Geobacillus thermodenitrificans YjbH recognizes the C-terminal end of Bacillus subtilis Spx to accelerate Spx proteolysis by ClpXP

Chio Mui Chan, Saurabh Garg,† Ann A. Lin and Peter Zuber

Division of Environmental and Biomolecular Systems, Institute of Environmental Health, Oregon Health and Science University, 20000 NW Walker Rd, Beaverton, OR 97006, USA

Proteolytic control can govern the levels of specific regulatory factors, such as Spx, a transcriptional regulator of the oxidative stress response in Gram-positive bacteria. Under oxidative stress, Spx concentration is elevated and upregulates transcription of genes that function in the stress response. When stress is alleviated, proteolysis of Spx catalysed by ClpXP reduces Spx concentration. Proteolysis is enhanced by the substrate recognition factor YjbH, which possesses a His–Cys-rich region at its N terminus. However, mutations that generate H12A, C13A, H14A, H16A and C31/34A residue substitutions in the N terminus of Bacillus subtilis YjbH (BsYjbH) do not affect functionality in Spx proteolytic control in vivo and in vitro. Because of difficulties in obtaining soluble BsYjbH, the Geobacillus thermodenitrificans yjbH gene was cloned, which yielded soluble GtYjbH protein. Despite its lack of a His–Cys-rich region, GtYjbH complements a B. subtilis yjbH null mutant, and shows high activity in vitro when combined with ClpXP and Spx in an approximately 30 : 1 (ClpXP/Spx : GtYjbH) molar ratio. In vitro interaction experiments showed that Spx and the protease-resistant SpxDD (in which the last two residues of Spx are replaced with two Asp residues) bind to GtYjbH, but deletion of 12 residues from the Spx C terminus (SpxΔC) significantly diminished interaction and proteolytic degradation, indicating that the C terminus of Spx is important for YjbH recognition. These experiments also showed that Spx, but not GtYjbH, interacts with ClpX. Kinetic measurements for Spx proteolysis by ClpXP in the presence and absence of GtYjbH suggest that YjbH overcomes non-productive Spx–ClpX interaction, resulting in rapid degradation.

INTRODUCTION

The global regulator Spx, which activates gene transcription in response to disulfide stress, is a substrate of the ATP-dependent protease ClpXP (Nakano et al., 2003). Proteolytic regulation is mediated by YjbH, a putative substrate recognition factor for ClpXP-catalysed Spx proteolysis, to maintain low or pre-stress levels of Spx (Larsson et al., 2007). As with Spx, YjbH is highly conserved in Gram-positive bacteria. Studies of YjbH have revealed its potential biological importance, for example, in control of haemolytic activity in Listeria monocytogenes (Zemansky et al., 2009) and glycopeptide sensitivity and desiccation tolerance in Staphylococcus aureus (Charbonnier et al., 2005; Chaibenjawong & Foster, 2011). Additionally, inactivation of YjbH leads to moderate resistance to oxacillin and other β-lactam antibiotics in S. aureus (Göhring et al., 2011). All of the studies suggested that YjbH plays important roles in pathogenicity, probably due to its contribution to the regulation of the oxidative stress response.

YjbH is functionally related to the adaptor proteins that confer substrate specificity to regulated proteolysis catalysed by ATP-dependent Clp proteases, such as ClpAP and ClpXP (Dougan et al. 2002; Levchenko et al., 2000). The proteases are composed of a hexameric, ATP-hydrolysing ‘unfoldase’ (ClpA or ClpX) and two heptameric rings of the ClpP subunit (Porankiewicz et al., 1999). The ClpP multimer forms the proteolytic chamber that receives the unfolded substrate protein from the hexameric ATPase component of the protease. ClpS and Spb are adaptor proteins that tether substrates to the protease ATPase component (ClpA or ClpX, respectively). This is accomplished through direct interaction of the adaptor with both substrate and protease.

YjbH protein of Bacillus subtilis has been obtained from an insoluble state by denaturation and renaturation during affinity column purification (Garg et al., 2009). Zinc was

1Present address: Vollum Institute, Oregon Health and Science University, Portland, OR, USA.

Abbreviations: CV, column volumes; NTA, nitrilotriacetic acid.

Two supplementary tables and eight supplementary figures are available with the online version of this paper.
required for renaturation and solubility, and studies of intact and N-terminal-truncated protein have suggested that the N-terminal His–Cys rich region was necessary for Zn atom coordination. A proposed model of YjbH control predicted that oxidation would result in Zn release and inactivation of YjbH with subsequent release of Spx from proteolytic control (Garg et al., 2009). However, the putative Zn-binding N-terminal region is not conserved among all YjbH orthologues. Recent studies of S. aureus YjbH have provided evidence that the Cys residues associated with the N-terminal region of YjbH are not necessary for activity (Engman et al., 2012). Alternative explanations for regulation of YjbH-mediated proteolysis of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered. For example, a small protein, YirB, has been shown to inhibit the Spx of Spx may have to be considered.

