Supporting Information

Gaballa et al. 10.1073/pnas.1000928107

SI Materials and Methods

Media and Growth Conditions. For selection, antibiotics were added as follows: erythromycin (1 μg/mL) and lincomycin (25 μg/mL) (for selecting for macrolide-lincosamide-streptogramin B resistance), spectinomycin (100 μg/mL), chloramphenicol (10 μg/mL), kanamycin (15 μg/mL), and neomycin (10 μg/mL). *Bacillus subtilis* was grown in LB, and unless otherwise indicated, liquid media were inoculated from an overnight preculture and incubated at 37 °C with shaking at 200 rpm. *Escherichia coli* DH5α was used for routine DNA cloning as described previously (1). Sporulation and germination efficiencies were measured according to ref. 2. Restriction enzymes, DNA ligase, and DNA polymerases were used according to the manufacturer's instructions (New England Biolabs).

Chemical Sensitivity Assays. *B. subtilis* strains were grown at 37 °C overnight in LB medium, diluted 1:100 into fresh LB or LS medium, and grown at 37 °C with shaking. Cells were grown to midlogarithmic phase, and 100 μL of culture was mixed with 4 mL of 0.7% soft LB medium agar and poured onto 15-mL bottom agar. After cooling, filter paper disks (6-mm diameter) carrying the test compounds were placed on the top of the agar, and the plates were incubated at 37 °C overnight. The next day, the diameter (in centimeters) of the zone of growth inhibition around the filter paper disk was measured under low magnification to achieve a precision of ±0.05 cm. Values shown are averages and SD ($n \geq 3$), and where no SD is indicated all measurements were identical within the precision of the assay. Effects of salt and pH on the growth of bacillithiol (BSH) mutants were measured in LB with different pH or salt concentrations in automated growth curves using a Bioscreen C.

Proteome and Thiol-Redox Proteome Analysis. The redox proteome analysis was performed as described previously (3) with the following modifications: cells were harvested by centrifugation at 7600 × *g* for 10 min at 4 °C. The pellet was resuspended in denaturing buffer [8 M urea, 1% CHAPS, 1 mM EDTA, and 200 mM Tris-HCl (pH 8.0)] containing 100 mM iodoacetamide. Cells were disrupted by sonication, and the lysate was incubated in the dark for 30 min to alkylate all reduced thiol groups. Proteins were precipitated with 75% acetone, and the resulting protein precipitate was washed twice with acetone, dried in a SpeedVac, and resuspended in denaturing buffer without IAM. The protein concentration was measured according to Bradford (4). Finally, all reversible thiol modifications (disulfide bonds and mixed disulfides) were reduced with Tris(2-carboxyethyl)phosphine and labeled with the fluorescence dye BODIPY FL C_1-IA as described previously (4). The labeling reaction was stopped by centrifugation through a Micro Bio-Spin 6 column. 2D PAGE of the fluorescence-labeled cell extract was performed in the dark, and the resulting 2D gels were scanned first using a Typhoon Scanner (GE Healthcare) followed by staining with SYPRO Ruby.

Northern Blot Experiments. Northern blot analyses were performed as described previously (5) using RNA isolated from cells of *B. subtilis* wild type, *bshA*, *bshB2*, and *bshB1bshB2* mutants before (control) and 10 min after the treatment with 5 μM benzoquinone, 0.5 mM methylhydroquinone, 1 mM diamide, 5.6 mM methylglyoxal, 100 μM H_2O_2, or 100 μM cumene hydroperoxide. Hybridizations specific for *nfrA*, *trxA*, and *ohrA* were performed with the digoxigenin-labeled RNA probes synthesized in vitro using T7 RNA polymerase from T7 promoter containing internal PCR products of the respective genes using the primers sets for *trxA*, *ohrA*, and *nfrA* as indicated in Table S2.

Expression and Purification of *B. subtilis* BshA. BshA was PCR amplified from *B. subtilis* JH642 using primers BshAN5 and BshAN3 (Table S2) and thermostable, proofreading Pfu DNA polymerase (Stratagene). The amplicon was cloned into pET151 TOPO vector (Invitrogen) and expressed in BL21 Star (DE3) pLysS *E. coli* strain as the N-terminal His-6 tagged protein. The protein was purified to homogeneity on a chelate chromatography nickel column.

