMN_2591	SAUSA300_2626	BA_2700 BA_1354 BA_2379 BA_2379 BA_2937 BA_2558 BA_4768 BA_2721 BA_3104 BA_2777 BA_3538 BA_2007 BA_3539 BA_2065 BA_2078 BA_2990	BSU08390 (<i>yfiT/bstA</i>) BSU31030 (<i>yuaE</i>) BSU05630 (<i>dinB</i>) [†] BSU10860 (<i>yisT</i>) [†] BSU13070 (<i>ykkA</i>) BSU10800 (<i>yizA</i>) BSU26780 (<i>yrdA</i>) BSU12410 (<i>yjoA</i>)

B. subtilis 168 ORF names are in parenthesis. STL proteins listed on the same line share 30% amino acid identity with 90% sequence coverage. ORF names in grey share homology with more than one *B. subtilis* protein.

[†] *B. subtilis* genes that share >30% amino acid sequence identity with 90% sequence coverage.

B. anthracis: *Bacillus anthracis*; *B. subtilis*: *Bacillus subtilis*; *S. aureus*: *Staphylococcus aureus*; ORF: Open reading frame.

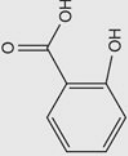
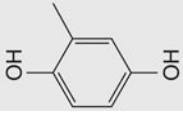
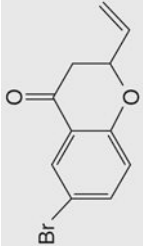
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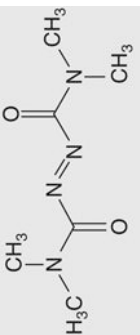
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Table 2 Transcriptomic and proteomic analyses of bacillithiol-related enzymes during treatment with stressors.

Compound	Structure	Organism	Gene	Transcriptome	Proteome
Salicylic acid		<i>Bacillus subtilis</i> 168 [†]	<i>yuaE</i> (STL) <i>yofG</i> (BshB2) <i>yhdN</i> (FosB)	6-8 3-4 3-4	5-6 - 3-4
2-methylhydroquinone		<i>Bacillus subtilis</i> 168 [†]	<i>yjoA</i> (STL) <i>yofG</i> (BshB2) <i>yuaE</i> (STL) <i>yqtW</i> (BrxB)	6 6-7 10-14 4-6	- - 4-10 -
6-brom-2-vinyl-chroman-4-on		<i>Bacillus subtilis</i> 168 [†]	<i>yjoA</i> (STL) <i>yofG</i> (BshB2) <i>yuaE</i> (STL) <i>yjIA</i> (BshC) <i>yqtW</i> (BrxB)	4-5 9-10 7-10 4 4-6	- - 3-8 - -
Hydrogen peroxide	H ₂ O ₂	<i>Staphylococcus aureus</i> MW2 [§]	MW1348 (BshA) MW2611 (BstA/STL) MW2442 ^{¶¶} (GlxA) MW1463 ^{¶¶} (GlxB) MW1467 ^{¶¶} (BrxB) BA_1558 (BshA) BA_1557 (BshB1) BA_3524 (BshB2) BA_4058 (BshC) BA_1515 ^{¶¶} (YpdA)	0.5 3 2 3 6 2 2 2 3 2	ND ND ND ND ND ND ND ND ND ND
		<i>Bacillus anthracis</i> Sterne [¶]			

Compound	Structure	Organism	Gene	Transcriptome	Proteome
Diamide		<i>Staphylococcus aureus</i> COL.#	SACOL1498 (BshA) SACOL1190 (BshC) SACOL2717 (BstA/STL) SACOL1553 ^{¶¶} (GlxB) SACOL1520 ^{¶¶} (YpdA)	2 5 0.3 2 4	ND ND ND ND ND
Nitric oxide	NO	<i>Bacillus subtilis</i> 168 ^{††}	<i>yuaE</i> (STL)	ND	2-3
		<i>Staphylococcus aureus</i> COL. ^{††}	SACOL0614 (BshB2)	ND	3
			SACOL1520 ^{¶¶} (YpdA)	ND	1-3
Nitrite	NO ₂ ⁻	<i>Staphylococcus aureus</i> N315 ^{††}	SA1291 (BshA) SA0525 (BshB2) SA1020 (BshC) SA2310 ^{¶¶} (GlxA) SA1311 ^{¶¶} (YpdA)	3 3 5 8 3	ND ND ND ND ND
Azurophilic granule proteins	-	<i>Staphylococcus aureus</i> MW2 [§]	MW1348 (BshA) MW0552 (BshB2) MW2611 (BstA/STL) MW1463 ^{¶¶} (GlxB) MW1318 ^{¶¶} (BrxA) MW1467 ^{¶¶} (BrxB)	0.6 0.5 2 0.7 0.6 2	ND ND ND ND ND ND
Hypochlorous acid	HOCl	<i>Staphylococcus aureus</i> MW2 [§]	MW1348 (BshA) MW2611 (BstA/STL) MW2442 ^{¶¶} (GlxA) MW1318 ^{¶¶} (BrxA) MW1467 ^{¶¶} (BrxB)	0.5 2 3 0.6 2	ND ND ND ND ND
Mammalian peptidoglycan recognition protein (PGLYRP4)	-	<i>Bacillus subtilis</i> 168 ^{§§}	<i>yuaE</i> (STL)	8	ND

Numbers < 1 show down-regulated genes, where 1 is no change.

- Van Duy *et al.* 2007; treatment with 4 mM salicylic acid for 10 min, range shown is from duplicate experiments.
- [‡]Van Duy *et al.* 2007; 100 µg/ml 6-brom-2-vinyl-chroman-4-on or 63 µg/ml of 2-methylhydroquinone (treatment for 10 min for both conditions), range shown is from duplicate experiments.
- [§]Palazzolo-Balance *et al.* 2008; treatment with 100 µg/ml azurophilic granule proteins, 5 mM H₂O₂, or 25 µM HOCl for 15 min for all conditions.
- [¶]Pohl *et al.* 2011; treatment with 1.0 mM H₂O₂ for 10 min.
- [#]Posada *et al.* 2014; treatment with 1 mM diamide for 20 min.
- ^{††}Hochgrafe *et al.* 2008; treatment with 500 µM MAHMA NONOate (nitric oxide donor) 10 min, range shown is from duplicate experiments.
- ^{‡‡}Schlag *et al.* 2007; treatment with 5 mM sodium nitrite for 6 h at 37°C.
- ^{§§}Kashyap *et al.* 2014; treatment with 100 µg/ml PGLYRP4 for 30 min at 37°C.
- ^{¶¶}Closest homolog to *B. subtilis* 168 gene.
- ND: Not determined.