In this study, the N-terminal His–Cys-rich region of the YjbH protein was subjected to amino acid substitutions to create mutant YjbH derivatives that were tested in vivo and in vitro for proteolysis-enhancing activity. The results indicate that the residues do not function in proteolytic control of Spx in B. subtilis. Because of the uncertainties associated with assaying activity of renatured protein preparations, a YjbH orthologue (GtYjbH) was identified that is produced by Geobacillus thermodenitrificans. GtYjbH was produced from an Escherichia coli expression system in soluble form. The gene encoding GtYjbH can complement a yjbH null mutant of B. subtilis and the complemented strain is responsive to oxidative stress by induction of Spx activity. This was observed despite the absence in GtYjbH of most of the His–Cys rich region that coordinates Zn in the YjbH orthologue of B. subtilis. Unlike renatured B. subtilis YjbH (BtYjbH), GtYjbH is highly active, mediating rapid substrate degradation in reactions containing approximately 30-fold less GtYjbH than ClpXP or Spx in terms of molar equivalents. Unlike other well-studied adaptors (Dougan et al., 2002; Levchenko et al., 2000; Persuh et al., 2002; Schlothauer et al., 2003), no evidence of YjbH–ClpX interaction was detected. However, like the adaptor SpxB (Levchenko et al., 2005), YjbH requires recognition of the C-terminal residues of Spx to enhance ClpXP-catalysed proteolysis.

**METHODS**

**Bacterial strains, plasmids and chemicals.** B. subtilis strains and plasmids are listed in Table S1 (available with the online version of this paper) and are all JH642 derivatives. G. thermodenitrificans was obtained from the Bacillus Genetic Stock Center (Columbus, Ohio). E. coli strain DH5α was used for general cloning procedures. Plasmid pET-23a and E. coli strain BL21(DE3)plyS were from Novagen, and were used in heterologous protein production. Medium components were from Difico. All restriction/modifying enzymes were from New England Biolabs. Oligonucleotide primers (Table S2) were from Invitrogen. The Ni-nitrilotriacetic acid (NTA) resin was from 5 PRIME (PerfectPro Ni-NTA agarose) and PCR/plasmid purification kits were from Qiagen. High-Q anion exchange and heparin prepack columns were from Bio-Rad. All analytical grade chemicals were from Sigma–Aldrich unless otherwise stated.

**Construction of yjbH mutants.** Codon substitutions introduced into the N-terminal coding end of the yjbH gene were generated by a two-step PCR procedure described previously (Nakano et al., 2010). Complementary oligonucleotides (Table S2) specifying each nucleotide substitution that would change YjbH codons to Ala codons were designed: for H12A, oligonucleotides SG-08-13 and SG-08-14; C13A, oligonucleotides SG-08-15 and SG-08-16; H14A, oligonucleotides SG-08-17 and SG-08-18; H16A, oligonucleotides SG-08-19 and SG-08-20; C31A/34A, oligonucleotides SG-08-23 and SG-08-24. The reverse primer of the mutagenic complementary pair was combined with SG-08-4 for PCR of the N-terminal coding end of the YjbH coding sequence, and the forward primer of the mutagenic complementary pair was combined with oligonucleotide SG-07-4 to amplify the C-terminal ~900 bp of the yjbH coding sequence. The two products of the separate PCRs were then combined together with oligonucleotides SG-08-4 and SG-07-4 (Table S2) in a PCR to generate the intact mutant yjbH coding sequence. For production of mutant YjbH protein, the fragments were cleaved with Nhel and Xhol and ligated into Nhel/Xhol-cleaved pET23a. The ligations were used to transform strain DH5α, and then in strain BL21(DE3)plyS to construct mutants versions of pDR111 plasmids bearing yjbH. PCR products were obtained with primers SG-08-8 and SG-08-9 (Table S2) for PCR amplification using pET23a plasmids carrying yjbH mutant alleles as templates. The PCR fragment generated contained the complete mutant yjbH coding sequence with a Hiso tag. The fragment was cleaved with HindIII and Nhel, followed by ligation with HindIII/Nhel-cleaved pDR111 (Britton et al., 2002). The ligations were used to transform strain DH5α. The resulting mutant plasmids (Table S1) were transformed into B. subtilis strains for complementation using the same method as for pEC-22 (GtyjbH) described below.

**Assay of β-galactosidase activity.** Cells were grown in Difico sporulation medium (DSM) (Nicholson & Setlow, 1990) to OD_600

Log_2 0.3–0.4 at 37 °C, followed by induction with 1 mM IPTG, and samples were withdrawn at 30 min intervals during growth. β-Galactosidase activity in each sample was determined as described previously (Nakano et al., 1988) and is presented as Miller units (Miller, 1972).

