The Bacillus subtilis monothiol bacilliredoxin BrxC (YtxJ) and the Bdr (YpdA) disulfide reductase reduce S-bacillithiolated proteins

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The bacterial cytosol is generally a reducing environment with protein cysteine residues maintained in their thiol form. The low molecular weight thiol bacillithiol (BSH) serves as a general thiol reductant, analogous to glutathione, in a wide range of bacterial species. Proteins modified by disulfide bond formation with BSH (S-bacillithiolation) are reduced by the action of bacilliredoxins, BrxA and BrxB. Here, the YtxJ protein is identified as a monothiol bacilliredoxin, renamed BrxC, and is implicated in BSH removal from oxidized cystolic proteins, including the glyceraldehyde 3-phosphate dehydrogenases GapA and GapB. BrcX can also debacillithiolate the mixed disulfide form of the bacilliredoxin BrxB. Bdr is a thioredoxin reductase-like flavoprotein with bacillithiol-disulfide (BSSB) reductase activity. Here, Bdr is shown to additionally function as a bacilliredoxin reductase. Bdr and BrxB function cooperatively to debacillithiolate OhrR, a transcription factor regulated by S-bacillithiolation that results from conditions of disulfide stress.

1. Introduction

Cells contain several systems to maintain cysteine residues and other thiols in their reduced form in the cytosol, and dedicated pathways for the controlled oxidation of cysteines to form disulfide bonds in secreted and periplasmic proteins. Maintaining the redox balance of cellular thiols is critical for cell physiology, and oxidation can lead to a specific type of oxidative stress known as disulfide stress [1–4]. Reduction of intracellular thiols ultimately relies on the reducing power of NADPH, which serves as a cofactor to reduce thiol groups in either protein-based thiold reductants like thioredoxin (Trx) or low molecular weight (LMW) thiols. Oxidized Trx is reduced by a dedicated NADPH-dependent thiol reductase. The best characterized LMW thiol is the cysteine-containing tripeptide, glutathione [5]. Under oxidizing conditions, protein thiols may form intra- or intermolecular protein disulfides or be modified by GSH in a process known as S-glutathionylation (or S-thiolation in general). S-glutathionylation is a common post-translation modification [6–8], and is reversed by disulfide exchange reactions with proteins with high reduction potential such as glutaredoxins (Grx) [9]. Oxidized Grx proteins are typically reduced by GSH, and the oxidized GSH (GSSG) is reduced by an NADPH-dependent glutathione disulfide oxidoreductase. Grx and Trx proteins share a common motif, known as the Trx-fold, consisting of four stranded β-sheets and surrounded by three α-helices [10]. Grx are classified into dithiols, with CPTC active site motifs, and monothiols, with a typical CGPS active site [11,12]. De-glutathionylation of substrate proteins is catalyzed by thiol transfer to the conserved Cys residue (the amino-terminal residue in the dithiol class proteins) followed, in a second step, by de-glutathionylation of the Grx protein. Most dithiol class Grx proteins use a monothiol mechanism in which the initially formed S-glutathionylated Grx (Grx-SSG) is reduced either by excess thiol or by a specific oxidoreductase [12,13]. Alternatively, in a dithiol mechanism the Grx-SSG intermediate may be resolved by the second (resolving) Cys residue resulting in a protein disulfide [12].

In Firmicutes bacteria, including Bacillus and Staphylococcus species, bacillithiol (Cys-GlcN-Malate; BSH) is the major LMW thiol and serves as a functional analog of GSH [7,14]. Although relatively recently discovered, comparative genomics suggests that BSH and its derivatives

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are widely distributed in Bacteria and Archaea [15–17]. We first identified BSH by virtue of its role in modification of the redox sensor protein OhrR in vivo, leading to a novel 398 Da adduct [18]. Subsequent chemical characterization revealed that BSH is a glycoside of L-cysteinyl-o-glucosamine with N-malic acid [19]. Similar to GSH, BSH has diverse roles in cell physiology including detoxification of reactive electrophiles and some antibiotics [7,8,20], FeS cluster biogenesis [21–23], buffering of thiophilic metal ions [24–26], and protection of protein thiols by oxidation to mixed disulfides (S-bacillithiolation) [27]. S-bacillithiolation is widespread in Bacillus subtilis under stress conditions including exposure to cumene hydroperoxide (CHP) and bleach [27]. In the case of OhrR, S-bacillithiolation is regulatory and controls the ability of OhrR to repress expression of the OhrA peroxiredoxin [18]. S-bacillithiolation may also regulate enzyme activity [28–30]. Modification of methionine synthase MetE results in a transient growth arrest in the absence of exogenous methionine [31,32], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is also modified by S-thiolation [29].

