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The DinB Superfamily Includes Novel Mycothiol, Bacillithiol, and Glutathione *S*-Transferases

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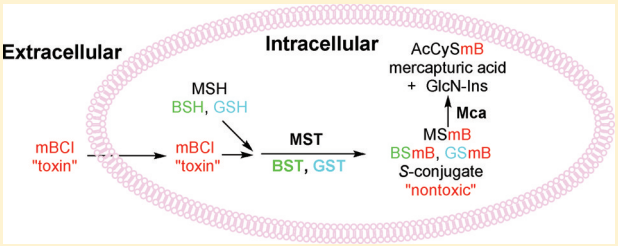
Supporting Information

ABSTRACT: The superfamily of glutathione *S*-transferases has been the subject of extensive study; however, Actinobacteria produce mycothiol (MSH) in place of glutathione, and no mycothiol *S*-transferase (MST) has been identified. Using mycothiol and monochlorobimane as substrates, an MST activity was detected in extracts of *Mycobacterium smegmatis* and purified sufficiently to allow identification of MSMEG_0887, a member the DUF664 family of the DinB superfamily, as the MST. The identity of the *M. smegmatis* and homologous *Mycobacterium tuberculosis* (Rv0443) enzymes was confirmed by cloning, and the expressed proteins were found to be active with MSH but not bacillithiol (BSH) or glutathione (GSH). *Bacillus subtilis* YfiT is another member of the DinB superfamily, but this bacterium produces BSH. The YfiT protein was shown to have *S*-transferase activity with monochlorobimane when assayed with BSH but not with MSH or GSH. *Enterococcus faecalis* EF_3021 shares some homology with MSMEG_0887, but *En. faecalis* produces GSH but not MSH or BSH. Cloned and expressed EF_0321 was active with monochlorobimane and GSH but not with MSH or BSH. MDMPI_2 is another member of the DinB superfamily and has been previously shown to have mycothiol-dependent maleylpyruvate isomerase activity. Three of the eight families of the DinB superfamily include proteins shown to catalyze thiol-dependent metabolic or detoxification activities. Because more than two-thirds of the sequences assigned to the DinB superfamily are members of these families, it seems likely that such activity is dominant in the DinB superfamily.



Most aerobic organisms generate a low molecular weight thiol that plays a role in protecting against oxidative stress and in the neutralization of electrophilic toxins. In eukaryotes, the thiol is glutathione [GSH (Figure 1)], and the glutathione *S*-transferases (GSTs) constitute a superfamily of enzymes that catalyze the reaction of GSH with a wide range of electron deficient substrates.[1−4] Chlorinated hydrocarbons make up one important class of GST substrates, and 1-chloro-2,4-dinitrobenzene (CDNB) is a convenient, but not universal, assay substrate.[5] GSTs also catalyze the reduction of organic hydroperoxides by GSH, the addition of GSH to enals, enones, and thiocarbamates, the conjugation of GSH with epoxides, and the GSH-dependent isomerization at carbon−carbon double bonds.[3] The GST superfamily includes at least 15 different enzyme classes,[3,6] and these enzymes are currently a common subject of research.

In prokaryotes, the situation is more complex. GSH is absent from many Gram-positive bacteria,[7] and those bacteria that possess GSH produce it in a variety of different ways. Some bacteria have similar independent genes encoding γ-glutamylcysteine ligase (GshA) and GSH synthetase (GshB) to produce γ-Glu-Cys-Gly (GSH), which occurs in most eukaryotes.[8] However, the genomes of halobacteria and some lactobacilli have only a *gshA* gene homologue, and the bacteria produce γ-Glu-Cys but no GSH.[9,10] In other bacteria, GSH is generated by a fused gene encoding both GshA and GshB activities. The fused gene and corresponding protein designated as γ-GCS-GS[11] were identified in *Streptococcus agalactiae*, and the protein designated GshF[12] was independently identified in *Listeria monocytogenes*. Phylogenetic analysis has suggested that the *gshA*[8] and *gshF*[11,12] genes have spread by horizontal gene transfer. Given the complexity of the GSH distribution and biosynthesis in bacteria, it follows that the biochemistry of bacterial GSTs is also predicted to be complex.

Bacterial GSTs have been less extensively studied but already have been shown to be involved in diverse chemical processes, including many involved in the metabolism of xenobiotic compounds.[13,14] The first bacterial GST was detected in *Escherichia coli* using the spectrophotometric assay with CDNB as a substrate.[15] Subsequently, bacterial GSTs associated with the beta, chi, theta, and zeta classes have been identified, and GSTs of the MAPEG (membrane-associated proteins involved in ecosanoid and glutathione metabolism) class have also been identified.[13] Most of these GSTs are found in proteobacteria and cyanobacteria, phyla generally found to produce GSH.[16−18] The rates for bacterial GSTs are substantially

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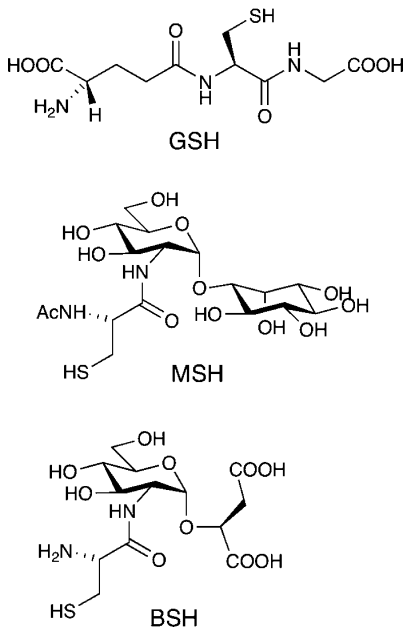

Figure 1. Structures for glutathione (GSH), mycothiol (MSH), and bacillithiol (BSH).

lower (5−200-fold) than those of mammalian liver GSTs.[19] Bacterial GSTs have been shown to catalyze a wide range of GSH-dependent activities; examples include detoxification of antibiotics such as fosfomycin,[20] double-bond isomerization of maleylpyruvate[21] and maleylacetoacetate,[22] reductive and hydrolytic dechlorination of organic chlorides,[23−25] and reduction of disulfides.[26]

In contrast with the dominance of GSH in Gram-negative bacteria, most Gram-positive bacteria do not produce GSH.[7,27] In Actinobacteria, the dominant thiol is mycothiol [MSH (Figure 1)],[27] and sufficient evidence indicates that MSH has functions in these bacteria similar to the role that GSH plays in Gram-negative bacteria.[28−30] However, no mycothiol *S*-transferases catalyzing MSH-dependent detoxification reactions have been described. Another bacterial thiol, bacillithiol [BSH (Figure 1)], has recently been found in Firmicutes and *Deinococcus radiodurans*.[31] Much less is known about the function of this newest bacterial thiol, but BSH was recently shown to be the preferred thiol substrate employed by FosB in the detoxification of fosfomycin, the first example of a bacillithiol *S*-transferase activity.[32]

In this study, we describe the purification of a mycothiol *S*-transferase (MST) from *Mycobacterium smegmatis* and its identification as MSMEG_0887. This enzyme is related by amino acid sequence to *Enterococcus faecalis* EF_3021 whose structure [Protein Data Bank (PDB) entry 3cex] places it in the DinB_2 family of the DinB superfamily of DNA damage-induced gene products.[33] The DinB_2 family also includes *Bacillus subtilis* YfiT (PDB entry 1rxq). Of the 15 proteins with structures related to that of *Geobacillus stearothermophilus* DinB (PDB entry 3gor),[33] the only one having an experimentally verified function is a mycothiol-dependent maleylpyruvate isomerase (MDMPI) from *Corynebacterium glutamicum*.[34,35] Here we show that *Mycobacterium tuberculosis* Rv0443, like its homologue, MSMEG_0887, is also a MST, YfiT is a bacillithiol *S*-transferase (BST), and EF_3021 is a novel DinB GST.

MATERIALS AND METHODS

Chemicals. Mycothiol[36] and bacillithiol[32] were produced as described previously. Azithromycin, cerulenin, dithiothreitol, glutathione, lincomycin, mitomycin C, *p*-nitrophenyl acetate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-α-*p*-tosyl-L-phenylalanylchloromethyl ketone (TPCK), and *N*-α-*p*-tosyl-L-lysinechloromethyl ketone (TLCK) were from Sigma. Antimycin A and streptozotocin were from Calbiochem; spectinomycin and methanesulfonic acid were from Fluka, and monochlorobimane was from Invitrogen. All other buffers and reagents were from Fisher except as noted.