Expression and Purification of *B. anthracis* BshB1. The codon-optimized gene for *B. anthracis* BshB1 (BA1557) was synthesized by GenScript and subcloned into pET28a(+) (Novagen). The gene was expressed with a C-terminal His-tag in *E. coli* BL21(DE3) cells using auto-induction media at 37 °C (6). All steps of the purification were carried out at 4 °C. Harvested cells were disrupted using an Avestin EmulsiFlex-C5 homogenizer, and nucleic acids were removed by addition of 2% streptomycin sulfate and centrifugation.

For *Ba*BshB, the immobilized metal-ion affinity chromatography column was first converted to the Zn^{2+} form as described previously [MshB purification (7)]. A clarified extract was loaded onto a 25-mL column in 50 mM sodium phosphate (pH 8.0) containing 0.3 M NaCl and 20 mM imidazole. *Ba*BshB was eluted by increasing the imidazole concentration to 250 mM. Peak fractions were pooled and loaded onto a Q-Sepharose HP column after dialysis against 25 mM potassium phosphate (pH 7.0). The protein was eluted with a 0 → 1 M NaCl gradient, and the purified *Ba*BshB was dialyzed against 25 mM Tris-HCl (pH 8.0) containing 50 mM NaCl, concentrated by ultrafiltration, and brought to a final concentration of 20% (vol/vol) glycerol before being frozen in aliquots at −80 °C.

Expression and Purification of BshC. The coding region of BshC was amplified from the *B. subtilis* CU1065 genome using oligonucleotides (Table S2). The resulting fragment was digested with *Nde*I and *Bam*HI, cloned into pET16B (Novagen), and used to transform *E. coli* DH5a (1). The sequence of the cloned fragment was checked by DNA sequencing in both orientations, and the resulting plasmid was used to transform *E.coli* BL21(DE3)(pLysS).

A single colony was grown overnight in LB containing ampicillin (200 μg/mL). The overnight culture was used to inoculate LB medium containing ampicillin (200μg/mL) and incubated at 37 °C with vigorous shaking to an OD_{600} of 0.4. Isopropyl-β-D-thiogalactopyranoside was added to 1 mM (final concentration), and the cells were harvested after further incubation for 3 h. The cell pellet was suspended in 10 mL resuspension solution [50 mM NaH_2PO_4, 5 mM Tris·Cl (pH 8.0), 20 mM imidazole, 300 mM NaCl, and 5% (vol/vol) glycerol], and the cells were broken by sonication. The soluble fraction was collected and purified using Ni-NTA beads (USB) according to the manufacturer's instruction. Samples were analyzed on 12% SDS/PAGE to identify the fractions that contained BshC and dialyzed overnight against 50 mM Tris·Cl (pH 8.0), 100 mM NaCl (pH 8.0), and 5% (vol/vol) glycerol. The protein was further purified by size exclusion chromatography on a Superdex 200 FPLC column using the same buffer. The fractions containing BshC were concentrated using ultrafiltration and stored at −20 °C.

Biological Synthesis of GlcNAc-Mal. The substrate for BshB, GlcNAc-Mal, was produced by a preparative reaction of *B. subtilis* BshA with the native substrates UDPGlcNAc and L-malate. BshA (0.2 mg) was incubated in 5 mL of 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 25 mM Hepes (pH 7.5),