Following their inactivation, S-bacillithiolated OhrR (OhrR-SSB) and MetE (MetE-SSB) can be reduced by two bacillirredoxin (BxR) proteins, BrxA and BrxB [32]. Both BrxA and BrxB are dithiol class enzymes and are detected in vivo in their S-bacillithiolated forms after oxidative stress. If BxR proteins functioned predominantly by an intramolecular (dithiol) mechanism, we would expect the oxidation to result in an intramolecular disulfide with release of free (reduced) bacillithiol. The observed accumulation of S-bacillithiolated BrxB protein after treatment with oxidants suggests that they work, in part, by a monothiol mechanism. However, mutation of the second (resolving) Cys residue leads to even greater accumulation of S-bacillithiolated BrxB proteins, suggesting that a dithiol mechanism may also be operative. It remains unclear, however, how the oxidized BrxA and BrxB proteins are recycled [32]. Bdr (formerly YpdA) is an NADPH-dependent disulfide oxidoreductase found in those organisms that encode the enzymes for BSH synthesis [33], suggesting a role in reduction of BSSB and/or bacillirredoxins. Indeed, the S. aureus Bdr ortholog (formerly YpdA, 63% identity) has NADPH-dependent BSSB reductase in vitro [34,35], and mutants have increased levels of intracellular BSSB and elevated sensitivity to oxidative stress [34,36]. BSSB reductase activity has also been determined for the B. cereus ortholog, and the structures of the B. cereus and S. aureus Bdr proteins have been recently reported [35]. Bdr can also reduce other BSH-derived disulfides. For example, Bdr can reduce BSH that has been modified by S-thioallylation by the reactive diallyl thiosulfinate allicin, a natural product associated with garlic, to form S-allylmercaptobacillithiol (BSSA) [37]. Bdr is probably not the only pathway for reduction of BSSB in vivo, since even null mutants still maintain a reduced BSH pool even after treatment with strong oxidants like HOCI [34,36].

Here, we report biochemical studies of a third bacillirredoxin, the monothiol-class protein BrxC (YtxJ). A proteomic survey identifies numerous candidate BrxC substrates, including BrxB and Bdr. We provide evidence that Bdr also functions as a NADPH-dependent bacillirredoxin reductase, and that this provides an additional pathway for reduction of S-bacillithiolated BrxB. By regeneration of BrxB, Bdr can function in a redox cascade to increase the efficacy of BrxB-mediated de-bacillithiolation of the OhrR-SSB mixed disulfide.

2. Results

2.1. Proteomics-based identification of potential substrates of BrxC

Previously, we identified several genes encoding thioredoxin family proteins that co-occur in genomes that also encode enzymes for the biosynthesis of BSH [35]. This type of statistical correlation suggests that these co-occurring proteins may recognize BSH or BSH-modified targets. Two of these proteins, BrxA and BrxB, have been shown to function as bacillirredoxins that de-bacillithiolate OhrR-SSB and MetE-SSB [32]. Here, we have investigated the biochemical activities of two other co-occurring proteins, BrxC (YtxJ) and Bdr (YpdA). BrxC contains a thioredoxin-like fold and a TCIPS motif characteristic of monothiol glutaredoxins [38].

