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Bacillithiol is an antioxidant thiol produced in *Bacilli*

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Abstract

Glutathione is a nearly ubiquitous low-molecular-weight thiol and antioxidant, although it is conspicuously absent from most Gram-positive bacteria. We identify here the structure of bacillithiol, a novel and abundant thiol produced by *Bacillus* species, *Staphylococcus aureus*, and *Deinococcus radiodurans*. Bacillithiol is the α-anomeric glycoside of L-cysteinyl-D-glucosamine with L-malic acid and likely functions as an antioxidant. Bacillithiol, like structurally similar mycothiol, may serve as a substitute for glutathione.

The presence of cysteine (**1**, Cys) in aerobic cells poses a problem owing to its rapid autoxidation as catalyzed by many transition metals with generation of peroxides that are toxic to cells[1]. For this reason the dominant low-molecular-weight thiol in most aerobes is a derivative of Cys in which the amino and carboxyl residues are blocked resulting in a marked decrease in metal catalyzed autoxidation. In most Eukaryota and many Gram-negative bacteria, the dominant low-molecular-weight thiol is glutathione (**2**, GSH)[2,3] in which the amino and carboxyl groups of Cys are blocked by the γ-glutamyl and glycine residues, respectively. Glutathione is maintained in the reduced state enzymatically by glutathione reductase. The development of this protective thiol system was a key step in the evolution of higher organisms[2].

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AUTHOR CONTRIBUTIONS MR and GLN prepared bacterial cultures for isolation of **8** and the survey of thiol distribution. GLN and TB purified **8**, GLN conducted the hydrolysis of **8** and the HPLC and enzymatic analyses. JJL and GLN conducted the NMR analysis of **8**, **11** and **12**. VKJ and CJH synthesized proposed hydrolysis intermediates **11** and **12**. GLN, MR, JJL, CJH, AC, JDH and RCF developed the project and interpreted the results. All authors contributed to the writing.

It was long ago recognized that many Gram-positive bacteria do not generate GSH but produce other low-molecular-weight thiols[4]. The most thoroughly studied of these alternative thiols is mycothiol (**3**, MSH) produced by the high G+C content Gram-positive Actinobacteria[5-7]. It is comprised of a Cys residue acetylated at the amine and linked via the carboxyl to a pseudodisaccharide comprised of glucosamine (**4**, GlcN) and *myo*-inositol (**5**). Mycothiol undergoes metal catalyzed autoxidation at a slower rate than GSH[8] and is maintained in the reduced state by mycothiol disulfide reductase[9].

In some low G+C content Gram-positive bacteria CoA (**6**) proved to be a major thiol[10]. It was found to undergo copper-catalyzed autoxidation at a four-fold slower rate than GSH[11] making it a suitable protective thiol for an aerobic organism. Although CoA is synthesized from Cys, the Cys residue is decarboxylated during the biosynthesis so CoA cannot function as a protected form of Cys from which Cys can be regenerated, as is the case for GSH and MSH. The gene encoding a CoA disulfide reductase was identified initially in *Staphylococcus aureus*[12] and homologs of this gene have been found in the genomes of a diverse collection of bacteria. Thus, CoA appears to function as a protective thiol in some bacteria but has the disadvantage that it cannot serve as a reservoir for regeneration of cysteine.

During studies of CoA related thiols in *Bacillus anthracis* an unidentified thiol was detected[13]. A thiol of the same molecular mass was found in *Bacillus subtilis* as a mixed disulfide with the redox controlled organic hydroperoxide resistance transcriptional regulator OhrR[14,15]. Recently this thiol was also detected in *Deinococcus radiodurans* at a sufficient level to permit isolation for structure determination.

Extraction of *D. radiodurans* in the presence of monobromobimane (**7**) generated the *S*-bimane derivative of the unknown thiol (**8**) that was purified by HPLC (Supplementary Methods). High-resolution mass spectral analysis of **8** gave a mass of 611.1648 Da corresponding to a formula for the positive ion of $C_{23}H_{32}O_{12}N_4SNa$ (theoretical mass 611.1630 Da). This is consistent with **8** originating from a thiol of mass 398 Da. We elucidated the composition of **8** by hydrolysis in HCl followed by HPLC analysis of the bimane derivatives and of glucosamine (Supplementary Methods). The studies indicated that the principal hydrolysis products contained Cys as its monobromobimane derivative CySmB (**9**), and GlcN (Fig. 1a). Assuming that **8** is a conjugate of CySmB and GlcN then the residual atoms $C_4H_6O_5$ could arise from malic acid attached to the GlcN via an ester or glycosidic link with loss of a water molecule. Using an enzymatic assay specific for L-malic acid (**10**, Supplementary Methods), we determined that one equivalent of L-malic acid was released per equivalent of **8** hydrolyzed, thereby confirming its composition (Fig. 1a).