**Diamide treatment.** Mid-exponential-phase cells in minimal TSS medium (Fouet et al., 1997) were treated with 0.5 mM diamide to induce mutant yjbH expression at 37 °C. After 30 min, cells were treated with 1 mM diamide (1 M stock). Culture samples were collected at 0, 10 and 25 min after diamide treatment. Whole-cell lysates were prepared by the protoplast isolation method (Harwood & Cutting, 1990). Protein samples (20 μg) were resolved by SDS-PAGE. The protein was transferred onto a nitrocellulose membrane and immunoblotting was performed to detect Spx, BsYjbH and GtYjbH with anti-Spx (Nakano et al., 2001), anti-BsYjbH (Kommieneni et al., 2011) and anti-His antibodies, respectively.

**In vitro proteolysis reactions.** In vitro proteolysis was carried out in 60 μl reaction mixtures containing 50 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, 5 mM DTT, 5 mM ATP, 5 mM creatine phosphate, 0.05 U creatine kinase ml⁻¹ (Sigma), and ClpX, ClpP, Spx and YjbH (concentrations are indicated in figure legends) at 37 °C. At the indicated time intervals (see figure legends), a 12 μl sample from each reaction mixture was collected, treated with 3 μl 5 x SDS loading dye (with 0.1 M DTT), and heated at 95 °C for 5 min. The protein samples were resolved on a 15 % SDS-PAGE gel,
visualized by Coomassie blue R-250 staining. Levels of Spx after proteolysis were determined as ratios of Spx to ClpP band intensities, since ClpP concentrations in each set of reactions were equal. The Spx:ClpP value in a reaction mixture at 0 min time point was defined as 100% (Zhang & Zuber, 2007).

**Protein purification.** For *G. thermodenitrificans* YjbH–His6, the yjbH coding sequence was amplified by PCR from *G. thermodenitrificans* genomic DNA, using the forward primer EC-26 and the reverse primer EC-28 (Table S2). PCR products were inserted into pET23a to construct pEC-21. The pEC-21-transformed *E. coli* BL21(DE3)pLysS cells were grown at 37°C in LB broth containing 50 μg ampicillin ml⁻¹ and 5 μg chloramphenicol ml⁻¹ to OD₆₀₀ 0.4–0.5, and expression was induced with 0.5 mM IPTG for 3 h at 37°C. The resulting amyrase-negative strain (ORB8166, amyE:: GtyjbH–His6, trxB:: trxB–lacZ) was transformed with chromosomal DNA of ORB86952 (JH642, yjbH:: tet) with selection on DSM agar containing 75 μg spectinomycin ml⁻¹ and 5 μg chloramphenicol ml⁻¹. To confirm the integration of GtyjbH–His6 at the amyE locus, clones were streaked on an LB-starch plate and grown for 16 h at 37°C. The resulting amylose-negative strain (ORB88169, amyE:: GtyjbH–His6, trxB:: trxB–lacZ) was transformed with chromosomal DNA of ORB86952 (JH642, yjbH:: tet) with selection on DSM agar containing 75 μg spectinomycin ml⁻¹, 12.5 μg tetracycline ml⁻¹ and 5 μg chloramphenicol ml⁻¹. The resulting strain (ORB88169) was used to determine if GtyjbH–His6 complements the yjbH-null mutation by testing Spx protein level in the absence and presence of diamide and by assaying for trxB-directed β-galactosidase activity as described above.

**In vitro affinity interaction assays using Ni-chelate chromatography.** Different combinations of proteins (indicated in figure legends) Spx or His₆–Spx (2.5 μM) or Spx²₀ (1.8 μM), GtyjbH (2.5 μM), ClpX (4 μM), ClpP (4 μM), ATP or ATPγS (80 μM) were incubated in buffer A (100 mM Tris/HCl, pH 8.5, 50 mM NaCl) for 10 min at room temperature. The protein mixtures were then applied to a 50 μl Ni²⁺–NTA affinity agarose column (5 PRIME) that was pre-equilibrated with buffer A. The column was washed with 20 CVs buffer W (20 mM Tris/HCl, pH 7.6, 200 mM NaCl, 30 μM imidazole). The bound protein was eluted in buffer E (20 mM Tris/HCl, pH 7.6, 200 mM NaCl, 250 μM imidazole). Eluted fractions that contain GtyjbH–His6, were pooled and diluted with buffer QA [20 mM Tris/HCl (pH 8.5), 2 % glycerol (v/v), 1 mM DTT] to lower the salt concentration to 40 mM. The diluted sample was applied to a High Q or heparin column equilibrated with buffer QA. The bound proteins were eluted with buffer QB (buffer QA containing 1 M NaCl) in a 0–100% gradient. Fractions containing GtyjbH–His6, were resolved on a 15 % SDS-PAGE gel, visualized by Coomassie blue staining, concentrated and stored in buffer (20 mM Tris/HCl, pH 7.6, 100 mM NaCl, 10 % glycerol) at −80°C. Protein concentration was measured using Bio-Rad protein assay, and BSA was used to generate the standard curve.