To identify substrates for BrxC, the purified protein was bound to CNBr-activated beads to generate an affinity column, as previously described for the identification of Grx substrates [39,40]. BSH concentration varies depending on the growth phase, reaching peak levels (3.5–5.2 mM) during stationary phase [41]. Therefore, cell lysates from late stationary phase cultures were incubated with the BrxC-bound beads with the expectation that S-bacillithiolated proteins might interact with BrxC to generate disulfide-linked protein-protein complexes. Retained proteins were eluted using DTT and identified by MS/MS-based analysis of tryptic peptides, and those identified in three independent biological replicas were tabulated. Encouragingly, the resultant list of proteins (Table S1) included many known targets of S-bacillithiolation (e.g. GapA, GapB, MetE, SerA, PpaC, GuaB, ArOa), bacillirredoxins (BrxA, BrxB), the BSSB reductase Bdr (YpdA), and enzymes with reactive thiols (AhpC, AhpF, AhpA, Tpx, TrxA, TrxB), as seen in prior proteomics studies [42]. However, this list was larger than expected (231 proteins) compared to the number of previously identified targets of S-bacillithiolation (~70 [42]), and included many protein chaperones and ribosomal subunits. Indeed, ~10% of the identified proteins lack Cys residues. We also recovered the E2 subunits (PdhC, OdhB, BkdB) of three dehydrogenases known to have lipoic acid as a covalently attached cofactor. We therefore suspected that some of the proteins bound to the BrxC column and eluted with DTT were retained non-specifically, possibly as part of protein complexes.

To identify those proteins that are likely to be direct targets for BrxC, the affinity purification was repeated with addition of a 6 M guanidine hydrochloride wash to unfold and remove non-covalently associated proteins prior to elution with DTT. Using this more stringent protocol, ~20 proteins were reproducibly retained (n = 3) and all contained at least one cysteine residue (Table 1). Among the putative BrxC-target proteins, BrxB and Bdr were detected together with several other proteins previously shown to be S-bacillithiolated in vivo under oxidative stress conditions. The latter include AbrB, PyrAB, PyrG, PyrE, PyrA, GapB, SucC and TutA [31,42]. These proteins are therefore candidates for in vivo substrates for de-bacillithiolation by BrxC. We chose four candidate substrates for further investigation: GapA, GapB, BrxB, and Bdr.

| Identified Proteins | cellular process |
|---------------------|------------------|
| BrxC [3]            | bacillirredoxin   |
| BdrA [3]            | acetoin/butanediol dehydrogenase |
| SalA [1]            | control of alkaline protease expression |
| PyrH [1]            | pyrimidine biosynthesis |
| PyrAA [6]           | pyrimidine biosynthesis |
| PyrAB [8]           | pyrimidine biosynthesis |
| PyrG [7]            | pyrimidine biosynthesis |
| PyrE [4]            | pyrimidine biosynthesis |
| GapB [4]            | glycolysis |
| PpRA [5]            | glycolysis |
| YtxJ [3]            | NADP-dependent malate dehydrogenase |
| SucC [5]            | tricarboxylic acid cycle |
| Bdr (YpdA) [3]      | oxidoreductase activity |
| FxnD [3]            | formylation of Met-tRNA(Met) |
| AbrB [1]            | transition state regulator |
| TutA [2]            | elongation factor Tu (translation) |
| RpsB [1]            | translation |
| GapA [3]            | translation |
| RpsK [1]            | translation |
| RpsL [1]            | translation |
2.2. *B. subtilis* encodes two glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoforms, GapA and GapB, which are required for glycolysis and gluconeogenesis, respectively [43]. GapB was recovered in our proteomics screen under stringent washing conditions (6 M guanidine HCl) and GapA was recovered reproducibly (3 biological replicates) under low stringency conditions, and in 2 of 3 replicates under high stringency conditions. This suggests that both GapA and GapB are $S$-bacillithiolated and are candidate substrates for BrxC.

To test whether BrxC might be involved in de-bacillithiolation, we expressed GapA-FLAG and GapB-FLAG proteins in *B. subtilis*. To monitor the effect of BrxC on de-bacillithiolation, we used a katA ahpCF (hpx) mutant strain lacking catalase and alkylhydroperoxidase and therefore deficient in the degradation of endogenously generated $H_2O_2$. This strain has elevated basal levels of oxidative stress, allowing the study of protein $S$-bacillithiolation without the addition of strong oxidants. Compared to the wild-type strain, the hpx mutant cells had little if any increase in GapA and GapB $S$-bacillithiolation. BrxC is a candidate monothiol bacilliredoxin encoded as part of a stress-inducible operon (ytxGH brxC) under the control of $\sigma^H$ and $\sigma^I$ (Fig. S1) [44]. When compared to hpx mutant cells, the hpx strain additionally carrying a brxC operon deletion (ytxGH brxC) had a substantial increase in the level of both GapA and GapB $S$-bacillithiolation (Fig. 1). As expected the signal seen with the anti-BSH antibodies is lost in cells additionally carrying a bshC mutation that abolishes BSH synthesis. These results support the hypothesis that the stress-inducible ytxGH brxC operon functions in de-bacillithiolation of GapA and GapB in vivo. The functions of YtxG and YtxH are unknown, and it is likely that this phenotype reflects the activity of BrxC as a bacilliredoxin.

