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

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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 Spx^{DD} (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 (Spx Δ C) 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.

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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 SspB 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

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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-proteolysis-enhancing activity of YjbH activity *in vivo* and *in vitro* through direct YjbH interaction (Kommineni *et al.*, 2011), although YirB's physiological role is uncertain at this time.

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 (BsYjbH), 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 SspB (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 DH5 α was used for general cloning procedures. Plasmid pET-23a and *E. coli* strain BL21(DE3)pLysS were from Novagen, and were used in heterologous protein production. Medium components were from Difco. 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 *NheI* and *XhoI* and ligated into *NheI/XhoI*-cleaved pET23a. The ligations were used to transform strain DH5 α , and then in strain BL21(DE3)pLysS for overexpression. The plasmids generated are listed in Table 1. To construct mutant 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 His₆ tag. The fragment was cleaved with *HindIII* and *NheI*, followed by ligation with *HindIII/NheI*-cleaved pDR111 (Britton *et al.*, 2002). The ligations were used to transform strain DH5 α . 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 Difco sporulation medium (DSM) (Nicholson & Setlow, 1990) to OD₆₀₀ 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.*, 1990) were treated with 0.5 mM IPTG 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 μ g) were resolved by 15% 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 (Kommineni *et al.*, 2011) and anti-His antibodies, respectively.

***In vitro* proteolysis reactions.** *In vitro* proteolysis was carried out in 60 μ l 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 μ l sample from each reaction mixture was collected, treated with 3 μ l 5 $\times$ 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,

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

Substrate	His-tag adaptor	$V_{\max}$ ($\mu\text{M min}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M s}^{-1}$)
BsSpx	– GtYjbH	0.24 ± 0.06	0.42 ± 0.17	0.0081 ± 0.0020	21324 ± 7956
BsSpx	+ GtYjbH	1.98 ± 0.48	1.79 ± 0.66	0.0659 ± 0.0161	38320 ± 8729

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 30 °C. Cells from a 1 l culture were suspended in 20 ml buffer A (20 mM Tris/HCl, pH 7.6, 200 mM NaCl). One tablet of EDTA-free protease inhibitor cocktail (Roche Applied Science) was added per 20 ml suspended cells. The cells were subjected to freeze–thaw cycles and disrupted by French press. The lysate was cleared by centrifugation at 15 000 r.p.m. (Sorvall, rotor SL-50T) for 30 min. The soluble fraction from the whole-cell lysate was applied to a 5 ml Ni²⁺–NTA affinity agarose (5 PRIME) column pre-equilibrated with buffer A. The column was incubated and gently rotated for 1 h at 4 °C. The column was washed with 20 column volumes (CVs) buffer W (20 mM Tris/HCl, pH 7.6, 200 mM NaCl, 30 mM imidazole). The bound protein was eluted in buffer E (20 mM Tris/HCl, pH 7.6, 200 mM NaCl, 250 mM 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 Spx^{DD} proteins were purified using a previously published procedure (Nakano *et al.*, 2003). The histidine tag on Spx was removed using γTEV (tobacco etch virus) protease (provided by Kelly Chacon, Ohio State University) (Nakano *et al.*, 2005). SpxHA and SpxΔCHA 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).

Complementation of *yjbH* with His-tagged *GtyjbH*. To obtain a *GtyjbH*–His₆ gene, including the ribosome-binding site, for complementation analysis, DNA was amplified by PCR from pEC-21 using the forward primer EC-4 and the reverse primer EC-29 (Table S2). PCR products were digested with *Hind*III and *Nhe*I and cloned at identical sites in plasmid pDR111 to generate pEC-22. pEC-22 was used to transform strain ORB4541 (JH642, *trxB::trxB-lacZ*), and transformants were selected 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 amylase-negative strain (ORB8166, *amyE::GtyjbH*–His₆, *trxB::trxB-lacZ*) was transformed with chromosomal DNA of ORB6952 (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 (ORB8169) 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 Spx^{DD} (1.8 µM), GtYjbH (2.5 µM), ClpX (4 µM), ClpP (4 µM), ATP or ATP-γ-S (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. The bound protein was eluted three times with 1 CV each time in 20 mM Tris/HCl, pH 7.6, 200 mM NaCl, 250 mM imidazole. The input, flow-through, wash and eluent samples were resolved on a 15% SDS-PAGE gel, followed by 'Blue silver' colloidal Coomassie G-250 protein staining: gels were washed with water, then gently shaken in washing/fixing solution (40% methanol, 10% phosphoric acid) twice for 30 min per wash and then in staining solution (0.12% Coomassie G-250, 10% phosphoric acid, 10% ammonium sulfate, 20% methanol) for a minimum of overnight to a maximum of 24 h.

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 C 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 (*P_{hyspank}*) in the *amyE* locus was used

as negative control. The Spx level was determined by monitoring the β -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 β -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*-His₆ 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 *BsYjbH* as shown in two *in vivo* assays: (1) both showed greatly decreased *trxB*-directed β -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*.

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 β -galactosidase activity encoded by the *trxB-lacZ* fusion in *yjbH* mutant cells bearing the $P_{hyspant}$ -*BsYjbH* (white) and mutants [coloured (see key)] or the $P_{hyspant}$ promoter (black) in a *yjbH*-null mutant background. β -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).