with 50 μmoles of UDPGlcNAc (Sigma) and 50 μmoles of L-malate (Sigma) at 37 °C. The reaction was followed at absorbance 260 nm by the consumption of UDPGlcNAc and production of UDP by HPLC as previously described (8) and was complete in 4 h (Fig. S3*A*). One volume of acetonitrile was added to the reaction, and the mixture was incubated for 15 min at 60 °C. The protein was pelleted by centrifugation for 15 min at 13,000 × *g*. The supernatant was reduced to 2 mL in a SpeedVac and adjusted to pH 3 with TFA. The product was purified by preparative HPLC on a Vydac 218TP1022 (10 × 250 mm) reverse phase column with A solvent = 0.1% TFA-H_20 and methanol as the B solvent. A linear gradient of 0–20% B in 40 min at (5 mL/min) gave separation of GlcN-Mal from the other reaction components, and fractions containing (ESP^-) m/z = 336 were combined. The separation was repeated to obtain a purified fraction GlcNAc-Mal (Fig. S3). The product was adjusted to pH 6 with NaOH and reduced to dryness on a SpeedVac. The product was pure by proton NMR and displayed the expected mass spectrum ESP^- m/z = 336 (Fig. S3*B*). The product was dissolved in water at 80 mM and contained ≈1 equivalent of NaTFA from the HPLC solvent.

Biological Synthesis and Assay of GlcN-Mal. GlcNAc-Mal was deacetylated by *Ba*BshB1 to yield the free amine GlcN-Mal for BshC assays. A 100-μL aliquot of 140 mM GlcNAc-Mal (pH 6) was mixed with 100 μL of 25 mM Hepes (pH 8.0), to give a solution of pH ≈7.3. *Ba*BshB1 (Ba1557, 43 μg) was added, and the reaction was monitored periodically by assaying a 1-μL aliquot with AccQ Tag (Waters) amine fluorescent labeling (see below) and HPLC for GlcN-Mal. The reaction was complete in 2 h at 37 °C. One volume of acetonitrile was added to the reaction, and the mixture was incubated for 15 min at 60 °C. The protein was pelleted by centrifugation for 15 min at 13,000 × *g*. The supernatant was concentrated to dryness in a SpeedVac and dissolved in 100 μL of water. The GlcN-Mal stock solution was assayed to be 138 mM and was used without further purification. The reaction was also monitored by mass spectrometry and showed a complete loss of ESP^- m/z = 336 (GlcNAc-Mal) and the appearance of GlcN-Mal, ESP^+ m/z = 296. The mass spectrum of the final product (Fig. S3*C*) shows Hepes salt as a major contaminant.

GlcN-Mal was assayed by precolumn derivitization with the fluorescent amine reagent AccQ Tag (Waters) by a minor modification of the method of Anderberg et al. (9). The GlcN-AccQ-Mal derivative elutes with a major hydrolysis product when the HPLC A buffer was 0.1% TFA as described previously (9). Partial ionization of the malate carboxylates allowed separation of the GlcN-Mal derivative from the reagent products. Substitution of 0.1% formic acid-water adjusted to pH 3.8 with NaOH for buffer A gives resolution of the GlcN-AccQ-Mal derivative from the reagents with the published gradient separation (9). The retention time of GlcN-AccQ and GlcN-AccQ-Mal with the modified protocol was 13.4 and 18.3 min, respectively. To generate a quantitative standard of GlcN-Mal, a minor BSH S-conjugate amidase activity in *Ba*BshB1 was used to cleave BSmB to 1 equivalent each of CySmB and GlcN-Mal. Two hundred microliters of 0.2 mM BSmB was incubated in 50 mM NaCl and 50 mM Hepes (pH 7.5) for 48 h with 10 μg of *B. anthracis* BshB1 at 37 °C. The reaction was stopped by the addition of one volume of acetonitrile, and the mixture was incubated for 15 min at 60 °C. The protein was pelleted by centrifugation for 15 min at 13,000 × *g*. The supernatant was concentrated to dryness in a SpeedVac and dissolved in 200 μL of water. The GlcN-Mal content of the standard solution was determined by the CySmB content as previously described (10).