2.3. BrxC functions as a bacilliredoxin in vitro

In previous work, we defined BrxA and BrxB as prototype bacilliredoxin proteins that can de-bacillithiolate mixed disulfides of BSH with OhrR and MetE [32]. Both proteins have a Trx-fold with a dithiol (CGC) active site motif (sequence alignments available in Fig. S1 of [34]). Genetic studies indicate that substrate de-bacillithiolilation generates BrxB-SSB (bacillithiolated on Cys52), and this intermediate accumulates to a greater extent in protein lacking the second (resolving) Cys54 residue [32]. Thus, the pathways that regenerate reduced BrxB appear to include both the protein disulfide (dithiol mechanism) and mixed disulfides (monothiol mechanism), but the subsequent reduction steps are not defined.

Since BrxB was recovered on our BrxC affinity column (Table 1), we hypothesized that BrxC might function as a monothiol bacilliredoxin (Fig. S1C) to catalyze de-bacillithiolilation of BrxB-SSB. Indeed, incubation of BrxB-SSB with BrxC results in the partial transfer of the BSH moiety from BrxB to BrxC (Fig. 2A, lane 2). BrxB-SSB was not de-bacillithiolated by excess BSH (Fig. 2A, lane 5).

2.4. BrxC de-bacillithiolitates Bdr in vitro

*S. aureus* Bdr (YpdA) functions as an NADPH-dependent BSSB reductase [34,35]. Since *B. subtilis* Bdr is a close homolog (63% identity), we hypothesized that this protein may also reduce BSSB. We overexpressed *B. subtilis* Bdr as a His-tagged fusion protein in *E. coli*. As expected, purified Bdr had the characteristic yellow color of FAD-containing enzymes, with absorbance maxima at 273, 374 and 453 and a shoulder at 484 nm (Fig. S2), similar to the reported spectrum of *S. aureus* Bdr [34]. To monitor BSSB reductase activity, we exchanged the protein into nitrogen-saturated buffers and monitored NADPH consumption with and without BSSB. Despite several attempts we did not detect any BSSB reductase activity under these conditions. Our protein preparation was active, since under aerobic conditions Bdr is an NADPH:O$_2$ oxidoreductase (NADPH oxidase) with formation of H$_2$O$_2$ as ultimate product (Figs. S3 and S4). When BSH was included in the reactions, H$_2$O$_2$ formation was diminished, as was the concentration of BSH, suggestive of H$_2$O$_2$-dependent oxidation of BSH (Fig. S4). As expected, H$_2$O$_2$ formation was observed in aerobic, but not in anaerobic buffers.

Since Bdr was reproducibly retained on our BrxC affinity column (Table 1), we wished to identify possible site(s) of *S*-bacillithiolation.

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![Fig. 1. GAPDH enzymes have increased $S$-bacillithiolation in strains lacking BrxC.](image)

Immunopurification and Western blot analysis of *B. subtilis* strains expressing FLAG-tagged GapA or GapB expressed in different mutant backgrounds. Wild-type (WT) is *B. subtilis* 168 strain CU1065, and the hydroperoxidase minus (hpx) derivative is a katA ahpCF triple mutant. Strains were grown in LB at 37 °C with vigorous shaking to early stationary phase. Cell extracts were generated from equal cell numbers (judged by OD$_{600}$) and lysates enriched using anti-Flag antibodies coupled to magnetic beads and then analyzed SDS-PAGE followed by Western blot analysis using anti-BSH antibodies. The red asterisk highlights the bands corresponding in size to GapA-FLAG (left) and GapB-FLAG (right). Other bands represent proteins cross-reactive with the anti-FLAG beads and anti-BSH antibodies. Representative results are shown from replicate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