Fig. 2. *GtYjbH* mutants show similar activities to *BsYjbH*. (a) *BsYjbH* and *GtYjbH* affect Spx-dependent expression of *trxB-lacZ*. The graph shows the time-course of β -galactosidase activity encoded by the *trxB-lacZ* fusion in cells bearing the *P_{hyspank}-BsYjbH* (white) and *GtYjbH* (grey) or the *P_{hyspank}* promoter (black) in a *yjbH*-null mutant background. (b) Immunoblot analysis of Spx level before and after 1 mM diamide treatment at 0 (DI0), 10 (DI10), 25 (DI25) min time points. UI denotes uninduced. Cells were induced by IPTG for 30 min before diamide treatment. Anti-Spx antiserum, anti-*BsYjbH* antiserum and anti-His antibodies were used to detect Spx, *BsYjbH* and *GtYjbH*-His₆ levels. Control is purified Spx, *BsYjbH*-Strep and *GtYjbH*-His₆ protein.

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 μ M) showed a much higher activity in terms of Spx (8 μ M) degradation. In 2 min, 90% of Spx was degraded by ClpXP (Fig. 3c). Interestingly, a low concentration (0.16 μ M) of *GtYjbH* added to the proteolysis reaction resulted in 90% degradation of Spx (5 μ M) 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 (Spx Δ C) 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 Spx Δ C resisted proteolysis by ClpXP in the presence or absence of *GtYjbH* (Fig. 4a). Moreover, in the presence of both Spx Δ C and Spx WT, the rate of Spx WT proteolysis was not affected (Fig. S4). These results suggested that Spx Δ C 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

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 μ M), ClpX (3 μ M) and ClpP (8 μ M) 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 μ M). (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 μ M *GtYjbH*.

effective *GtYjbH* interaction. *In vitro* affinity interaction experiments were performed using *GtYjbH*-His₆ and Ni-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 Spx Δ C was bound to and co-eluted with *GtYjbH*-His₆ (Fig. 4d). A negative control with only WT Spx and Spx Δ C 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 Spx Δ C 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. Spx Δ CHA, 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 Spx Δ CHA 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. 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 SpxHA or 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.

Fig. 6. *In vitro* Ni-affinity interaction experiments to detect interaction between GtYjbH-His₆, Spx and ClpX, in the absence (a) or presence (b) of ClpP; between His₆-Spx and ClpX, in the absence or presence (c) of ClpP, all reactions contain ATP- γ -S; between GtYjbH-His₆, Spx^{DD}, ClpX and ClpP, in the presence or absence (d) of ATP- γ -S. Column fractions were applied to SDS-PAGE gels that were stained with 'Blue silver' colloidal Coomassie G-250 protein. M, marker; I, input; FT, flowthrough; W, last wash; E, elution.

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 Spx Δ CHA 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. *spx^{DD}* 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 ($K_m=0.42\pm0.17\ \mu\text{M}$, $V_{\max}=0.24\pm0.06\ \mu\text{M min}^{-1}$) or presence ($K_m=1.79\pm0.66\ \mu\text{M}$, $V_{\max}=1.98\pm0.48\ \mu\text{M min}^{-1}$) of $0.5\ \mu\text{M}$ *GtYjbH*. In the presence of *GtYjbH*, 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 *GtYjbH*, 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 overwhelms 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*. *GtYjbH* 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 *GtYjbH* 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 *GtYjbH* binding and for effective proteolysis by ClpXP (Fig. 6). While *GtYjbH*-His₆ binds to Spx in the presence of ClpX or ClpXP, there is negligible binding of ClpX with *GtYjbH*. In other binding experiments utilizing His₆-Spx and ClpX or ClpXP, a small amount of ClpX was found co-eluted with His₆-Spx, which suggested interaction between His₆-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 Δ C 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 *GtYjbH*, 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 *GtYjbH*, a weaker K_m and increased $V_{\max}$ value ($K_m=1.79\pm0.66\ \mu\text{M}$, $V_{\max}=1.98\pm0.48\ \mu\text{M min}^{-1}$) were observed, while in the absence of *GtYjbH*, proteolysis was characterized by a lower K_m and lower $V_{\max}$ ($K_m=0.42\pm0.17\ \mu\text{M}$, $V_{\max}=0.24\pm0.06\ \mu\text{M min}^{-1}$). However, the turnover rate was approximately eightfold enhanced when *GtYjbH* was present. The increased $V_{\max}$ value, but increased K_m may be explained as an uncompetitive 'inhibitor' effect of *GtYjbH*. Our *in vitro* interaction experiments showed that *GtYjbH* binds Spx tightly, but ClpX only shows weak interaction with Spx, and both *GtYjbH* and ClpX bind to the C terminus of Spx. This resulted in a weaker K_m when *GtYjbH* is present. Our data support the hypothesis that *GtYjbH* binds the C terminus of Spx, altering Spx structure to expose a structural element for productive recognition

Fig. 7. A model for the mechanism of YjbH-enhanced proteolysis is proposed. In the absence of YjbH, the Spx C terminus contacts ClpX, but ClpXP proteolysis is slow. YjbH binds to the Spx C-terminal region, which may alter Spx folding, expose a structural element of Spx or eliminate Spx–ClpX contacts that might be inhibitory, thus accelerating Spx proteolysis.

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 GfYjbH 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 GfYjbH 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.

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