Enzyme Assays for BshA, BshB1, and BshC. *B. subtilis* BshA was assayed in 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 25 mM Hepes (pH 7.5) at 37 °C by HPLC with detection at Abs 260 nm for UDPGlcNAc and UDP as previously described for MshA (8). *B. anthracis* BshB1 was assayed for conversion of GlcNAc-Mal to GlcN-Mal in 140 mM NaTFA and 13 mM Hepes (pH 7.3), as described in *Biological Synthesis of GlcN-Mal* above. *Staphylococcus aureus* Cys tRNA synthetase and *B. subtilis* BshC (YllA) were assayed for BshC activity using a modification of the MshC HPLC assay previously described (11). The protein (42 μg of Cys tRNA synthetase or 8.4 μg of BshC) was assayed in 125 μL of 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM GlcN-Mal (see above), 1 mM L-cysteine, 1 mM ATP, and 50 mM Hepes (pH 7.5). The reaction was initiated with the addition of protein and quenched at various times by removing a 10-μL aliquot and mixing with 10 μL prewarmed (60 °C) 5 mM monobromobimane (Invitrogen) in acetonitrile. The sample was incubated for 10 min at 60 °C and diluted for HPLC. HPLC analysis of the labeled samples showed less than 0.44 pmol of BSmB formed in 90 min at 37 °C using either enzyme. This gives an estimate of <0.007 nmol/min per mg BshC activity for *B. subtilis* BshC (YllA) and <0.002 nmols/min per mg BshC activity for *S. aureus* Cys tRNA synthetase using the above conditions. Genetic studies indicate that BshC is required for the cysteine-adding step in BSH synthesis (see main text), although purified BshC is inactive under these in vitro conditions. BshC as purified may be inactive, may require other protein partners or cosubstrates, or may require different reaction conditions from those tested here.

1. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Plainview, NY), 2nd Ed.
2. Nicholson WL, Setlow P (1990) Sporulation, germination and outgrowth. *Molecular Biological Methods for Bacillus*, eds Harwood CR, Cutting SM (John Wiley and Sons, Chichester, UK), pp 391–450.
3. Hochgräfe F, Mostertz J, Albrecht D, Hecker M (2005) Fluorescence thiol modification assay: Oxidatively modified proteins in *Bacillus subtilis*. *Mol Microbiol* 58:409–425.
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
5. Wetzstein M, et al. (1992) Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. *J Bacteriol* 174:3300–3310.
6. Studier FW (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* 41:207–234.
7. Newton GL, Ko M, Ta P, Av-Gay Y, Fahey RC (2006) Purification and characterization of *Mycobacterium tuberculosis* 1D-myo-inosityl-2-acetamido-2-deoxy-alpha-D-glucopyranoside deacetylase, MshB, a mycothiol biosynthetic enzyme. *Protein Expr Purif* 47:542–550.
8. Newton GL, Ta P, Bzymek KP, Fahey RC (2006) Biochemistry of the initial steps of mycothiol biosynthesis. *J Biol Chem* 281:33910–33920.
9. Anderberg SJ, Newton GL, Fahey RC (1998) Mycothiol biosynthesis and metabolism. Cellular levels of potential intermediates in the biosynthesis and degradation of mycothiol in *Mycobacterium smegmatis*. *J Biol Chem* 273:30391–30397.
10. Fahey RC, Newton GL (1987) Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. *Methods Enzymol* 143:85–96.
11. Newton GL, Ta P, Sareen D, Fahey RC (2006) A coupled spectrophotometric assay for l-cysteine:1-D-myo-inosityl 2-amino-2-deoxy-alpha-D-glucopyranoside ligase and its application for inhibitor screening. *Anal Biochem* 353:167–173.

Fig. S1. Multiple sequence alignment of orthologs to *B. subtilis* BSH biosynthesis enzymes. Aligments are shown for (*A*) BshA, (*B*) BshB1 and BshB2, and (*C*) BshC, with (*D*) a table of percentage identities and similarities and data on related structures. The bacterial sequences shown are from *B. subtilis* (BS), *B. anthracis* (BA), *B. cereus* (BC), *Deinococcus radiodurans* (DR), *Myxococcus xanthus* (MX), *and Staphylococcus aureus* (SA). Alignments were generated using MatGAT [Campanella JJ, Bitincka L, Smalley J (2003) MatGAT: An application that generates similarity/identity matrices using protein or DNA sequences. *BMC Bioinformatics* 4:29] with a BLOSUM50 similarity matrix.