![Fig. 2. BrxC is a monothiol bacilliredoxin and Bdr is bacilliredoxin reductase.](image)

Bacillithiolated BrxB (2 μM) was incubated with BrxC (A: 1 μM and B: 0.4 μM increments), and when indicated Bdr (0.4 μM) with 0.1 mM NADPH, or 1 mM BSH, and the level of protein $S$-bacillithiolation was monitored by immunoblot analysis using anti-BSH antibodies.
B. subtilis Bdr contains 3 cysteine residues (C14, C122 and C220), with C14 conserved in Bdr orthologs from other Firmicutes [34] (Fig. S3D), but not in more distantly related homologs [35]. We purified all possible single, double, and triple Cys to Ala mutant proteins (Fig. S3A). All seven mutant proteins retain NADPH oxidase activity, although some with modestly reduced activity compared to the wild-type protein (Fig. S3B).

When assayed with the thiol modification reagent methyl methanethiosulfonate (MMTS), a single adduct was detected at C220 (Fig. 3A). Incubation of Bdr with BSH under aerobic conditions revealed formation of an S-bacillithiolated species (Bdr-SSB) as detected using anti-BSH antibodies. Bdr S-bacillithiolation was also seen for the C14A and C122A single mutants, but not for the C220A mutant (Fig. 3B). This suggests that Bdr can be S-bacillithiolated on C220, which is surface-exposed [35]. By homology modeling on the related B. cereus structure [35], we predict that the C14 and C122 residues are too distant (11.7 Å) to form a disulfide bond (Fig. S3E), and their lack of reactivity may be due to steric constraints. Consistent with the hypothesis that Bdr-SSB is a BrxC substrate, addition of BrxC leads to efficient de-bacillithiolation of Bdr, with concomitant appearance of the S-bacillithiolated BrxC product. As expected, this activity requires the active site C31 residue in BrxC (Fig. 3C).

2.5. Bdr (YpdA) functions as a bacilliredoxin reductase

We next hypothesized that Bdr might act as a bacilliredoxin reductase. Indeed, Bdr in the presence of NAPDH de-bacillithiololated BrxB-SSB, but did not appear to strongly affect the level of BrxC-SSB (Fig. 2A, lanes 3 and 4). Even when present at comparable amounts in the reaction, BrxC did not fully reverse the S-bacillithiollation of BrxB-SSB (Fig. 2B). However, Bdr and NAPDH efficiently reduced both BrxB-SSB and BrxC-SSB, with BrxB a better substrate than BrxC (Fig. 2B). To determine if Cys residues in Bdr are required for bacilliredoxin reductase activity, we purified variant proteins with one or more Cys residues replaced with Ala (Fig. S3A). These variant proteins all retain NADPH oxidase activity, although those variants with a C14A substitution had up to a two-fold reduction in activity (Fig. S3B).

2.5. Bdr (YpdA) functions as a bacilliredoxin reductase

As a bacilliredoxin reductase, Bdr functions catalytically with the native protein reducing the level of BrxB-SSB by 73% even when this substrate was in 5-fold molar excess over Bdr (Fig. 3C). Mutation of any of the three Cys residues led to reduced activity, and the triple mutant was essentially inactive. Whether this loss of activity indicates a role for thiol chemistry in catalysis, or simply a defect in protein conformation or substrate-binding, is not clear.

Previously, BrxA and BrxB were shown de-bacillithiolate the DNA-binding transcription factor OhrR [32], which is regulated by S-bacillithiolation [18]. Since BrxB works stoichiometrically to de-bacillithiolate OhrR, and Bdr can catalytically de-bacillithiolate BrxB-SSB, we tested whether BrxB could function catalytically to reduce OhrR-SSB if it was regenerated by Bdr. OhrR-SSB was incubated with BrxB and Bdr with and without NADPH and the bacillithiolated peptides were measured by MS/MS analysis. As previously shown [32], in the absence of NADPH, BrxB was able to partially de-bacillithiolate OhrR-SSB and this resulted in the formation of BrxB-SSB. Addition of Bdr and NADPH resulted in the near complete de-bacillithiololation of both OhrR-SSB and BrxB-SSB (Fig. 4). Thus, Bdr can act as bacilliredoxin reductase to recycle...
BrxB-SSB, thereby increasing the efficiency of regulatory de-bacillithiolation (Fig. 5).