Assay of MST Activity. An assay of MST activity during purification of MST was conducted by mixing 25 μL of sample with 25 μL of a mixture of 2 mM NADPH, 20 μM mycothiol disulfide, and 5.8 μg of mycothiol disulfide reductase in 20 mM HEPES buffer (pH 7.4) containing 100 mM NaCl. The mixture was incubated at 37 °C for 5 min to generate 20 μM MSH and reaction initiated by addition of 2.5 μL of 0.5 mM monochlorobimane in DMSO. After reaction for 30 min, a 20 μL sample was quenched by being mixed with 20 μL of 50 mM methanesulfonic acid in acetonitrile and heated at 60 °C for 10 min to precipitate protein. After the sample had been iced for 15 min, the precipitate was removed by centrifugation at 13000*g* for 3 min in a microcentrifuge. A 25 μL aliquot of the supernatant was mixed with 100 μL of 10 mM aqueous methanesulfonic acid and the mixture analyzed by high-performance liquid chromatography (HPLC). HPLC analysis was conducted using a 4.6 mm × 250 mm Beckman Ultrasphere C18 column with a linear gradient from 0% solvent A [0.25% aqueous acetic acid (pH 4.0)] to 100% solvent B (methanol) over 35 min. MST activity was calculated from the sum of the amount of MSmB (elution time of 23 min) and AcCySmB (elution time of 25 min) produced.

For an assay of the purified MST, the protocol was modified. A 75 μL aliquot containing 50 μM thiol, 100 μM DTT, and 0.1 M NaCl in 25 mM HEPES (pH 7.0) and 3.2 μL of enzyme stock or buffer (control) was prewarmed at 37 °C for 5 min. Reaction was initiated by addition of 2 μL of 2 mM mBCl in DMSO and incubation continued at 37 °C. Reaction mixtures were sampled three times at intervals up to a maximum of 30−40 min, and the 20 μL aliquot was mixed with 20 μL of 50 mM methanesulfonic acid in acetonitrile. After the mixture had been heated at 60 °C for 10 min, iced for 15 min, and centrifuged for 3 min at 13000*g* in a microcentrifuge, 25 μL of the supernatant was mixed with 100 μL of 10 mM methanesulfonic acid for HPLC analysis on a 4.6 mm × 250 mm Beckman Ultrasphere IP C18 column using a linear gradient from 0.25% aqueous acetic acid (pH 3.8) to 100% methanol over 35 min. Rates were calculated for each time point and were linearly extrapolated to time zero to compensate for the decline in enzyme activity with time because of substrate depletion and enzyme inactivation. Reported values represent the mean and standard deviation of triplicate experiments.

Purification of *M. smegmatis* MST (*Ms*MST). *M. smegmatis* mc^{2}155 was grown to late exponential phase in Middlebrook 7H9 medium containing 0.05% Tween 80 and 1% glucose and harvested by centrifugation. A 100 g wet weight cell sample was suspended in 600 mL of 50 mM HEPES (pH 7.4) containing 150 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 35 μM TPCK, and 35 μM TLCK. The suspension was sonicated on ice for 20 min with the temperature maintained below 16 °C and centrifuged at

10000 rpm and 12000*g* for 30 min at 10 °C in a Sorvall RC5 centrifuge. Ammonium sulfate was added to the supernatant (680 mL), and pellets were collected at 0–20%, 20–45%, 45–65%, and 65–80% saturation. Most of the activity was found in the 45–65% fraction that was taken up in 20 mL of 50 mM HEPES (pH 7.4) containing 2 mM $MgCl_2$, 2 mM DTT, 35 μM TPCK, and 35 μM TLCK. Bradford protein assay (Biorad) indicated a protein concentration of 5 mg/mL. After dialysis against 50 mM HEPES (pH 7.4) containing 35 μM TPCK, 35 μM TLCK, and 1 mM 2-mercaptoethanol, this fraction was loaded on a 55 mm × 190 mm Toso-Haas DEAE 650M column and eluted with 50 mM HEPES (pH 7.4) containing 35 μM TPCK, 35 μM TLCK, and 1 mM 2-mercaptoethanol at 1.5 mL/min with a linear gradient from 1 mM to 1 M NaCl. A total of 96 fractions (20 mL each) were collected with the MST activity eluting in 0.34–0.40 M NaCl. The fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.8) containing 100 mM NaCl, 5 mM $MgCl_2$, 35 μM TPCK, and 35 μM TLCK. The dialyzed sample was loaded onto a 30 mm × 330 mm hydroxylapatite (Bio-Rad) column and eluted with a linear gradient from 10 mM sodium phosphate with 100 mM NaCl to 200 mM sodium phosphate with 0 mM NaCl (pH 6.8) in the presence of 5 mM $MgCl_2$, 35 μM TPCK, and 35 μM TLCK at a flow rate of 1.5 mL/min. A total of 130 fractions (20 mL each) were collected, and activity eluted at 58–76 mM sodium phosphate. Ammonium sulfate was added to each active fraction to 65% saturation and the protein pelleted by centrifugation at 12000*g* for 30 min at 10 °C in a Sorvall RC5 centrifuge. Each pellet was solubilized in 1 mL of 50 mM HEPES (pH 7.0) containing 2 mM 2-mercaptoethanol, and adjacent samples were combined to generate five 2 mL samples. Each pooled sample was chromatographed on a 20 mm × 140 mm Sephacryl S200 gel filtration column eluted at 0.2 mL/min with 25 mM HEPES (pH 7.5) containing 100 mM NaCl and 1 mM 2-mercaptoethanol. Fractions (2.5 mL) were collected, and activity was detected from fractions 56–63, corresponding to a molecular mass of ~40 kDa based upon calibration with known standards. Active MST fractions were concentrated with a Centricon 10 kDa Ultra Filter to ~200 μL. Glycerol was added to a final concentration of 10%, and the samples were stored at −70 °C.

Identification, Cloning, and Expression of *Ms*MST. The peak of MST activity on the Sephacryl S200 column eluted in fraction 59 and produced two major protein bands at ~20 kDa on a 10 to 20% Tris-Tricine Criterion (Bio-Rad) SDS gel (Figure 2A, lane 7). The two major bands at ~20 kDa were identified by the University of California at San Diago Molecular Proteomics Laboratory using an in-gel digest with trypsin and sequencing by tandem high-resolution mass spectrometry as previously described.[37] The upper band was identified by 19 peptides (95% confidence) with 71% sequence coverage as MSMEG_1680, which is annotated as a conserved hypothetical protein, is found in a limited group of mycobacterial strains and was not pursued. The lower band was identified by 19 peptides (95% confidence) with 88% sequence coverage as MSMEG_0887, a member of the DinB superfamily found in most Gram-positive bacteria. The *M. smegmatis mst* (*MSMEG_0887*) gene was codon optimized for expression in *E. coli* and synthesized by GenScript. The synthetic gene was subcloned into pET28a using NdeI and XhoI cloning sites generating a thrombin cleavable N-terminal His_6-tagged protein. *E. coli* BL21(DE3) (Novagen) was transformed with the pET28a-*Msmst* expression vector. *Ms*MST was produced in Luria Broth containing kanamycin (30 μg/mL) and 1 mM IPTG at 37 °C for 2 h. The cells were collected by centrifugation at 4 °C. The cells were lysed by sonication, and the cell free extract was purified on a Zn-liganded PrepEase (USB) His_6-tagged protein purification kit according to the manufacturer's instructions. *Ms*MST was eluted in 100 mM imidizole and was concentrated on a Centricon 10 (10 kDa) ultrafilter. The buffer was exchanged on the ultrafilter with 25 mM HEPES (pH 7.4) containing 100 mM NaCl and 1 mM 2-mercaptoethanol; 10% glycerol was added prior to storage at −70 °C. *Ms*MST was >90% pure on the basis of 10 to 20% Tris-Tricine Criterion (Bio-Rad) SDS gel analysis (Figure 2B, lane 1). The protein concentration was estimated using the calculated A_{280} value of 1.78 for a concentration of 1 mg/mL (ProtParam tool at http://ca.expasy.org).