Fig. S1

Fig. S2. Organization and expression of the *bshA-bshB1-, bshB2-*, and *bshC*-containing operons. Transcriptional activity in the regions corresponding to the three operons as determined using a DNA-tiling microarray for cells grown in LB medium [data adapted from Rasmussen S, Nielsen HB, Jarmer H (2009) The transcriptionally active regions in the genome of *Bacillus subtilis*. *Mol Microbiol* 73:1043–1057]. Tick marks indicate 2-kb intervals. Higher-intensity signals (*y* axis) correspond to higher levels of RNA hybridization (blue and red indicate the different strands of DNA). These data identify putative transcription start sites (arrows) located upstream of the *ypjD*, *panB*, *ylbQ*, *yojE*, and *ypyC* genes. Likely terminators (T) at the ends of transcription units are also shown. Note the apparent cotranscription of the *panBCD* genes with the upstream *ypjD* operon (*Top*) and *panE(ylbQ)* with *bshC(yllA)* (*Bottom Left*). Note that the tiling array contained iso-thermal probes (45–65 nt) in 22-nt intervals on each strand and an 11-nt offset between the strands.

Fig. S2

Fig. S3. Biosynthesis of GlcNAc-Mal and of Glc-Mal using BshA and BshB1(Ba). (*A*) Progress curve for preparative synthesis of GlcNAc-Mal as followed by UDP release and consumption of the UDP-GlcNAc substrate. (*B*) Mass spectral analysis (ESP–) of purified GlcNAc-Mal. (*C*) Mass spectrum (ESP+) of unpurified GlcN-Mal synthesized from GlcNAc-Mal using BshB1 from *B. anthracis*.

Fig. S3

Fig. S4. Dual-channel images of the thiol-redox proteome (Bodipy fluorescence images, red) compared with the Sypro-ruby–stained proteome (protein amount images, green) at control conditions (*Left*) and after exposure to 1 mM diamide (*Right*) in *B. subtilis* wild type (*A* and *B*), *bshA* (*C* and *D*), *bshB2* (*E* and *F*), and *bshB1bshB2* mutants (*G* and *H*). For labeling of reversible thiol-oxidations, cytoplasmic protein extracts of the *B. subtilis* wild type, *bshA, bshB2*, and *bshB1bshB2* mutants were harvested from untreated control cells and cells exposed to 1 mM diamide for 15 min (see *SI Materials and Methods* for details). Proteins with reversible thiol-oxidations under control conditions are marked in white, and those in which oxidation is increased after diamide treatment are labeled in red.

Fig. S4

Table S1. Strains used in the study

Strain	Genotype	Source or reference
CU1065	*trpC2 attSPβ*	Laboratory stock
JH642	*trpC2 pheA1*	Laboratory stock
HB8498	CU1065 *mgsA::kan*	This study
HB8499	CU1065 *mgsA bshB1::kan*	This study
HB11000	CU1065 *bshB1::cm*	This study
HB11001	CU1065 *mgsA bshB1 bshA* Kan^R	This study
HB11002	CU1065 *bshA::mls*	This study
HB11042	CU1065 *bshB2::spc*	This study
HB11051	HB11001 *amyE::P_{xyl}-bshA*	This study
HB11052	HB11002 *amyE::P_{xyl}-bshA*	This study
HB11053	CU1065 *bshB1::spc bshB2::cm*	This study
HB11079	CU1065 *bshC::kan*	This study
HB11089	HB11002 *bshC::kan*	This study
HB11091	HB11079 *amyE::P_{xyl}-bshC*	This study
HE8256	*E. coli* BL21 DE3(pLysS) contains *bshC* in pET16b	This study