3. Discussion

Bacillithiol (BSH) is a major LMW thiol that functions in redox buffering, metal homeostasis, and resistance to reactive electrophiles. As a reactive nucleophile, BSH can serve as a cofactor for conjugation to reactive electrophilic compounds, including the antibiotic fosfomycin [33]. In this role, conjugation often requires the activity of bacillithiol S-transferases, including FosB [45,46] and several additional proteins of unknown specificity [20]. However, in other cases conjugation is spontaneous, as with the reaction of BSH with methylglyoxal to generate the BSH-hemithioacetal that is further processed by a specific glyoxalase [47]. BSH also serves as an important thiol buffer, and reacts with protein thiols to form disulfides, presumably proceeding through a transient sulfenic acid intermediate [6,7,27]. In B. subtilis, at least 70 proteins have been defined as targets of S-bacillithiolation in cells treated with oxidants [42], and many of these modifications are seen in other organisms and with other LMW thiols [29,48]. Reduction of the resultant S-bacillithiolated proteins is thought to depend on the action of bacilliredoxins, including the previously described BrxA and BrxB proteins [32,34].

During our initial studies of the BSH biosynthesis pathway, we used phylogenomic analyses to identify several putative redox-active proteins that are correlated with BSH biosynthesis genes across bacterial genomes [33]. Two of these proteins, with redox active CXC motifs, were subsequently shown to function as bacilliredoxins and named BrxA and BrxB [32]. Here, we have defined new functions for two additional members of this group, BrxC (YtxJ) and Bdr (YpdA). BrxC is a monothiol bacilliredoxin and functions similarly to glutaredoxins: small proteins that can reduce GSH-mixed disulfides in proteins as well as glutathionylated small molecules [49,50]. A related class of proteins, designated mycoredoxins, functions with proteins modified by a different LMW thiol, mycothiol [51,52].

To define substrates for BrxC we used affinity trapping, which relies on the in vitro interaction of the oxidoreductase with its substrates followed by proteomics analysis [39,40]. Candidate BrxC substrates include proteins from multiple metabolic pathways including carbon metabolism, amino acid synthesis, sporulation, translation, and oxidative damage response (Table 1). Many of these targets are S-bacillithiolated under oxidative stress conditions [31,42]. Here, we provide evidence that deletion of the operon encoding BrxC leads to increased S-bacillithiolation of two GAPDH isoforms (GapA and GapB) in cells with increased oxidant levels due to mutation of catalase and alkylhydroperoxide reductase (Fig. 1). This suggests that BrxC is involved in protein de-bacillithiolation in vivo. BrxC also functions in vitro to de-bacillithiolate BrxB (Fig. 2) and Bdr (Fig. 3). All three bacilliredoxins are expressed across a range of growth conditions [53], but only BrxC is induced as part of the σE-dependent general stress response.

Bdr (YpdA) is a flavin-containing oxidoreductase, and the S. aureus and B. cereus orthologs reduce BSSB back to BSH [34,35]. It is likely that B. subtilis Bdr also has BSSB reductase activity, but this can be difficult to detect due to a high background of oxygen-dependent NADPH oxidase activity [35] (Fig. S3B). Bdr is itself S-bacillithiolated on C220 (Fig. 3), and was identified as a candidate substrate for the BrxC bacilliredoxin (Table 1). Indeed, BrxC can de-bacillithiolate Bdr in vitro (Fig. 3). The regulatory role, if any, of Bdr S-bacillithiolation is unknown.