The hydrolytic process generated transient species that were examined further. The bimane labeled fluorescent intermediates U8mB/U9mB (**11**), eluting at HPLC retention times of 8 and 9 min, respectively (Fig. 1b), were produced early during the hydrolysis but reached a maximum level at 8 h after which their level declined (Fig. 1a). When the 4 h hydrolysis sample was chromatographed and the individual U8mB, U9mB and **8** peaks were collected and reanalyzed the results showed that **8** produced a single peak corresponding to the original peak collected (Supplementary Fig. 1). However, purified U8mB and U9mB each

generated the same two peak HPLC profile and this was identical to that obtained for the synthetic bimane derivative of *N*-(L-cysteinyl)-D-glucosamine (**11**, L-CySmB-D-GlcN, Fig. 1b). Authentic L-CySmB-D-GlcN was mixed with the 4 h hydrolysis sample and was found to coelute with U8mB and U9mB (Fig. 1b). Synthetic *S*-bimanyl-*N*-(D-cysteinyl)-D-glucosamine (**12**, D-CySmB-D-GlcN) produced a completely different HPLC profile (Fig. 1b) and this result also rules out L-CySmB-L-GlcN as the structure for U8mB and U9mB. The HPLC analysis does not rule out U8mB and U9mB being the α- and β-anomers of *N*-(D-cysteinyl)-L-glucosamine (D-CySmB-L-GlcN) but this was excluded using chiral HPLC to show that the CySmB produced in the hydrolysis of **8** is L-CySmB and not D-CySmB (Supplementary Fig. 2 and Supplementary Methods). Thus, U8mB and U9mB represent the α- and β-anomers of L-CySmB-D-GlcN (**11**, Fig. 2a). Compound **8** is therefore a conjugate of L-malic acid with L-CySmB-D-GlcN (**11**).

The site of L-malic acid attachment was determined by nuclear magnetic resonance studies. A series of 1D and 2D NMR spectra were collected (Supplementary Figs. 3a-m) and each carbon and proton residue was assigned by examining a combination of gCOSY, TOCSY, HSQC and HMBC data (Fig. 2b; Supplementary Table 1). HMBC correlations were key to identifying each component and its linkage within these systems. The amide bond between Cys and glucosamine was established by an HMBC correlation from the 2′ proton of glucosamine to the 1″carbon of Cys (Supplementary Fig. 3i). The glycosidic bond was supported by a weak HMBC correlation from the 1′ proton of glucosamine and the 2‴ carbon of malic acid (Supplementary Fig. 3i). We then applied a rotating-frame Nuclear Overhauser Effect spectroscopy (ROESY) to establish that the L-malate 2-hydroxyl is attached in a glycosidic linkage to C1′ of glucosamine, and the H(1′)-H(2′) coupling constant of 3.7 Hz established the alpha configuration at C1′ (Supplementary Figs. 3a, e-g). A summary of the NOE interactions observed in the ROESY spectra is provided in Fig. 2c. This identifies the structure of **8** as the bimane derivative of the naturally produced thiol **13**, 2-[2-{S-L-cysteinyl}amino-2-deoxy-α-D-glucopyranosyloxy]-(2S)-butanedioic acid (Fig. 2d).

Compound **13** appears to be the first example of L-malate glycosidically linked to glucosamine in a natural product. The only other malyl-glycoside so far reported is L-malic acid-β-D-glucopyranoside (**14**), isolated as a taste component from morel mushrooms[16] and also from the medicinal plant *Synadenium pereskiifolium*[17]. MSH is related to **13** in that both thiols are based upon the L-Cys-D-GlcN moiety but MSH is acetylated at the α-amino group of L-Cys and has the GlcN linked α(1-1) to *myo*-inositol rather than α(1-2) to L-malate (Fig. 2d,e).