ClpX and ClpP were purified as described in previous studies using the intein-mediated purification with an affinity chitin-binding tag (IMPACT) system (Liu et al., 1999; Nakano et al., 2002). His₆-tagged wild-type Spx and Spx²₀ proteins were purified using a previously published procedure (Nakano et al., 2003). The histidine tag on Spx was removed using TeV (tobacco etch virus) protease (provided by Kelly Chacon, Ohio State University) (Nakano et al., 2005). SpxHA and SpxACHA were purified using the IMPACT system and RNA polymerase was purified using a Ni affinity column as described in previous studies (Nakano et al., 2005).

**Table 1.** Kinetic measurements of Spx proteolysis by ClpXP in the presence and absence of GtyjbH

<table>
<thead>
<tr>
<th>Substrate</th>
<th>His-tag adaptor</th>
<th>Vₘₐₓ (μM min⁻¹)</th>
<th>Kₘ (μM)</th>
<th>k-cat (s⁻¹)</th>
<th>k-cat/Kₘ (μM s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BsSpx</td>
<td>− GtyjbH</td>
<td>0.24 ± 0.06</td>
<td>0.42 ± 0.17</td>
<td>0.0081 ± 0.0020</td>
<td>21324 ± 7956</td>
</tr>
<tr>
<td>BsSpx</td>
<td>+ GtyjbH</td>
<td>1.98 ± 0.48</td>
<td>1.79 ± 0.66</td>
<td>0.0659 ± 0.0161</td>
<td>38320 ± 8729</td>
</tr>
</tbody>
</table>

**RESULTS**

**Mutagenesis studies on the His–Cys-rich domain of *B. subtilis* YjbH**

Sequence alignments show that *B. subtilis* and *G. thermodenitrificans* share a conserved YjbH [identities=150/273 (55%), similarities=199/273 (73%)] with a putative redox CxxC motif, but *Geobacillus* lacks a His–Cys-rich region at the N terminus (Fig. S1). Previous studies suggested that the His–Cys-rich region is important for Zn²⁺ binding, for interaction between Spx and YjbH, and also for the enhancement of ClpXP-dependent Spx proteolysis (Garg et al., 2009). To study the roles of the N-terminal His and Cys residues in the BsYjbH, mutants bearing amino acid substitutions (H12A, C13A, H14A, H16A and C31A/34A) were constructed and tested by complementation in a yjbH-null strain. Mutated yjbH encoding a product with six histidines appended to the N terminus was introduced into the amyE locus as a single-copy, IPTG-inducible construct in the *B. subtilis*. A yjbH-null strain with only the IPTG-inducible promoter (*P¹hsyn*) in the amyE locus was used.
as negative control. The Spx level was determined by monitoring the $\beta$-galactosidase activity of a promoterless lacZ gene, which is fused with the promoter region of trxB (thioredoxin reductase gene) – a gene that requires Spx for optimal expression. The $\beta$-galactosidase activities of all strains producing the BsYjbH mutants upon IPTG induction were similar to those of strains producing wild-type (WT) BsYjbH (Fig. 1a). Also, immunoblot analysis of samples collected 1 h after IPTG induction showed decreased levels of Spx in WT and in strains producing mutant BsYjbH (Fig. 1b), suggesting that the mutant YjbH proteins are functional in ClpXP-dependent proteolytic control of Spx following recovery from oxidative stress. These results indicate that all the mutants showed little if any defect in YjbH-dependent enhancement of Spx proteolysis. An oxidant treatment experiment in which the thiol-specific oxidant, diamide (1 mM) was added to the cultures showed that BsYjbH mutants responded to oxidant in the same fashion as WT BsYjbH as is evident by the increase in the Spx level (data not shown). In vitro proteolysis assays showed that WT and mutant BsYjbH enhanced ClpXP-catalysed Spx proteolysis, and addition of the YjbH inhibitor YirB reduced proteolysis (Fig. S2). Taken together, the data support the conclusion that the His and Cys residues in the N terminus are not essential for function of BsYjbH in the enhancement of ClpXP-catalysed Spx proteolysis.