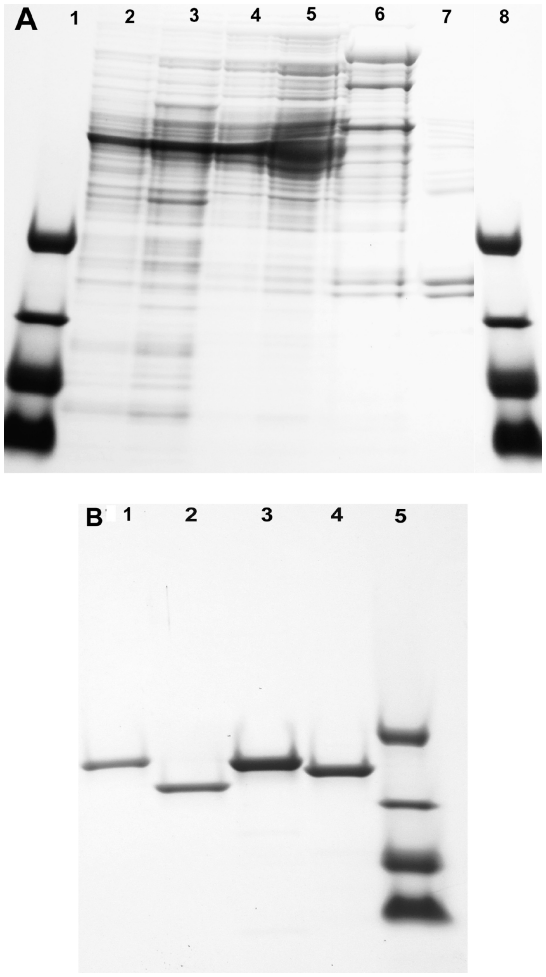

Figure 2. Gradient SDS–PAGE gels (10 to 20%) containing MST proteins. (A) MST purification: lanes 1 and 8, polypeptide standards containing triosephosphate isomerase (26.6 kDa), myoglobin (17 kDa), α-lactalbumin (14.4 kDa), and aprotinin (6.5 kDa); lane 2, crude extract of *M. smegmatis* mc^2155 protein; lane 3, 45–65% ammonium sulfate fraction; lane 4, DEAE peak fraction; lane 5, DEAE activity pool; lane 6, hydroxylapatite activity pool; lane 7, gel filtration peak fraction. (B) MST cloned proteins: lane 1, recombinant purified *Ms*MST; lane 2, recombinant purified *Mt*MST; lane 3, recombinant purified *B. subtilis Bs*BST (YfiT); lane 4, recombinant purified *En. faecalis Ef*GST; lane 5, polypeptide standards as in panel A.

Cloning and Expression of *M. tuberculosis mst* (*Rv0443*) and *En. faecalis gst* (EF_3021). The *Rv0443* gene was amplified from *M. tuberculosis* H37Rv genomic DNA using forward (CATATGATGGCAAGCACCGAC) and reverse (AAGCTTGGCTATCCCCCGCAGGTA) primers with the forward primer containing an NdeI restriction site and the reverse primer having a HindIII restriction site for cloning into pet22B. The amplified fragment was first cloned into TA cloning vector pcr2.1, and the resulting plasmid was digested with NdeI and HindIII. The insert was ligated to a similarly digested pet22B vector followed by transformation into *E. coli* BL21(DE3). The strain harboring the recombinant pet22B vector was induced for 4−6 h at 37 °C with 0.5 mM IPTG, and the culture was refrigerated overnight. The pelleted cells were sonicated and centrifuged; the supernatant was applied to a PrepEase Zn affinity column that was washed and eluted with 250 mM imidazole according to the manufacturer's instructions. The eluted protein was desalted on a Sephadex 25 column and stored at −70 °C in 25 mM HEPES (pH 7.4) containing 100 mM NaCl, 1 mM 2-mercaptoethanol, and 10% glycerol. The purified *Mt*MST produced a single band on SDS gel electrophoresis (Figure 2B, lane 2). The *En. faecalis gst* gene was amplified from *En. faecalis* ATCC 19433 genomic DNA using the forward primer CATATGATGAAAGTCACTCAA and the reverse primer AAGCTTTTTTCCAATGACTAATCT, expressed, and purified according to the protocol described for *Mt*MST. SDS gel electrophoresis of the purified protein produced a single band (Figure 2B, lane 4). The protein concentration was based upon an A_{280} value of 1.93 mg^{-1} mL^{-1} calculated for both the *M. tuberculosis* and the *En. faecalis* enzymes (http://ca.expasy.org).

Bacillithiol *S*-Transferase (BST) Production and Assay of Activity. A clone of *B. subtilis* YfiT was kindly provided by W. F. Anderson (Northwestern University, Evanston, IL) and used to produce His_6-tagged Yfit.[38] The protein was expressed in Luria Broth containing 100 μg/mL ampicillin and 1 mM IPTG for 2 h at 37 °C. The protein was purified as described for *Ms*MST over a Zn PrepEase affinity column and was shown to be >90% pure by SDS gel electrophoresis (Figure 2B, lane 3). *Bs*BST was shown to be active with BSH when assayed as described above for purified MST. The protein concentration was estimated using an A_{280} of 2.1 mg^{-1} mL^{-1} (http://ca.expasy.org).

Survey of *Bs*MST, *Bs*BST, and *Ef*GST Substrate Specificity. Except as noted below, the survey of substrate activity was conducted at 23 °C with 0.15 mM thiol and 0.15 mM substrate in 0.1 mM NaCl and 25 mM HEPES (pH 7.0); the reaction was followed at 5 min intervals by titration of 50 μL aliquots by addition to an equal volume of 0.4 mM DTNB in 100 mM Tris-HCl (pH 8.0) and determination of the ΔA_{412} value. Rates were calculated using an ε_{412} of 14.1[39] after correction for background rates measured in controls lacking enzyme or lacking substrate and represent the mean and standard error of at least three determinations.

The rates with CDNB were determined at 1 mM CDNB and 1 mM thiol by the method of Habig and Jakoby.[40] The rates with *p*-nitrophenyl acetate were determined at 400 nm in 0.1 M NaCl and 25 mM HEPES (pH 7.0) with 0.15 mM thiol and 0.15 mM *p*-nitrophenyl acetate as previously described.[40] The rate for *Ms*MST with mitomycin C (100 μM) was determined with 200 μM MSH at 37 °C in 0.1 M NaCl and 25 mM HEPES (pH 7.0) following the loss of mitomycin C by HPLC analysis. Reaction mixtures (250 μL) were sampled by mixing 50 μL with 50 μL of acetonitrile containing 50 mM methanesulfonic acid. The sample was heated at 60 °C for 10 min, iced, and then centrifuged for 5 min at 13000*g* to remove protein. The supernatant was dried in a Speed Vac and resuspended in a 0.1% TFA/water mixture for HPLC analysis. Mitomycin C was assayed by HPLC with monitoring at 360 nm using a linear gradient from 0 to 100% B over 40 min (0.05% TFA/water mixture, buffer A; acetonitrile, buffer B). The 4.6 mm × 250 mm Beckman Ultrasphere IP C18 column was operated at 23 °C and 1 mL/min. Mitomycin C eluted at 12.5 min, and the product eluted at 13.8 min. The rate for *Bs*BST with cerulenin (200 μM) was determined with 200 μM BSH at 37 °C in 0.1 mM NaCl and 25 mM HEPES (pH 7.3) following the loss of cerulenin by HPLC analysis. Cerulenin was assayed by HPLC monitored at 220 nm using the method described above for mitomycin C except that buffer A was a 0.1% TFA/water mixture and buffer B was methanol. Cerulenin eluted at 32.2 min and the product at 27.5 min.

RESULTS

Assay of MST Activity. Monochlorobimane (mBCl) was found to be a useful reagent for estimating cellular GSH levels because the chemical reaction of mBCl with GSH to produce fluorescent GSmB is slow at physiological pH but is catalyzed by cellular GSTs causing cells to become fluorescent.[41,42] However, variation in GST activity between different mammalian cell lines can complicate the quantitation.[43] Treatment of a *M. smegmatis* cell suspension with mBCl also produces fluorescence in both the extracellular and intracellular compartments. This results from the reaction of MSH with mBCl to produce fluorescent MSmB, analogous to the previously demonstrated process with the more reactive monobromobimane.[44] The speed of the reaction with mBCl implied the presence of an MST catalyzing the reaction with MSH to produce MSmB (Figure 3). MSmB is rapidly cleaved by mycothiol *S*-conjugate amidase (Mca) to generate the bimane derivative of *N*-acetylcysteine (AcCySmB, fluorescent), a mercapturic acid that is excreted from the cell (Figure 3), as established by independent HPLC analysis of the cellular and extracellular fractions [data not shown (see ref 44 for the analogous process with monobromobimane)]. This process can also be observed in dialyzed cell-free extracts of *M. smegmatis*. Combining cell extract with a mixture of mBCl and MSH led to the production of a large amount of AcCySmB and only a small amount of MSmB as determined by HPLC (Figure 4A). The sum of AcCySmB and MSmB, designated RSmB, represents the activity of MST catalyzing the production of MSmB. This assay was used to purify the MST activity from *M. smegmatis*.