By virtue of its ability to function as a bacilliredoxin reductase, Bdr can increase the efficiency of Brx proteins that might otherwise accumulate in their oxidized states. Here, we document this effect for the reactivation of the regulatory protein OhrR, a repressor inactivated in vivo and in vitro by S-bacillithiolation [18,54]. The bacillithiolated OhrR can be reactivated by either BrxA or BrxB [32]. Bacillithiolated BrxB is in turn reactivated by the bacilliredoxin reductase Bdr, which couples its reduction to NADPH, or by the bacilliredoxin BrxC (Fig. 5). These findings highlight the complexity of the intertwined thiol-disulfide exchange reactions that sustain the activity of many cysteine-containing enzymes.
4. Material and methods

4.1. Strains and growth conditions

*B. subtilis* and *Escherichia coli* strains were grown on LB or MOPS-based minimal media [55]. All *B. subtilis* strains are derivatives of the CU1065 parent strain (168 trpC2 attSP1; Bacillus genetic stock center #1A100) (Table S2). Unless otherwise indicated, liquid media were inoculated from an overnight pre-culture and incubated at 37 °C (for macrodilincoamidine-streptogramin B (MLS) resistance), spectinomycin (100 μg/ml), chloramphenicol (10 μg/ml), kanamycin (15 μg/ml) and neomycin (10 μg/ml).

4.2. DNA manipulations

Routine molecular biology procedures were done according to Ref. [56]. Restriction enzymes, DNA ligase, Klenow fragment and DNA polymerase were used according to the manufacturer’s instructions (New England Biolabs). Mutants were constructed using long-flanking-homology PCR as described [57]. Site-directed mutagenesis of *brxC* strains carrying expression plasmids were cultured in 1 L LB medium, and cells were incubated overnight at 14 °C. For BrxC and BrxB expression, constructs were transformed using pET16b as follows: coding sequences were amplified by PCR and overlapped extension according to Ref. [58]. Site-directed mutagenesis of *bdr* was done using QuickChange II (Agilent) according to the manufacturer’s instructions. Constructs for expression of Bdr mutant proteins were generated using the following primers: ypdA-C14A-F 5′GCTGGACATCTGCTGCCATTC3′, ypdA-C14A-R 5′GAATGGCACAGATAGTCCACGCTCCCGCATTAATA3′, ypdA-C122A-F 5′ATGCTATCATCGCCACAGGCTATTAT3′, ypdA-C122A-R 5′ATAATAGCCGTGCGGATGATGACATATGGGCTGTGTAATCTC3′, ypdA-C20A-F 5′GCTTGTGAAAATTACCCGGAAT3′, and ypdA-C20A-R 5′ATTTCTGGTATTTTTTCTGCAGCAGGCTTCAAATTTCCATAAGGAT3′.

4.3. Expression and purification of His-tagged BrxB, BrxC and Bdr

*E. coli* BL21(DE3) pLysS was used for overproduction of His-tagged BxRB (NP_390279.1), BxRC (NP_390854.1), and Bdr (NP_390176.2) proteins. Expression plasmids for protein overproduction were constructed using pET16b as follows: coding sequences were amplified by PCR with *B. subtilis* chromosomal DNA as template, and the products were cloned in the Ndel-BamHI sites of pET16b. The resulting plasmids were confirmed by DNA sequencing and transformed into *E. coli* BL21 (DE3) pLysS. For BrxB and BrxC expression, *E. coli* BL21(DE3)pLysS strains carrying expression plasmids were cultured in 1 L LB medium, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added at mid-log phase (OD600 of 0.5) for 2 h. For Bdr, IPTG was added at 0.3 mM and cells were incubated overnight at 14 °C to avoid inclusion body formation. Recombinant His-tagged proteins were purified using PrePase™ His-Tagged High Yield purification Resin (Life Technologies) under native conditions according to the instructions of the manufacturer. His-tagged proteins were eluted in 50 mM NaH2PO4, 300 mM NaCl, 1 mM EDTA, 10% glycerol and 250 mM imidazole pH 8.0. A second step of purification was performed using a size exclusion Superdex 200 column using 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT and 1 mM EDTA 10% glycerol (v/v) and stored at −80 °C. Protein purity was assessed using SDS-PAGE.

4.4. Immunoprecipitation and western blotting

Strains containing FLAG-tagged GapA or GapB under xylose induction were used in immunoprecipitation with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich) and quantified by Western blotting analysis using Anti-FLAG or Anti-BSH antibodies as described [32,59], except that goat anti-rabbit IgG-horse radish peroxidase (Santa Cruz Biotechnology; sc-2004) was used as a secondary antibody and the blots were developed using the Clarity Western ECL Substrate (Bio-RAD; 1705060).