**Complementation of GtYjbH in a B. subtilis yjbH strain**

As stated earlier, GtYjbH lacks the putative Zn-binding motif at the N terminus, but still shares significant sequence similarities with BsYjbH, including the putative redox-sensing CxxC motif. Experiments were conducted to test if GtYjbH was able to complement the B. subtilis yjbH-null strain, and to determine if GtYjbH activity was responsive to oxidant treatment. A strain was constructed that bears an IPTG-inducible GtYjbH–His$_6$ construct at the amyE locus. A complementation assay was conducted in the same way as described in the previous section. GtYjbH proteins behaved in the same fashion as described in the previous section. GtYjbH proteins behaved in the same fashion as BsYjbH as shown in two *in vivo* assays: (1) both showed greatly decreased trxB-directed $\beta$-galactosidase activity compared with yjbH-null mutation (Fig. 2a); (2) both were sensitive to oxidant, with increased Spx concentration observed after treatment with 1 mM diamide (Fig. 2b). The data show that although GtYjbH lacks the His–Cys-rich motif, it exhibits similar functionality to BsYjbH with respect to control of Spx proteolysis *in vivo*.

![Graph showing $\beta$-Galactosidase activity over time after IPTG induction](a)

**Fig. 1.** BsYjbH mutants show similar activity to wild-type BsYjbH. (a) Effect of the wild-type and mutant on Spx-dependent expression of trxB–lacZ. The graph shows the IPTG induction time-course of $\beta$-galactosidase activity encoded by the trxB–lacZ fusion in yjbH mutant cells bearing the $P_{\text{hyspank}}$–BsYjbH (white) and mutants [coloured (see key)] or the $P_{\text{hyspank}}$ promoter (black) in a yjbH-null mutant background. $\beta$-Galactosidase activity is expressed as Miller units. (b) Immunoblot analysis of Spx level using anti-Spx antiserum (Nakano *et al.*, 2002). Cell samples were collected before IPTG induction and at 1 h after IPTG induction from the strains in (a).
GtYjbH enhances ClpXP-catalysed proteolysis of Spx

In vivo studies showed that GtYjbH was functional in B. subtilis. Expression of GtYjbH complemented a B. subtilis yjbH-null mutant and was responsive to diamide treatment. To test the ability of GtYjbH to facilitate Spx proteolysis in vitro, a proteolysis assay reaction composed of Spx, ClpXP, ATP and a creatine kinase/creatine-phosphate ATP-regenerating system was performed in the presence and absence of GtYjbH (Fig. 3a, b). Notably, in contrast with BsYjbH, GtYjbH was partially soluble, and could be purified using a Ni-column and HiQ or heparin column chromatography to reach >95% purity (Fig. S3). Compared with renatured BsYjbH, the same concentration of GtYjbH (4 μM) showed a much higher activity in terms of Spx (8 μM) degradation. In 2 min, 90% of Spx was degraded by ClpXP (Fig. 3c). Interestingly, a low concentration (0.16 μM) of GtYjbH added to the proteolysis reaction resulted in 90% degradation of Spx (5 μM) in 4 min (Fig. 3d). This result suggested that the adaptor GtYjbH may act catalytically to enhance Spx proteolysis by ClpXP. As expected, this activity is also ATP- and ClpX-dependent (Fig. 3c).

The C terminus of Spx is required for YjbH binding

A series of experiments were carried out to uncover the determinants mediating interaction between Spx and GtYjbH. Previous studies showed that a mutant Spx having a 12 amino acid deletion of its C-terminal end (SpxΔC) was present at high concentration in vivo (Lin & Zuber, 2012), suggesting that removal of the C-terminal residues renders Spx resistant to proteolysis. An in vitro proteolysis assay showed that SpxΔC resisted proteolysis by ClpXP in the presence or absence of GtYjbH (Fig. 4a). Moreover, in the presence of both SpxΔC and Spx WT, the rate of Spx WT proteolysis was not affected (Fig. S4). These results suggested that SpxΔC is unable to interact with GtYjbH or ClpX, and thus did not compete with wild-type Spx for contact with YjbH or protease in the proteolysis reaction. We hypothesized that the C terminus of Spx is required for
effective GtYjbH interaction. In vitro affinity interaction experiments were performed using GtYjbH–His6 and nickel-chelate chromatography to detect the interaction between GtYjbH and Spx. As expected, most of the WT Spx was bound to immobilized GtYjbH–His6 and co-eluted with GtYjbH after application of imidazole to the Ni column (Fig. 4c). On the other hand, little SpxD was bound to and co-eluted with GtYjbH–His6 (Fig. 4d). A negative control with only WT Spx and SpxD showed no non-specific Spx binding to the Ni column (Fig. S5a, b and Fig. 5a). WT Spx is observed in the wash fractions and was not detected in elution or as immobilized protein on the column beads (data not shown). In vitro competition affinity interaction experiments with reactions containing WT Spx and SpxD showed that only WT Spx co-eluted with GtYjbH–His6, indicating interaction between GtYjbH and WT Spx and loss of recognition by GtYjbH when the 12 C-terminal residues of Spx are deleted (Fig. 5b).