Purification and Identity of *Ms*MST. A cell-free extract of *M. smegmatis* was fractionated with ammonium sulfate and the 45−65% ammonium sulfate fraction found to contain the majority of the MST activity. This fraction was dialyzed and further purified by chromatography on DEAE-650M, followed by chromatography on hydroxylapatite. Final purification by size exclusion chromatography on Sephacryl S200 indicated that the native protein had a molecular mass of ∼40 kDa. Following the gel filtration step, Mca activity was separated, and the product of the assay was solely MSmB. The purified MST was analyzed by SDS gel electrophoresis (Figure 2A, lane 7). The lower major band at ∼20 kDa was identified by high-resolution mass spectrometry as MSMEG_0887 and confirmed as *Ms*MST by cloning (see below). Its monomer molecular

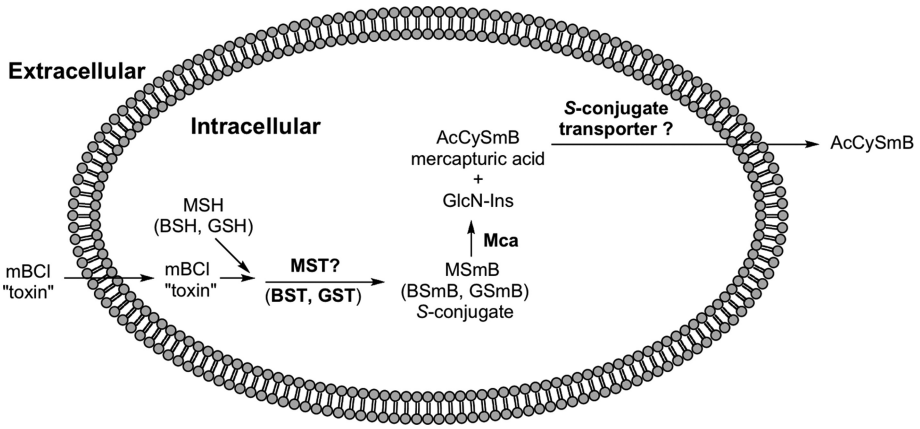

Figure 3. Thiol detoxification scheme showing production of thiol *S*-conjugates (MSmB, BSmB, and GSmB) catalyzed by *S*-transferases (MST, BST, and GST) and cleavage of MSmB by mycothiol *S*-conjugate amidase (Mca) to generate mercapturic acid AcCySmB and GlcN-Ins. The former is exported from the cell, possibly mediated by a putative *S*-conjugate transporter, and the latter is utilized for resynthesis of MSH.[44]

weight of 20383 together with native protein size indicates that the active protein is a dimer.

Cloning and Expression of MSMEG_0887 Confirms Its Assignment as *Ms*MST. The *MSMEG_0887* gene was cloned and expressed in pET28a as an N-terminal His_6-tagged protein and affinity purified to generate *Ms*MST, producing a single band via SDS gel electrophoresis (Figure 2B, lane 1).

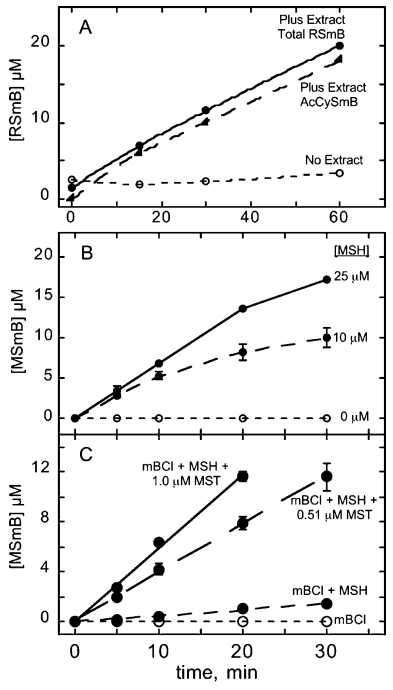

Figure 4. MST-catalyzed conversion of mBCl and MSH to MSmB. (A) Assay used to purify MST activity from *M. smegmatis* involving reaction of 20 μM MSH with 25 μM mBCl as catalyzed by a crude extract diluted 5-fold. The main product is AcCySmB generated from MSmB (present at a low steady-state level of 1−2 μM) by mycothiol *S*-conjugate amidase (Mca) that has high activity in the extract.[44] (B) Activity of *Ms*MST (0.51 μM) from cloned and expressed MSMEG_0887 as a function of MSH concentration with 50 μM mBCl and 0.51 μM *Ms*MST. (C) Activity with an increasing *Ms*MST concentration at 25 μM MSH and 50 μM mBCl. Data in panels B and C represent the mean of triplicate determinations. Error bars show the standard deviation when it is larger than the symbol. Experimental details are given in Materials and Methods.

Purified *Ms*MST exhibited substantial activity with 50 μM mBCl in the presence of an equal concentration of MSH but produced no detectable activity in the presence of BSH or GSH (Table 1). *Ms*MST produces only MSmB from MSH and mBCl

Table 1. Activities of Thiol *S*-Transferases with 50 μM mBCl and 50 μM Thiol

enzyme	thiol	activity (nmol min^{-1} mg^{-1})
*Ms*MST	MSH	340 ± 36
	BSH	<2
	GSH	<0.7
*Mt*MST	MSH	83 ± 10
	BSH	<0.4
	GSH	<0.4
*Bs*BST	MSH	<0.4
	BSH	2.5 ± 0.4
	GSH	<0.4
*Ef*GST	MSH	<0.04
	BSH	<0.04
	GSH	0.4 ± 0.06

in a mycothiol-dependent (Figure 4B) and protein-dependent (Figure 4C) manner. This enzyme activity facilitates the production of mycothiol *S*-conjugates that are cleaved by mycothiol *S*-conjugate amidase to generate a mercapturic acid that can be excreted from the cell (Figure 3).[44] It is therefore a key enzyme in the mycobacterial defense against cellular toxins.

Rv0443 Is the MST of *M. tuberculosis*. The gene encoding the MST of *M. tuberculosis* (*Mt*MST) was identified as *Rv0443*; its product is 77% identical in sequence to *Ms*MST. *Rv0443* was cloned and expressed in *E. coli* BL21(DE3) as an N-terminally His_6-tagged protein and purified on a zinc affinity column to generate pure protein (Figure 2B, lane 2). *Mt*MST was highly active toward mBCl with MSH, but activity with BSH or GSH was at least 200-fold lower (Table 1).

***B. subtilis* YfiT Is a Bacillithiol *S*-Transferase (BST).** A search of the pfam Web site at http://pfam.sanger.ac.uk with the *Ms*MST sequence revealed that it is a member of DUF664 (PF04978), an uncharacterized family of potential enzymes of the DinB superfamily. The later is comprised of eight families and includes 30 proteins with reported crystal structures, many from organisms known to produce BSH rather than MSH.[31] The expression vector for one of these (PDB entry 1rxq,

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B. subtilis yfit) was kindly provided by W. F. Anderson (Northwestern University). The purified protein (Figure 2B, lane 3) was assayed with mBCl and exhibited substantial activity with BSH but at least 60-fold lower activity with MSH and GSH (Table 1). Thus, *B. subtilis* YfiT is identified as a bacillithiol *S*-transferase and redesignated *Bs*BST. *Bs*BST is a member of the DinB_2 (PF12867) family of the DinB superfamily (http://pfam.sanger.ac.uk).

Identification of EF_3021 as a Glutathione *S*-Transferase (GST). A search of the GenBank with the *Ms*MST sequence identified numerous homologues among MSH producing Actinobacteria but also identified a gene in *En. faecalis* V583 (*EF_3021*) encoding a protein with a sequence 34% identical to that of *Ms*MST. Because *En. faecalis* produces GSH but not MSH or BSH,[31] we undertook the cloning and expression of this gene. Assay of the purified protein (Figure 2B, lane 4) with mBCl showed low but definite activity with GSH but at least 10-fold lower activity with MSH and BSH (Table 1). On the basis of this, we assign EF_3021 as the glutathione *S*-transferase *Ef*GST. Like *Bs*BST, *Ef*GST belongs to the DinB_2 (PF12867) family of the DinB superfamily and has no homology to other bacterial GST proteins.