The production of **13** was surveyed in selected bacteria. It was found in all 7 strains of *Bacillus* examined and at a low level in *Geobacillus* (Supplementary Table 2); we therefore propose the common name “bacillithiol” and the abbreviation BSH for **13**. BSH is present in other, but not all, members of the class *Bacilli*, including the genera *Staphylococcus* and *Streptococcus*, and in *D. radiodurans* (class *Deinococci*). A major unknown thiol previously detected in *Staphylococcus aureus* Strain SH1000[18] very likely corresponds to bacillithiol. The intracellular level of GSH or MSH in bacteria is estimated to be 3-7 mM (Supplementary Table 3), ~30 times the BSH level in the Bacilli. The ability of the aerobic Firmicutes to tolerate environmental oxidative stress with such low levels of BSH suggests

that BSH may have characteristics not shared by GSH or MSH. Of the 9 bacteria surveyed that produce significant levels of either GSH or MSH none was found to have a significant level of BSH (Supplementary Table 2) suggesting that BSH, like GSH and MSH, may also have an antioxidant function in addition to that provided by CoA.

Intracellular redox ratios such as GSH/GSSG and MSH/MSSM are maintained by specific disulfide reductases generating a substantially reduced thiol redox status. Analysis of the thiol and disulfide forms present in exponential phase *D. radiodurans* and *B. subtilis* gave respective redox ratios of 100 ± 20 and 400 ± 140 for bacillithiol and 46 ± 6 and 120 ± 80 for cysteine (n = 3), Supplementary Table 3. Thus, BSH is substantially reduced in the cell with a redox ratio comparable to that established for GSH in *E. coli* (320) and MSH in *M. bovis* BCG (100), *M. smegmatis* (400), and *M. tuberculosis* Erdman (360), Supplementary Table 3.

The reductase system responsible for maintaining BSH in the reduced state is not readily identified in *B. subtilis*. As pointed out previously *B. subtilis* lacks homologs of the coenzyme A disulfide reductase (CoADR) found in other *Bacillus* species[19]. *A* blastp search of the *B. subtilis* strain 168 genome sequence with the mycothiol disulfide reductase (Mtr) and glutathione reductase (Gor) sequences identified three homologous proteins annotated as dihydrolipoamide dehydrogenases (Supplementary Table 4). One of these proteins, PdhD, has been functionally verified as a dihydrolipoamide dehydrogenase[20] but either of the other two proteins, YqiV or YfjH, could be a bacillithiol disulfide reductase. Alternatively the redox status of bacillithiol may be maintained by the thioredoxin/thioredoxin reductase system[21,22].

The finding that *B. subtilis* treated with cumene hydroperoxide (**15**) accumulates mixed disulfide forms of the Ohr repressor OhrR with a 398 mass thiol (BSH) suggests a function for bacillithiol in redox sensing by OhrR[14]. Although OhrR also formed mixed disulfides with Cys and CoA, the single largest observed peak was with BSH. Since these low-molecular-weight thiols are present at approximately equal levels in the cell, this suggests that BSH may be the dominant redox active thiol during oxidative stress in *B. subtilis*. Metal ion complexation can play a role in redox sensing and control, and the position of the cysteinyl- and malyl-moieties of BSH on adjacent carbons of glucosamine suggests that complexation with metals is feasible as modeled in Fig. 2f.

Determination of the function of BSH in bacteria will ultimately require production of mutants deficient in its biosynthesis. Since BSH is found at substantial levels in a spectrum of bacteria, including the significant pathogens *B. anthracis* and *S. aureus*, elucidation of its biochemistry and function is an important undertaking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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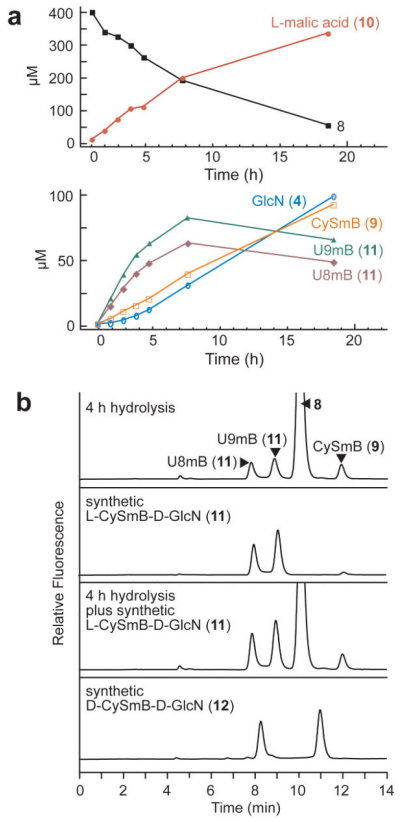

FIG. 1.