A far-Western blot was performed to confirm that the C terminus of Spx is required for interaction between Spx and YjbH. For these experiments, an influenza haemagglutinin (HA) epitope-tagged version of Spx was utilized. An allele of spx was constructed that encodes a product with a C-terminal HA tag. SpxDHA, with the HA tag appended to the Spx deletion derivative, resists proteolysis. SpxHA, full-length Spx with the C-terminal HA tag, behaves similarly to Spx WT with respect to YjbH-mediated proteolysis in vivo (Fig. S6). Both SpxHA and SpxDCHA were used as far-Western substrates. Each was incubated with filters onto which gel-resolved GtYjbH, ClpX and RNA polymerase were electro-blotted. RNA polymerase was used as a positive control, since both epitope-tagged Spx derivatives were expected to show interaction with the RNA polymerase α subunit. The filters that were incubated with the Spx derivatives were then treated with anti-HA monoclonal antiserum, and Western analysis was

**Fig. 3.** GtYjbH enhanced Spx proteolysis mediated by ClpXP, which is ATP- and ClpX-dependent. (a, b) SDS-PAGE shows the effect of GtYjbH on ClpXP-catalysed proteolysis of Spx in vitro in a time-course experiment. Spx (8 μM), ClpX (3 μM) and ClpP (8 μM) with an ATP-generating system (creatine kinase) were incubated at 37 °C for the times (min) indicated in the absence (a) or presence (b) of GtYjbH (4 μM). (c) Plot of Spx band intensities against the reaction time in the experiments shown in the graph. The intensities of ClpP protein in each lane were used as internal controls (Zhang & Zuber, 2007). The Spx/ClpP ratio at 0 min was defined as 100 %. (d) Proteolysis assay under the same conditions as in (a, b) but in the presence of 0.16 μM GtYjbH.
**Fig. 4.** GtYjbH recognizes the C terminus of Spx. Deletion of 12 amino acids at the Spx C terminus (SpxΔC) greatly diminished GtYjbH binding. (a) SDS-PAGE shows the effect of GtYjbH on ClpXP-catalysed proteolysis of SpxΔC in vitro in a time-course experiment. SpxΔC (4 μM), ClpX (3 μM) and ClpP (8 μM) with an ATP-generating system (creatine kinase) were incubated at 37 °C for the times (min) indicated in the absence (a) or presence (b) of GtYjbH (4 μM). (b) Far-Western blotting to detect Spx interaction with GtYjbH. Proteins that interact with SpxΔCHA were detected by anti-HA antibodies. RNA polymerase α subunit was used as a positive control for Spx interaction. (c, d) In vitro Ni-affinity interaction experiments to detect contact between GtYjbH–His₆ and Spx (c) or SpxΔC (d). M, marker; I, input; FT, flowthrough; W, last wash; E1–3, elution fractions 1–3.

**Fig. 5.** Competition in vitro Ni-affinity interaction experiments show Spx outcompetes SpxΔC for GtYjbH binding. (a) Spx WT and SpxΔC proteins applied to a Ni affinity column. Column fractions separated by SDS-PAGE and stained with ‘Blue silver’ colloidal Coomassie G-250 protein. (b) Spx WT, SpxΔC and GtYjbH–His₆ reaction applied to Ni affinity column. Fractions separated by SDS-PAGE with staining as in (a). M, marker; I, input; FT, flowthrough; W, last wash; E1–3, elution fractions 1–3.
conducted. The far-Western results indicated that only SpxHA showed strong binding to GtYjbH–His6, as evident by reaction of the GtYjbH band with anti-HA antibodies after SpxHA treatment (Fig. 4b). The other two membranes incubated with and without SpxCHA showed minimal interaction and little non-specific binding of anti-HA antibodies, respectively. Overall, the data presented herein indicate that the deletion of 12 amino acids from the Spx C terminus mostly abolished the binding to GtYjbH, and this region increases the specificity of the GtYjbH–Spx interaction.

In vitro affinity interaction shows that Spx binds to ClpX in the absence of GtYjbH

Further in vitro affinity interaction assays were performed to test interactions between Spx, GtYjbH, ClpX and ClpP. Protein mixtures composed of GtYjbH–His6, Spx and ClpX with or without ClpP were applied to a Ni-NTA column in the presence of non-hydrolysable ATP-γ-S, but only GtYjbH–His6 and Spx co-eluted from the column, which suggested again strong interaction between GtYjbH and its substrate (Fig. 6a, b). In a reaction containing His₆-Spx, ATP-γ-S and ClpX with and without ClpP, the result showed that a small amount of ClpX co-eluted with His₆-Spx (Fig. 6c). Similar affinity interaction experiments performed with or without ATP produced the same results (Fig. S7). From these in vitro interaction studies, we concluded that Spx establishes a weak interaction with ClpX and that GtYjbH does not contact ClpX.

spxDD is an allele encoding a product in which the last two amino acid residues of Spx (Ala and Asn) are replaced with Asp (Nakano et al., 2003). This change renders the protein impervious to ClpXP-catalysed degradation. An affinity interaction assay showed that the change does not affect Spx interaction with GtYjbH (Fig. 6d).