Substrate Specificity of *Ms*MST, *Bs*BST, and *Ef*GST. A survey of potential substrates was undertaken using *Ms*MST, *Bs*BST, and *Ef*GST (Table 2). The data should be viewed only as qualitative indicators of possible activity because conditions vary widely between different assays. Also, all of these *S*-transferases were isolated as His_6-tagged proteins, and it is possible that the presence of the His_6 tag has influenced their activity. The most commonly used substrate for measurement of GST activity is CDNB,[40] and both *Ms*MST and *Bs*BST gave positive assays with this substrate using MSH and BSH, respectively. However, *Ef*GST failed to give detectable GSH-dependent activity in this assay.

Table 2. Activities of *Ms*MST, *Bs*BST, and *Ef*GST with Various Substrates

	enzyme activity (nmol min^{-1} mg^{-1})[a]		
substrate	*Ms*MST	*Bs*BST	*Ef*GST
mBCl[b]	340 ± 36	2.5 ± 0.4	0.40 ± 0.06
CDNB	5.6 ± 1.1	54 ± 4	≤0.03
CuOOH	<3[c]	5 ± 2	<2[c]
H_2O_2	<2[c]	<80[c]	<2[c]
cerulenin	<1[c]	40 ± 9	<0.2[c]
mitomycin C	17 ± 3	<1[c]	nd[d]

[a]Determined as described in Materials and Methods except as noted. [b]From Table 1. [c]Detection of activity limited by the indicated rate of the control reaction lacking enzyme. [d]Not determined.

The only previously reported activity for *Bs*BST (YfiT) was an esterase activity (results not shown) with *p*-nitrophenyl acetate in the absence of thiol.[38] Esterase activity with *p*-nitrophenyl acetate was observed with *Ms*MST (6.5 ± 0.1 nmol min^{-1} mg^{-1}), *Ef*GST (2.0 ± 0.1 nmol min^{-1} mg^{-1}), and *Bs*BST (4.4 ± 0.4 nmol min^{-1} mg^{-1}) in the absence of thiol. The respective addition of 0.15 mM MSH, GSH, or BSH did not significantly increase the rate.

Some bacterial GSTs have activity with peroxides,[13,45] but neither *Ms*MST nor *Ef*GST gave measurable rates with CuOOH or H_2O_2 (Table 2). *Bs*BST produced a low but significant rate with CuOOH that was greater than the control value. However, the control rate without enzyme was very high for H_2O_2. This suggests that BSH alone, or complexed with a suitable metal (ref 31 and unpublished results), may function together with a bacillithiol disulfide reductase (currently unidentified) to protect *B. subtilis* against peroxide toxicity. *Bs*BST, but neither *Ms*MST nor *Ef*GST, exhibited significant activity with the antibiotic cerulenin produced by the fungus *Sarocladium oryzae*.[46] Other antibiotics, including antimycin A, azithromycin, lincomycin, spectinomycin, and streptozocin, had little or no detectable activity (<2 nmol min^{-1} mg^{-1}) with the three *S*-transferases in the thiol depletion assay. For *Bs*BST, the result was similar with rifamycin S, but higher limits were obtained with *Ms*MST (<6 nmol min^{-1} mg^{-1}) and *Ef* GST (<4 nmol min^{-1} mg^{-1}) because of the higher control rates for the uncatalyzed reaction.

The positive assays for *Ms*MST with mitomycin C and for *Bs*BST with cerulenin indicate that antibiotic detoxification may be a function of these *S*-transferases. A more detailed survey of native substrates and the detailed kinetics of these reactions will have to await the preparation and isolation of the native form of these enzymes.

Phylogenetic Analysis of *Ms*MST, *Bs*BST, and *Ef*GST Homologues. The DinB superfamily is comprised of eight families, all but one of which have been labeled domains of unknown function (DUF) (http://pfam.sanger.ac.uk). One family is designated MDMPI_N (pfam11716) based upon the identification of one member as a mycothiol-dependent maleylpyruvate isomerase (MDMPI) in *C. glutamicum*[34] and subsequent determination of its structure.[35] To elaborate the relationship between MDMPI and the enzymes identified here, we searched the GenBank for sequence homologues of *Ms*MST, *Bs*BST, and *Ef*GST. Results were limited to those organisms for which low-molecular weight thiol analyses were available,[27,31,47,48] and a phylogenetic tree was generated for representative species (Figure 5). The *S*-transferase sequences fall into three groups associated with the major low-molecular weight thiol found in the organism. The organisms that produce MSH belong to the DUF664 family of the DinB superfamily (http://pfam.sanger.ac.uk).

The proteins related to *Bs*BST (YfiT) are members of the DinB_2 family (PF12867) of the DinB superfamily (Figure 5, http://pfam.sanger.ac.uk) and are produced in bacteria known to generate bacillithiol. It should be noted that FosB, the previously identified example of a BST,[32] is not a member of the DinB superfamily. The two GST sequences belong to different families; *Ef*GST is assigned to DinB_2, but the *Streptococcus mutans* enzyme is categorized with DUF664.

The three families that have been found to include thiol-dependent biochemical processes (MDMPI_N, DUF664, and DinB_2) contain 69% of the total sequences assigned to the DinB superfamily.

Function of DinB Superfamily Proteins. The one demonstrated physiologic function of a DinB superfamily protein is that for *C. glutamicum* MDMPI that plays an essential role in the metabolism of gentisate and other aromatic compounds.[34,35,49] Substantial primary sequence homologues are found in many Actinobacteria and likely have the same function. Other members of the MDMPI_N family have lower levels of primary sequence homology, and their function remains to be established.

The *B. subtilis* YfiT (*Bs*BST) structure (PDB entry 1rxq) is an example of a DinB crystal structure that was probably of early interest because of its genome context. In the *B. subtilis* genome, *yfiT* is flanked by genes encoding efflux transport

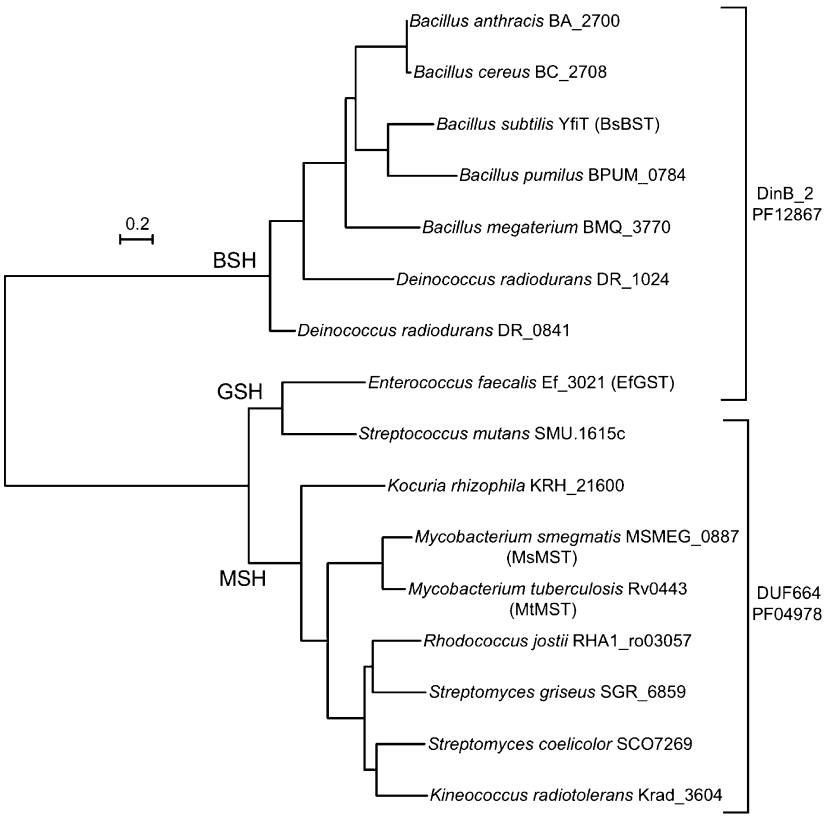

Figure 5. Phylogenetic tree of selected *S*-transferase sequences. Branches are labeled with the thiol that the bacteria of the group are known to produce. Proteins belonging to the DinB_2 and DUF664 families are designated by brackets.

proteins. Orthologs of *yfiS* in *Bacillus* are annotated [Kyoto Encyclopedia of Genes and Genomes (KEGG)] as macrolide transport proteins (http://www.genome.jp/dbget-bin/www_bget?bsu:BSU08380), and orthologs of *yfiU* are annotated as multidrug resistance transporters or drug resistance transporters (http://ssdb.genome.jp/ssdb-bin/ssdb_best?org_gene=bsu:BSU08400). The efflux transporters for mercapturic acids in MSH-producing species (Figure 3 and ref 29) and for cysteine *S*-conjugates in BSH-producing species have not been identified, but *yfiS* and *yfiU* are candidate genes for these transporters. The genome context of *yfiT* is consistent with its proposed function in the detoxification of antibiotics. Thus, the genome context of *yfiT* and the results listed in Table 2 are consistent with the hypothesis that antibiotic detoxification is a physiological function of *Bs*BST.