The hydrolysis of **8** defines its composition. (**a**) Hydrolysis of **8** in 5 N HCl at 60°C initially results in the release of L-malic acid (**10**), as determined enzymatically (top), and production of the transient species U8mB and U9mB (**11**) (bottom). Further hydrolysis of U8mB and U9mB leads to the release of CySmB (**9**) and GlcN (**4**). A representative example of three independent experiments is shown. (**b**) HPLC analysis of the bimane derivatives from a 4 h hydrolysis sample of **8** (panel 1), and of the bimane derivative of synthetic L-CySmB-D-GlcN (**11**, panel 2) and D-CySmB-D-GlcN (**12**, panel 4). Synthetic L-CySmB-D-GlcN was mixed with the 4 h hydrolysis sample and coinjected showing that U8mB and U9mB are α, β anomers of L-CySmB-D-GlcN (panel 3) and not D-CySmB-D-GlcN (panel 4).

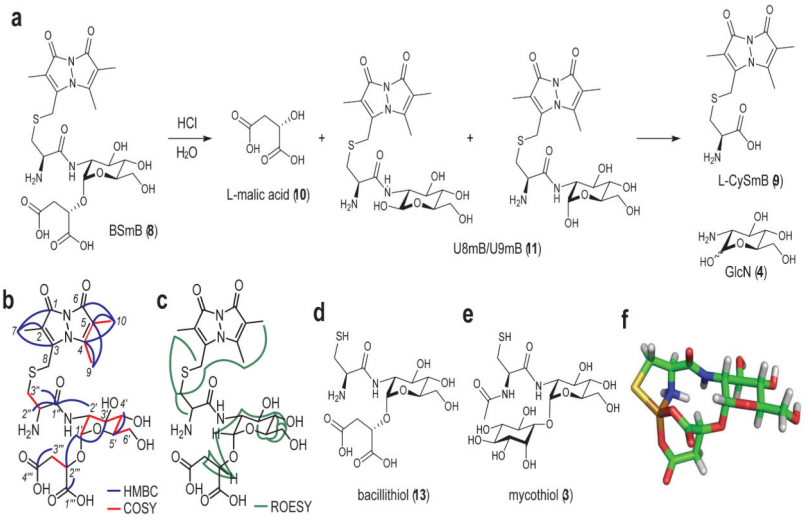

FIG. 2.

NMR studies confirm the composition of **8**, establish linkage in **8** and define the structure of bacillithiol (**13**): (**a**) Structure of **8** and of products from acid hydrolysis of **8**; (**b**) HMBC and gCOSY interactions depicting connectivity within **8** and carbon numbering (Supplementary Figs. 3c, 3d, 3i and 3j); (**c**) NOE interactions in **8** as identified in ROESY spectra (Supplementary Figs. 3e, 3f and 3g); (**d**) structure of bacillithiol (**13**); (**e**) structure of mycothiol (**3**); (**f**) a model depicting a complex of a metal (brown) by bacillithiol: H, white; C, green; N, blue; O, red; and S, yellow.

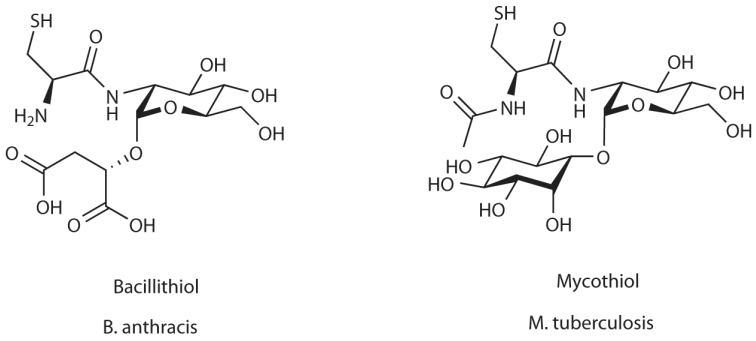

FIG. 3.