Table S2. Primers used in the study

Name	Sequence	Use
mgsA-up-F	TTACACAGATATCCATGCCTG	Construction of HB8498
mgsA-up-R-K_m	CCTATCACCTCAAATGGTTCGCTGCTTGTCATGCGCGATCAAAG	
mgsA-down-F-K_m	CGAGCGCCTACGAGGAATTTGTATCGTTCGAATTCCGTGACCTTCTT	
mgsA-down-R-K_m	ATATACCCATTCGTCAGAGGA	
mgsA-up-F	TTACACAGATATCCATGCCTG	Construction of HB8499
mgsA-up-R-K_m	CCTATCACCTCAAATGGTTCGCTGCTTGTCATGCGCGATCAAAG	
ypjG-down-F-km	CGAGCGCCTACGAGGAATTTGTATCGGCGTGGAGTATGCCGAAGT	
ypjG-down-R-km	AGCCGCAAGCGCTGAGGA	
ypjG-up-F-Cm	CTTGCACAGCGTCCGTCTT	Construction of HB11000
ypjG-up-R-Cm	CTTGATAATAAGGGTAACTATTGCCTCATCACTGTGGGCGCCAA	
ypjG-down-F-Cm	GGGTAACTAGCCTCGCCGGTCCACGGGGCGTGGAGTATGCCGA	
ypjG-down-R-Cm	GATACACGCGCTCGTCTATA	
mgsA-up-F	TTACACAGATATCCATGCCTG	Construction of HB11001
mgsA-up-R-K_m	CCTATCACCTCAAATGGTTCGCTGCTTGTCATGCGCGATCAAAG	
ypjH-down-F-km	CGAGCGCCTACGAGGAATTTGTATCGGCGGCAATAGAGATGCTTGA	
ypjH-down-R-km	GCGCATCCTCTTGAAATCTAT	
ypjH-up-f-mls	TCGGAATGGGCGGCACAAT	Construction of HB11002
ypjH-up-R-mls	GAGGGTTGCCAGAGTTAAAGGATCGAGCCTCCAACGCTCGGA	
ypjH-down-f-mls	CGATTATGTCTTTTGCGCAGTCGGCTTGTCAGCCAGTATGAACAGA	
ypjH-down-R-mls	CGCCTGGCGAGAAGCTCT	
yojG-up-F	GGAGCAAGACGATGGACAAT	Construction of HB11042
yojG-up-R	CGTTACGTTATTAGCGAGCCAGTCGGCCTGCCACTCCATATGA	
yojG-down-F	CAATAAACCCTTGCCCTCGCTACGCGGCGAAGCTGATGTCGTA	
yojG-down-R	GAAACAATCGAGGAGACTGAT	
ypjh-F-comp	cgcgcgatCGGATGCTGATGCTTGATCA	Cloning of *bshA* in pSWEET
ypjh-R-comp	GCGggatCCTGATGGCCCGCTTCGAT	
yllA-Up-F	AGAACAGGCTGCGGCCATT	Construction of HB11079
yllA-Up-R	CCTATCACCTCAAATGGTTCGCTGATCTATATAGTGCTGTACAAACA	
yllA-down-F	CGAGCGCCTACGAGGAATTTGTATCGATTTGAACAGGTACGGTCCAA	
yllA-down-R	GCCGAGTGTGCAGTCTACA	
yllA-comp-F	gcgcttaatTAACTATTTATTGGAGAAAGGAAG	Cloning of *bshC* in pSWEET
yllA-comp-R	GCGAgaTCTACAACTTCATTTTACACTGT	
yllA-pet16	GAGCTCGAGATGCAGCTAACTGAACTTTC	Cloning of *bshC* in pET16b
yllA-comp-R	GCGAgaTCTACAACTTCATTTTACACTGT	
trxA-for	ggctatcgtaaaagcaac	trxA Northern probe
trxA-rev	CTAATACGACTCACTATAGGGAGAtcaactacttctccgtct	
ohrA-for	TTTCAGCGGTAGGAGGAAGA	ohrA Northern probe
ohrA-T7-rev	CTAATACGACTCACTATAGGGAGATCAATATTTCCGGAGGTTGC	
nfrA_for	gttgagaagaacggacactt	nfrA Northern probe
nfrA_rev	CTAATACGACTCACTATAGGGAGAccctttttctttcacgtagtc	
BshAN5	CACCATGAGAAAACTAAAAATAGGA	Overproduction of BshA
BshAN3	TCACTCCGGTTCTGCTAAATCGGC	