The DinB superfamily as determined by structural parameters (SCOP, http://sufam.cs.bris.ac.uk/SUPERFAMILY/) appears to be much larger than simple sequence searches suggest, and the variation of DinB protein content with genera provides some support for their involvement with antibiotics. The DinB superfamily is overrepresented in the genomes of the Bacillales (9.1 sequences per species or strain) and Actinobacteria (9.3 sequences per species or strain) relative to the Proteobacteria (1.9 sequences per species or strain) and other genomes (http://pfam.sanger.ac.uk). The content is particularly high in those bacteria known to produce secondary metabolites. The secondary metabolite biosynthesis potential of marine actinomycetes has been assessed by the number of polyketide synthesis (PKS) and nonribosomal peptide synthesis (NRPS) operons found in the genome.[50] Taking the secondary metabolite potential as the total of the PKS and NRPS genes found in the NCBI database for bacteria, we find a significant correlation between the content of genes encoding DinB proteins and the secondary metabolite potential (Table S1 of the Supporting Information). In the Firmicutes, the bacilli have up to 22 genes for DinB proteins per species and are rich in PKS and NRPS secondary metabolite synthesis genes. The *Streptococcus*, *Enterococcus*, and *Staphylococcus* genera have genes encoding only one or two DinB proteins and one or two genes for secondary metabolites. The largest numbers of genes (20−38 per species) encoding DinB proteins occur in the Actinobacteria, which also contain the largest number of PKS and NRPS genes, 24−66 per species. Especially notable are *Amycolatopsis mediteranei*, a rifamycin producer with 38 DinB protein-encoding genes, and *Saccharopolyspora erythraea*, an erythromycin producer with 34 such genes, correlating with 29 and 33 PKS and NRPS genes, respectively. It is interesting that *M. tuberculosis*, an obligate human pathogen, has 11 DinB-encoding genes, approximately half of which are found in *M. smegmatis*, a soil bacterium. The lowest number of genes for DinB proteins (one to four) among Actinobacteria are found in *Kocuria rhizophila*, *Micrococcus luteus*, and *Rubrobacter xylanophilus*, which have only one or two secondary metabolite genes. The apparent correlation of DinB genes with secondary metabolite genes suggests that these proteins may function in the production of secondary metabolites, or in self-protection against toxic effects of these metabolites, rather than in protection against secondary metabolites produced by other competing bacteria in the environment.

The small number of DinB proteins encoded by Proteobacterial genomes, and in those of the green sulfur and green non-sulfur bacteria, correlates with the few PKS or NRPS genes present in these bacteria. Exceptions include the Gram-negative

bacteria *Nostoc muscorum* and *Pseudomonas aeruginosa* with only one or two genes for DinB proteins and multiple PKS and NRPS genes (Table S1 of the Supporting Information). *N. muscorum*[51] and *P. aeruginosa*[19] have the canonical CDNB-utilizing GST that may function in place of DinB enzymes related to *Ef*GST.

DISCUSSION

The identification of four new thiol-dependent *S*-transferase enzymes as members of the DinB superfamily substantially expands our understanding of this large and important group of proteins. As pointed out previously,[52] the DinB superfamily proteins are not to be confused with the *E. coli dinB*-encoded type IV DNA polymerase and homologous enzymes that are involved in error prone DNA repair.[53,54] The first DinB superfamily protein to have a function assigned was the mycothiol-dependent maleylpyruvate isomerase from *C. glutamicum*,[34,35] and this defines the MDMPI_N family. The MDMPI_N family currently includes 779 sequences from 144 species and strains (http://pfam.sanger.ac.uk), nearly all from Actinobacteria where MSH has been generally found to be the dominant low-molecular weight thiol. The primary sequences of these proteins have only distant homology to that of *Ms*MST, but like other DinB proteins,[52] MDMPI has a binding site for divalent metals.[35]

The finding that *Ms*MST and *Mt*MST belong to the DUF664 (PF04978) family (Figure 5) provides the first identification of function for this family. Both proteins recognize mBCl as a substrate and are specific for MSH as the thiol cofactor (Table 1), while *Ms*MST was also shown to have activity with CDNB and mitomycin C (Table 2). The activities are modest compared with those for other bacterial GSTs[6,45] but sufficient to establish the validity of the catalytic activity. Quantitative assessment of these MSTs will have to await preparation of the native forms for the enzymes. Close homologues (>65% identical) are found in all mycobacterial genomes with the exception of that for *Mycobacterium leprae*, and substantial homologues (>40% identical) are found in approximately half of 88 genomes for other Actinobacteria. There can be little doubt that these proteins also have MSH-dependent enzyme activities. The DUF664 family includes 453 sequences from 186 species and strains, ~70% of which are Actinobacteria; most of the remainder are *Bacillus* (http://pfam.sanger.ac.uk). It seems likely that many of the proteins encoded by these genes are also MSH-dependent enzymes. The *M. smegmatis* genome contains two other members of the DUF664 family (MSMEG_1356 and MSMEG_3923) that are 27% identical in primary sequence but are only 16% identical to *Ms*MST (MSMEG_0887). It is possible that these two related proteins are also MSH-dependent enzymes, but this needs to be established experimentally.

*Bs*BST (Yfit) belongs to the DinB_2 family (PF12867) and has BSH-specific *S*-transferase activity, but how good a model is it for other DinB_2 family proteins? This family contains 2153 sequences from 735 species, of which ~50% are Firmicutes, ~14% Proteobacteria, and ~10% each Actinobacteria and Bacteriodetes (http://pfam.sanger.ac.uk). Close primary sequence homologues of *Bs*BST are found in most members of the class Bacilli and in several species of Deinococci, so it seems likely that these are BSH-dependent enzymes. More distant homologues are present in the Thermaceae, the strongest having been found in the genome of *Meiothermus silvanus* (Mesil_2528); however, thiol analyses have not been conducted on members of this order, so whether they produce BSH is uncertain. More surprising was the finding of strong homologues of *Bs*BST among the Bacteroidetes, including in ~60% of the completed genomes of Flavobacteria, Sphingobacteria, and Cytophagia. The thiols produced by these bacteria have not been studied, but those Bacteroidetes with strong *Bs*BST homologues also have strong homologues of the *B. subtilis* protein YllA proposed to encode BshC, the BSH synthase;[55] therefore, it seems likely that these bacteria are capable of producing BSH. It would thus appear that a substantial portion of the DinB_2 family proteins could prove to be BSH-dependent enzymes.

The novel GST enzyme identified here in *En. faecalis* appears to have a substantially more restricted distribution than MST or BST. The genomes of *S. mutans* and *En. faecalis* contain a gene that encodes an unusual protein that catalyzes both steps of GSH biosynthesis, designated either GSHF[12] or γ-GCS-GS.[11] The fused gene for GSH synthesis appears in the genomes of diverse Gram-positive and Gram-negative bacteria, suggesting that it was spread by lateral gene transfer.[11,12] However, of the 13 Gram-positive bacteria other than *En. faecalis* identified as having the *gshF* (*gshAB*) gene, only the *S. mutans* genome encodes a protein (SMU.1615c) that is substantially homologous to *Ef*GST (Figure 5). There are weaker homologues of *Ef*GST in bacteria producing MSH and identified as MSTs, but it seems clear that this unusual GST has a very limited distribution.

Approximately three-quarters of these sequences contain only the DinB domain, while the others contain one or more additional domains of varying type. In addition to *Bs*BST, the *B. subtilis* genome has two additional sequences that belong to the DinB_2 family, YkkA (BSU13070) and YuaE (BSU32030), containing only the DinB_2 domain. The sequences of YkkA and YuaE are only 12% identical, as are those of *Bs*BST (YfiT) and YkkA, whereas the sequences of *Bs*BST and YuaE are 14% identical. Whether these two additional *B. subtilis* sequences assigned to the DinB_2 family are also BSH-dependent proteins requires experimental verification.