Kinetics of ClpXP-catalysed Spx proteolysis with and without GtYjbH

Kinetic measurements were conducted to provide insight into the mechanism of YjbH-enhanced Spx proteolysis. Increasing concentrations of Spx were incubated with GtYjbH, ClpXP and creatine kinase, an ATP-generating
system, and the remaining Spx was quantified from SDS-PAGE in order to obtain initial rates (Fig. S8). Experiments were performed in triplicate to obtain a standard deviation. The results are listed in Table 1 as two sets of data, in the absence \((K_m=0.42 \pm 0.17 \mu M, V_{max}=0.24 \pm 0.06 \mu M \text{ min}^{-1})\) or presence \((K_m=1.79 \pm 0.66 \mu M, V_{max}=1.98 \pm 0.48 \mu M \text{ min}^{-1})\) of 0.5 \(\mu M\) GyYjbH. In the presence of GyYjbH, the turnover rate is approximately eightfold higher with an increased \(K_m\) value. The increased \(V_{max}\) value but increased \(K_m\) value may be explained as an uncompetitive 'inhibitor' effect of GyYjbH, suggesting that YjbH overcomes low \(K_m\), non-productive Spx binding to ClpX to enhance the rate of proteolysis.

**DISCUSSION**

Previous studies (Garg et al., 2009; Kommineni et al., 2011; Larsson et al., 2007) showed that YjbH acts as a substrate recognition factor to enhance Spx proteolysis by ClpXP. YjbH bears a His–Cys-rich region on the N terminus which is important for Zn metal binding and for enhancement of proteolysis of Spx by ClpXP (Garg et al., 2009). To investigate the roles of the histidines and cysteines in the YjbH N terminus, YjbH mutants bearing amino acid substitutions (H12A, C13A, H14A, H16A and C31A/C34A) were tested for *in vivo* activity and responsiveness to diamide treatment. Results showed that all the mutants behaved like the wild-type YjbH, indicating that mutation on the N terminus did not affect YjbH functionality with respect to Spx regulation. The loss of function when YjbH lacks the 23 aa N terminus may be caused by gross changes in YjbH protein structure. At present, we do not know if YjbH activity is regulated in response to stress. The small protein YirB can exert a negative effect on YjbH activity when overproduced, and has been shown to interact with YjbH in *vivo* and *in vitro* (Kommineni et al., 2011). However, no Spx-related phenotype has been uncovered in the *yirB* deletion mutant. Response to thiol stress that proteolytically controls Spx concentration might be accomplished through oxidation of the ClpX protein itself, which bears a Cys-coordinated Zn atom in its N-terminal domain that can undergo oxidation (Zhang & Zuber, 2007). Alternatively, YjbH might be titrated by increasing concentrations of Spx upon disulfide stress, leading to further accumulation of Spx protein. Such a mechanism has been proposed for the SspB–ClpXP substrate of E. coli, RecN, which overwhemls the proteolytic complex responsible for its degradation by titration when cells undergo DNA damage (Neher et al., 2006). The conserved CxxC motif of YjbH, while not essential for Spx proteolytic control, may play a role in some other function fulfilled by YjbH that is, perhaps, unrelated to Spx turnover.

Expression of *B. subtilis yjbH* produced insoluble product in an *E. coli* expression system. In previous studies, renatured BsYjbH was used for biochemical studies including *in vitro* proteolysis assay, Zn measurement (Garg et al., 2009). In order to study YjbH in a native form, we cloned and expressed *yjbH* from *G. thermodenitrificans*, which yielded soluble protein when produced in *E. coli*. GyYjbH possesses a similar protein function to BsYjbH both *in vivo* (lacZ assay, responses to diamide) and *in vitro* (proteolysis assay). No His–Cys-rich region is found at the N terminus of GyYjbH except the putative redox CxxC motif, but both YjbH orthologues share high sequence similarity (Fig. S1).