4.5. Preparation of BrxC affinity column and identification of target proteins

Target proteins that interact with BrxC were identified as described [39,40]. Briefly, three milligrams of BrxC protein in sodium carbonate buffer (pH 8.3) was incubated with 0.5 g of CNBr-sepharose activated beads (Sigma) according to the manufacturer’s instructions. The CNBr reacted sites were blocked by incubation for 1 h with 1 M Tris-HCl, pH 8.0 and the beads were stabilized in binding buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5% glycerol and 1 mM EDTA). Wild type *B. subtilis* cell lystate from a stationary phase culture was incubated with BrxC-immobilized beads in binding buffer overnight at 4 °C. Beads were washed three times with binding buffer and three times with binding buffer that contain 0.3 M NaCl. In the first attempts, proteins that interact with the BrxC affinity column were eluted using binding buffer containing 20 mM DTT. This wash method resulted in the recovery of proteins that interact with BrxC directly and those that were in complexes retained on the column. To identify proteins that interact covalently with BrxC, the experiment was repeated and a third wash was done using binding buffer with 6 M guanidine HCl before elution with DTT. Proteins in the eluate were TCA precipitated, washed with cold acetone, and separated on 4–20% gradient SDS-PAGE. Proteins were identified using mass spectrometry (MS/MS) analysis to sequence tryptic peptides in the sample (Biotechnology Resource Center, Cornell University). Only proteins that were detected in three biologically independent experiments were further considered.

4.6. Preparation and quantitation of S-bacillithiolated proteins in vitro

S-bacillithiolation of BrxC, BrxB and Bdr was done by incubation of 50 μM protein in the presence of 0.5 mM BSH in 50 mM air-saturated Tris pH 8.0, 150 mM NaCl, 1 mM EDTA and 5% glycerol at room temperature for 1 h. OhrR (50 μM) was bacillithiolated by treatment with cumene hydroperoxide (0.5 mM) in the presence of BSH (1 mM). Bdr and BrxB were reduced and subjected to buffer exchange to remove excess BSH or the reducing agent. Buffer exchange was done at least twice using micro-spin columns (Bio-Rad) pre-washed three times with 50 mM Tris pH 8.0, 150 mM NaCl and 10% glycerol.

For quantitation, S-bacillithiolated proteins were detected by immunoblots using anti-BSH antibodies or by excision of proteins bands from coomassie-stained, non-reducing SDS-PAGE gels followed by MS/MS analysis to quantify S-bacillithiolated peptides as previously described [32].

4.7. Anaerobic bacilliredoxin assays

De-bacillithiolation assays were done under anaerobic conditions in a nitrogen glove box. All buffers were bubbled with nitrogen gas for 30 min and equilibrated in the anaerobic chamber overnight. To exchange all proteins into anaerobic buffer, Micro-Spin columns were washed once with anaerobic buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 10% glycerol). The spin columns were incubated overnight in the anaerobic chamber and then washed three times with anaerobic buffer. All proteins were buffer-exchanged twice in the anaerobic equilibrated Micro-Spin columns. S-bacillithiolated proteins (at 2 μM) were incubated with different concentrations of bacilliredoxins at room temperature for 15 min and the reaction was stopped by addition of anaerobically equilibrated, non-reducing SDS-loading buffer. Samples were separated by 4–20% gradient SDS-PAGE and S-bacillithiolation levels were detected using western blot analysis using anti-BSH antibodies.
4.8. NADPH oxidase assay

Bdr was reduced by addition of 1 mM DTT and incubated on ice for 5 min. Bdr was buffer exchanged twice using buffer exchange micro-spin columns (Bio-RAD) that were pre-washed three times with 50 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 10% glycerol. Protein concentration was determined using the Bradford assay with BSA as standard. Bdr (typically at 5 μM) concentration was determined using the Bradford assay with BSA as standard. Bdr was reduced by addition of 1 mM DTT and incubated on ice for 5 min.

Declaration of competing interest

The authors declare that they have no competing interests relative to the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.101935.

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