The crystal structure of *Bs*BST (YfiT, PDB entry 1rxq) provides some insights into how this protein may function. YfiT (*Bs*BST) was crystallized as a dimer with the Ni binding site facing away from the dimer interface and toward the solvent.[38] The Ni could be readily exchanged with Zn by dialysis, and we assume the Zn form is the native form of the enzyme. In MshC, Zn binds the cysteine sulfhydryl in the mycothiol ligase reaction that links Cys with GlcN-Ins producing Cys-GlcN-Ins, an intermediate in MSH biosynthesis.[56,57] The Zn ligand in YfiT (*Bs*BST) is a possible binding site for the cysteine sulfhydryl of bacillithiol. Rajan et al.[38] noted that the metal binding site is surrounded within 10 Å by three pairs of hydrophobic amino acids (Tyr8 and Tyr93, Tyr54 and Tyr157, and Trp59 and Trp 159) that may define a hydrophobic binding site for aromatic xenobiotics. These amino acids are fully conserved in the proteins of the BSH branch of Figure 5, with the exception of DR_1024 where the positions corresponding to Tyr93 and Tyr157 have Ala in place of Tyr. The hydrophobic site generated by these residues could provide a binding site for hydrophobic substrates such as mBCl, CDNB, CuOOH, and cerulenin (Table 2).

CONCLUSIONS

In this work, we have identified three new types of *S*-transferase enzymes that involve three different thiol cofactors. These

include the first mycothiol *S*-transferase to be reported, a bacillithiol *S*-transferase unrelated to FosB, recently reported to have BST activity, and a glutathione *S*-transferase unrelated to the superfamily of known GSTs. These enzymes belong to two families (DinB_2 and DUF664) of the DinB superfamily that also includes the MDMPI_N family previously shown to have mycothiol-dependent maleylpyruvate isomerase activity. These three families include more than two-thirds of the sequences comprising the DinB superfamily, indicating that thiol-dependent metabolic and detoxification processes represent a major activity of this superfamily. The full range of natural substrates for these enzymes remains to be determined. Such studies will not be pursued in the Fahey laboratory that was recently closed, but it is hoped that this study provides key insights and methods to aid such studies by our collaborators and other investigators.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

BSH, bacillithiol; BST, bacillithiol *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; GSH, glutathione; GST, glutathione *S*-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl *β*-D-1-thiogalactopyranoside; mBCl, monochlorobimane; MSH, mycothiol; MDMPI, mycothiol-dependent maleylpyruvate *S*-transferase; MST, mycothiol *S*-transferase; TLCK, *N*-*α*-*p*-tosyl-L-lysinechloromethyl ketone; TPCK, *N*-*α*-*p*-tosyl-L-phenylalanyl-chloromethyl ketone.

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■ REFERENCES

(1) Eaton, D. L., and Bammler, T. K. (1999) Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci. 49*, 156−164.

(2) Frova, C. (2006) Glutathione transferases in the genomics era: New insights and perspectives. *Biomol. Eng. 23*, 149−169.

(3) Hayes, J. D., Flanagan, J. U., and Jowsey, I. R. (2005) Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol. 45*, 51−88.

(4) Josephy, P. D., and Mannervik, B. (2006) Glutathione transferases. In *Molecular Toxicology*, 2nd ed., pp 333−364, Oxford University Press, Oxford, U.K.

(5) Clark, A. G., Smith, J. N., and Speir, T. W. (1973) Cross specificity in some vertebrate and insect glutathione-transferases with methyl parathion (dimethyl p-nitrophenyl phosphorothionate), 1-chloro-2,4-dinitro-benzene and s-crotonyl-N-acetylcysteamine as substrates. *Biochem. J. 135*, 385−392.

(6) Stourman, N. V., Branch, M. C., Schaab, M. R., Harp, J. M., Ladner, J. E., and Armstrong, R. N. (2011) Structure and function of YghU, a nu-class glutathione transferase related to YfcG from *Escherichia coli*. *Biochemistry 50*, 1274−1281.

(7) Fahey, R. C., Brown, W. C., Adams, W. B., and Worsham, M. B. (1978) Occurrence of glutathione in bacteria. *J. Bacteriol. 133*, 1126−1129.

(8) Copley, S. D., and Dhillon, J. K. (2002) Lateral gene transfer and parallel evolution in the history of glutathione biosynthesis genes. *Genome Biol 3*, research0025.

(9) Malki, L., Yanku, M., Borovok, I., Cohen, G., Mevarech, M., and Aharonowitz, Y. (2009) Identification and characterization of *gshA*, a gene encoding the glutamate-cysteine ligase in the halophilic archaeon *Haloferax volcanii*. *J. Bacteriol. 191*, 5196−5204.

(10) Newton, G. L., and Javor, B. (1985) *γ*-Glutamylcysteine and thiosulfate are the major low-molecular-weight thiols in halobacteria. *J. Bacteriol. 161*, 438−441.

(11) Janowiak, B. E., and Griffith, O. W. (2005) Glutathione synthesis in *Streptococcus agalactiae*. One protein accounts for *γ*-glutamylcysteine synthetase and glutathione synthetase activities. *J. Biol. Chem. 280*, 11829−11839.

(12) Gopal, S., Borovok, I., Ofer, A., Yanku, M., Cohen, G., Goebel, W., Kreft, J., and Aharonowitz, Y. (2005) A multidomain fusion protein in *Listeria monocytogenes* catalyzes the two primary activities for glutathione biosynthesis. *J. Bacteriol. 187*, 3839−3847.

(13) Allocati, N., Federici, L., Masulli, M., and Di Ilio, C. (2009) Glutathione transferases in bacteria. *FEBS J. 276*, 58−75.

(14) Vuilleumier, S., and Pagni, M. (2002) The elusive roles of bacterial glutathione S-transferases: New lessons from genomes. *Appl. Microbiol. Biotechnol. 58*, 138−146.

(15) Shishido, T. (1981) Glutathione *S*-Transferase from *Escherichia coli*. *Agric. Biol. Chem. 45*, 2951−2953.

(16) Fahey, R. C., Buschbacher, R. M., and Newton, G. L. (1987) The Evolution of Glutathione Metabolism in Phototrophic Microorganisms. *J. Mol. Evol. 25*, 81−88.

(17) Fahey, R. C., and Newton, G. L. (1983) Occurrence of low molecular weight thiols in biological systems. In *Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects* (Larsson, A., Orrenius, S., Holmgren, A., and Mannervik, B., Eds.) pp 251−260, Raven Press, New York.

(18) Newton, G. L., and Fahey, R. C. (1989) Glutathione in Procaryotes. In *Glutathione: Metabolism and Physiological Functions* (Viña, J., Ed.) pp 69−77, CRC Press, Boca Raton, FL.

(19) Piccolomini, R., Di Ilio, C., Aceto, A., Allocati, N., Faraone, A., Cellini, L., Ravagnan, G., and Federici, G. (1989) Glutathione transferase in bacteria: Subunit composition and antigenic characterization. *J. Gen. Microbiol. 135*, 3119−3125.

(20) Arca, P., Hardisson, C., and Suarez, J. E. (1990) Purification of a glutathione *S*-transferase that mediates fosfomycin resistance in bacteria. *Antimicrob. Agents Chemother. 34*, 844−848.

(21) Fang, T., Li, D. F., and Zhou, N. Y. (2011) Identification and clarification of the role of key active site residues in bacterial glutathione *S*-transferase *ζ*/maleylpyruvate isomerase. *Biochem. Biophys. Res. Commun. 410*, 452−456.

(22) Anandarajah, K., Kiefer, P. M. Jr., Donohoe, B. S., and Copley, S. D. (2000) Recruitment of a double bond isomerase to serve as a reductive dehalogenase during biodegradation of pentachlorophenol. *Biochemistry 39*, 5303−5311.

(23) Federici, L., Masulli, M., Di Ilio, C., and Allocati, N. (2010) Characterization of the hydrophobic substrate-binding site of the bacterial beta class glutathione transferase from *Proteus mirabilis*. *Protein Eng., Des. Sel. 23*, 743−750.

(24) Kiefer, P. M. Jr., and Copley, S. D. (2002) Characterization of the initial steps in the reductive dehalogenation catalyzed by tetrachlorohydroquinone dehalogenase. *Biochemistry 41*, 1315−1322.