Seeking to understand the substrate recognition mechanism of YjbH, *in vitro* affinity interaction assays and far-Western blotting experiments were conducted and showed that the 12 aa C terminus of Spx is important for GyYjbH binding and for effective proteolysis by ClpXP (Fig. 6). While GyYjbH–His\(_{6}\) binds to Spx in the presence of ClpX or ClpXP, there is negligible binding of ClpX with GyYjbH. In other binding experiments utilizing His\(_{6}\)-Spx and ClpX or ClpXP, a small amount of ClpX was found co-eluted with His\(_{6}\)-Spx, which suggested interaction between His\(_{6}\)-Spx and ClpX (Fig. 6). To date, there are five classes of degradation tags proposed that are recognized by ClpXP: two at the C terminus and three at the N terminus (Flynn et al., 2003). *In vivo* and *in vitro* data showed that Spx\(_{DD}\) and Spx\(_{AC}\) resist proteolysis, which indicates C terminus recognition by ClpXP. The data we provide here suggest that the C terminus of Spx is not only important for ClpX recognition, but also important for YjbH binding. Interestingly, the Spx\(_{DD}\) product was found to interact with GyYjbH, but was not susceptible to proteolysis. By analogy with the ClpXP system of *E. coli*, the extreme C-terminal residues may be required to engage the ClpX unfoldase, while the adjacent region contacts YjbH. This is similar to the way in which SspB–ClpXP interacts with peptides bearing the C-terminal SsrA tag, which is appended during clearance from ribosomes of products resulting from interrupted translation (Chien et al., 2007; Levchenko et al., 2000). SspB and ClpX contact adjacent amino acid sequence motifs on the SsrA tag, with ClpX recognizing the terminal 3 aa. Unlike SspB, which interacts with the N-terminal Zn-binding domain of ClpX (Bolon et al., 2004), no evidence for ClpX–YjbH interaction could be uncovered in the current study.

We present (Table 1) the kinetic measurements of YjbH-enhanced Spx proteolysis. In the presence of GyYjbH, a weaker \(K_m\) and increased \(V_{max}\) value \((K_m=1.79 \pm 0.66 \mu M, V_{max}=1.98 \pm 0.48 \mu M \text{ min}^{-1})\) were observed, while in the absence of GyYjbH, proteolysis was characterized by a lower \(K_m\) and lower \(V_{max}\) \((K_m=0.42 \pm 0.17 \mu M, V_{max}=0.24 \pm 0.06 \mu M \text{ min}^{-1})\). However, the turnover rate was approximately eightfold enhanced when GyYjbH was present. The increased \(V_{max}\) value but increased \(K_m\) may be explained as an uncompetitive 'inhibitor' effect of GyYjbH. *Our in vitro* interaction experiments showed that GyYjbH binds Spx tightly, but ClpX only shows weak interaction with Spx, and both GyYjbH and ClpX bind to the C terminus of Spx. This resulted in a weaker \(K_m\) when GyYjbH is present. Our data support the hypothesis that GyYjbH binds the C terminus of Spx, altering Spx structure to expose a structural element for productive recognition.
by ClpX, and reducing non-productive binding of Spx with ClpX. Hence the weaker $K_m$ would result in faster degradation, since the unfolding of native protein is a rate-limiting step for protein degradation by ClpX (Kenniston et al., 2003). The strong interaction of GtYjbH and Spx could increase the effective concentration of Spx to the pore of ClpX, and then the equilibrium would shift towards the generation of degraded products (Davis et al., 2009). Finally, a model mechanism is proposed of how GtYjbH is an adaptor mediating Spx proteolysis by ClpXP. In the absence of YjbH, ClpXP recognizes Spx, but degrades the substrate slowly due to the lower $K_m$ value, non-productive interaction. However, YjbH binds to the C-terminal region of Spx, elevating the $K_m$ value, possibly reflecting the elimination of inhibitory Spx–ClpX interactions, and resulting in accelerated Spx proteolysis (Fig. 7).

In vitro studies of adaptor-mediated proteolysis catalysed by Clp proteases involve reactions in which the adaptor, such as SspB and ClpS, is present at equimolar or near equimolar amounts with respect to protease and substrate (Levchenko et al., 2000). In one case, SspB was present in 10-fold molar excess over ClpXP (Chowdhury et al., 2010), while in another study, ClpS was present in a ClpAP-catalysed proteolytic reaction in 0.5 molar equivalent to protease and substrate (Dougan et al., 2002). Thus, the adaptor–protease complex acts catalytically in the reaction that causes substrate degradation, but the adaptor acts stoichiometrically with respect to interaction with the protease. Importantly, YjbH behaves quite differently, in that concentrations approximately 30-fold less than those of substrate or protease subunit result in rapid, efficient elimination of Spx substrate. This suggests a novel mechanism of proteolytic enhancement carried out by YjbH, which is unlike that of other Clp protease adaptors. The mechanism of YjbH-mediated proteolytic enhancement warrants further investigation.

ACKNOWLEDGEMENTS

The authors wish to thank Kelly Chacon and Ninian Blackburn for providing TEV protease and Michiko M. Nakano and members of the Zuber lab for helpful discussions. The research reported herein was supported by the National Institutes of Health grant GM045898 to P. Z.

REFERENCES


Edited by: V. Eijsink