(25) Vuilleumier, S., Ivos, N., Dean, M., and Leisinger, T. (2001) Sequence variation in dichloromethane dehalogenases/glutathione S-transferases. *Microbiology 147*, 611−619.
(26) Xun, L., Belchik, S. M., Xun, R., Huang, Y., Zhou, H., Sanchez, E., Kang, C., and Board, P. G. (2010) S-Glutathionyl-(chloro)-hydroquinone reductases: A novel class of glutathione transferases. *Biochem. J. 428*, 419−427.
(27) Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., delCardayré, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) Distribution of thiols in microorganisms: Mycothiol is a major thiol in most actinomycetes. *J. Bacteriol. 178*, 1990−1995.
(28) Jothivasan, V. K., and Hamilton, C. J. (2008) Mycothiol: Synthesis, biosynthesis and biological functions of the major low molecular weight thiol in actinomycetes. *Nat. Prod. Rep. 25*, 1091−1117.
(29) Newton, G. L., Buchmeier, N., and Fahey, R. C. (2008) Biosynthesis and functions of mycothiol, the unique protective thiol of *Actinobacteria*. *Microbiol. Mol. Biol. Rev. 72*, 471−494.
(30) Rawat, M., and Av-Gay, Y. (2007) Mycothiol-dependent proteins in actinomycetes. *FEMS Microbiol. Rev. 31*, 278−292.
(31) Newton, G. L., Rawat, M., La Clair, J. J., Jothivasan, V. K., Budiarto, T., Hamilton, C. J., Claiborne, A., Helmann, J. D., and Fahey, R. C. (2009) Bacillithiol is an antioxidant thiol produced in Bacilli. *Nat. Chem. Biol. 5*, 625−627.
(32) Sharma, S. V., Jothivasan, V. K., Newton, G. L., Upton, H., Wakabayashi, J. I., Kane, M. G., Roberts, A. A., Rawat, M., La Clair, J. J., and Hamilton, C. J. (2011) Chemical and Chemoenzymatic Syntheses of Bacillithiol: A Unique Low-Molecular-Weight Thiol amongst Low G + C Gram-Positive Bacteria. *Angew. Chem., Int. Ed. 50*, 7101−7104.
(33) Cooper, D. R., Grelewska, K., Kim, C. Y., Joachimiak, A., and Derewenda, Z. S. (2010) The structure of DinB from *Geobacillus stearothermophilus*: A representative of a unique four-helix-bundle superfamily. *Acta Crystallogr. F66*, 219−224.
(34) Feng, J., Che, Y., Milse, J., Yin, Y. J., Liu, L., Ruckert, C., Shen, X. H., Qi, S. W., Kalinowski, J., and Liu, S. J. (2006) The gene ncgl2918 encodes a novel maleylpyruvate isomerase that needs mycothiol as cofactor and links mycothiol biosynthesis and gentisate assimilation in *Corynebacterium glutamicum*. *J. Biol. Chem. 281*, 10778−10785.
(35) Wang, R., Yin, Y. J., Wang, F., Li, M., Feng, J., Zhang, H. M., Zhang, J. P., Liu, S. J., and Chang, W. R. (2007) Crystal structures and site-directed mutagenesis of a mycothiol-dependent enzyme reveal a novel folding and molecular basis for mycothiol-mediated maleylpyruvate isomerization. *J. Biol. Chem. 282*, 16288−16294.
(36) Unson, M. D., Newton, G. L., Davis, C., and Fahey, R. C. (1998) An immunoassay for the detection and quantitative determination of mycothiol. *J. Immunol. Methods 214*, 29−39.
(37) Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem. 68*, 850−858.
(38) Rajan, S. S., Yang, X., Shuvalova, L., Collart, F., and Anderson, W. F. (2004) YfiT from *Bacillus subtilis* is a probable metal-dependent hydrolase with an unusual four-helix bundle topology. *Biochemistry 43*, 15472−15479.
(39) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) Reassessment of Ellman's reagent. *Methods Enzymol. 91*, 49−60.
(40) Habig, W. H., and Jakoby, W. B. (1981) Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol. 77*, 398−405.
(41) Cook, J. A., Pass, H. I., Russo, A., Iype, S., and Mitchell, J. B. (1989) Use of monochlorobimane for glutathione measurements in hamster and human tumor cell lines. *Int. J. Radiat. Oncol., Biol., Phys. 16*, 1321−1324.
(42) Shrieve, D. C., Bump, E. A., and Rice, G. C. (1988) Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J. Biol. Chem. 263*, 14107−14114.
(43) Ublacker, G. A., Johnson, J. A., Siegel, F. L., and Mulcahy, R. T. (1991) Influence of glutathione S-transferases on cellular glutathione determination by flow cytometry using monochlorobimane. *Cancer Res. 51*, 1783−1788.
(44) Newton, G. L., Av-Gay, Y., and Fahey, R. C. (2000) A novel mycothiol-dependent detoxification pathway in mycobacteria involving mycothiol *S*-conjugate amidase. *Biochemistry 39*, 10739−10746.
(45) Kanai, T., Takahashi, K., and Inoue, H. (2006) Three distinct-type glutathione *S*-transferases from *Escherichia coli* important for defense against oxidative stress. *J. Biochem. 140*, 703−711.
(46) Ayyadurai, N., Kirubakaran, S. I., Srisha, S., and Sakthivel, N. (2005) Biological and molecular variability of *Sarocladium oryzae*, the sheath rot pathogen of rice (*Oryza sativa* L.). *Curr. Microbiol. 50*, 319−323.
(47) Buchmeier, N. A., Newton, G. L., and Fahey, R. C. (2006) A mycothiol synthase mutant of *Mycobacterium tuberculosis* has an altered thiol-disulfide content and limited tolerance to stress. *J. Bacteriol. 188*, 6245−6252.
(48) Johnson, T., Newton, G., Fahey, R. C., and Rawat, M. (2008) Unusual production of glutathione in *Actinobacteria*. *Arch. Microbiol. 191*, 89−93.
(49) Shen, X. H., Jiang, C. Y., Huang, Y., Liu, Z. P., and Liu, S. J. (2005) Functional identification of novel genes involved in the glutathione-independent gentisate pathway in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol. 71*, 3442−3452.
(50) Gontang, E. A., Gaudencio, S. P., Fenical, W., and Jensen, P. R. (2010) Sequence-based analysis of secondary-metabolite biosynthesis in marine actinobacteria. *Appl. Environ. Microbiol. 76*, 2487−2499.
(51) Galhano, V., Gomes-Laranjo, J., and Peixoto, F. (2011) Exposure of the cyanobacterium *Nostoc muscorum* from Portuguese rice fields to Molinate (Ordram): Effects on the antioxidant system and fatty acid profile. *Aquat. Toxicol. 101*, 367−376.
(52) Cooper, D. R., Grelewska, K., Kim, C. Y., Joachimiak, A., and Derewenda, Z. S. (2010) The structure of DinB from *Geobacillus stearothermophilus*: A representative of a unique four-helix-bundle superfamily. *Acta Crystallogr. F66*, 219−224.
(53) Benson, R. W., Norton, M. D., Lin, I., Du Comb, W. S., and Godoy, V. G. (2011) An active site aromatic triad in *Escherichia coli* DNA Pol IV coordinates cell survival and mutagenesis in different DNA damaging agents. *PLoS One 6*, e19944.
(54) Hori, M., Yonekura, S., Nohmi, T., Gruz, P., Sugiyama, H., Yonei, S., and Zhang-Akiyama, Q. M. (2010) Error-Prone Translesion DNA Synthesis by *Escherichia coli* DNA Polymerase IV (DinB) on Templates Containing 1,2-dihydro-2-oxoadenine. *J. Nucleic Acids,*, 807579.
(55) Gaballa, A., Newton, G. L., Antelmann, H., Parsonage, D., Upton, H., Rawat, M., Claiborne, A., Fahey, R. C., and Helmann, J. D. (2010) Biosynthesis and functions of bacillithiol, a major low-molecular-weight thiol in Bacilli. *Proc. Natl. Acad. Sci. U.S.A. 107*, 6482−6486.
(56) Fan, F., Luxenburger, A., Painter, G. F., and Blanchard, J. S. (2007) Steady-state and pre-steady-state kinetic analysis of *Mycobacterium smegmatis* cysteine ligase (MshC). *Biochemistry 46*, 11421−11429.
(57) Tremblay, L. W., Fan, F., Vetting, M. W., and Blanchard, J. S. (2008) The 1.6 Å Crystal Structure of *Mycobacterium smegmatis* MshC: The Penultimate Enzyme in the Mycothiol Biosynthetic Pathway. *Biochemistry 47*, 13326−13335.