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A Conserved ClpP-like Protease Involved in Spore Outgrowth in *Bacillus subtilis*

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Abstract

Germination and outgrowth of endospores of the Gram-positive bacterium *Bacillus subtilis* involves the degradation and conversion to free amino acids of abundant proteins located in the spore core known as small acid-soluble proteins (SASP). This degradation is mediated primarily by the germination protease Gpr. Here we show that YmfB, a distant homolog of ClpP serine proteases that is highly conserved among endospore-forming bacteria, contributes to SASP degradation but that its function is normally masked by Gpr. Spores from a *ymfB gpr* double mutant were more delayed in spore outgrowth and more impaired in SASP degradation than were spores from a *gpr* single mutant. The activity of YmfB relied on three putative active site residues as well as on the product of a small gene *ylzJ* located immediately downstream of, and overlapping with, *ymfB*. We propose that YmfB is an orphan ClpP protease that is dedicated to the degradation of a specialized family of small protein substrates.

Keywords

Bacillus subtilis; spore outgrowth; protease; SASP

Introduction

Members of the low-GC phylum of Gram-positive bacteria (Firmicutes) produce spores (or, more properly, endospores) that are highly resistant to environmental extremes. These spores consist of an inner poorly hydrated core in which water has been largely replaced with a large depot of pyridine-2, 6-dicarboxylic acid (dipicolinic acid (DPA)). The core is surrounded by a thick layer of peptidoglycan known as the cortex, which is, in turn, encased in a protein shell known as the coat. Spores monitor the environment by means of receptors embedded in the membrane surrounding the core. These receptors detect small molecule germinants in the environment. Binding of germinants to the receptors triggers the release of the DPA, allowing water to enter the spore core. This re-hydration activates spore enzymes that hydrolyze the spore cortex, allowing the spores to germinate. Cortex hydrolysis and subsequent cortex expansion allows full hydration of the spore core. This enables the restart of metabolism and macromolecular synthesis in a process termed outgrowth, eventually leading to the resumption of vegetative growth (Setlow, 2003).

Soon after the initiation of spore germination, 10 to 20% of the total protein in the spore core is degraded to amino acids, the majority of which belong to a group of small, acid-soluble spore proteins (SASP) that make up approximately 5–15% of the total protein

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content of the core of dormant spores (Setlow, 1988, Setlow, 2007). Three major SASP (α, β, and γ) are produced during a late stage of spore formation in *Bacillus subtilis* and are encoded by multiple *ssp* genes (Mason *et al.*, 1988, Wang *et al.*, 2006). Resistance to heat, toxic chemicals, and UV radiation is principally conferred by the α- and β-type SASP, which are DNA-binding proteins with significant sequence similarity to each other. The third SASP type, SASP-γ, does not exhibit DNA-binding activity and does not significantly contribute to spore resistance (Hackett & Setlow, 1988, Setlow, 1988, Moeller *et al.*, 2009). Early during outgrowth, SASP are rapidly degraded to single amino acids, freeing up the DNA of the spore for transcription, and providing a reservoir of amino acids for *de novo* protein synthesis as macromolecular synthesis resumes (Setlow, 1988). The germination protease Gpr initiates the degradation of SASP by cleaving these DNA-binding proteins at one or two sites. Gpr is an atypical aspartic acid protease produced as a zymogen during a late stage of sporulation. Through an autocleavage event, the N-terminus of Gpr is processed to activate the protease immediately prior to spore dormancy. Due to the low water content of the spore core, Gpr does not cleave its substrates until hydration takes place during germination (Sussman & Setlow, 1991, Sanchez-Salas & Setlow, 1993, Illades-Aguiar & Setlow, 1994, Carroll & Setlow, 2005).

We recently identified *ymfB* as a previously unrecognized *B. subtilis* sporulation gene that is highly conserved among endospore-forming bacteria (Traag *et al.*, 2013). The *ymfB* gene product has some similarity to caseinolytic proteases (ClpP-type proteases) and was previously suggested to be involved in translocation and processing of the α-amylase AmyQ ((Bolhuis *et al.*, 1999); *ymfB* was therein named *tepA*). The initial results, however, could not be reproduced using a *ymfB* null mutation (Westers *et al.*, 2004). *ymfB* is activated by the forespore-specific sigma factor σ^G and repressed by the modulator of σ^G-dependent gene expression SpoVT (Traag et al., 2013). Cells mutant for *ymfB* exhibit no apparent defect under sporulation-inducing conditions, even in competition with the wild type (Traag et al., 2013). Here we show that the function of YmfB is masked by redundancy with the germination protease Gpr and that spores from a *ymfB gpr* double mutant are markedly delayed in spore outgrowth. The activity of YmfB was found to depend on three putative active site residues as well as on YlzJ, a small protein encoded by the gene immediately downstream of, and overlapping with, *ymfB*. Evidence indicates that YmfB and YlzJ contribute to SASP degradation during spore outgrowth. ClpP proteases by themselves have little activity against native or denatured substrates and typically rely on AAA+ unfoldases that prepare protein substrates for degradation (Thompson *et al.*, 1994, Thompson & Maurizi, 1994, Striebel *et al.*, 2009). We propose that YmfB is an orphan ClpP protease that does not depend on an unfoldase for the degradation of the inherently unstable SASP during spore outgrowth.

Results

The function of YmfB is masked by the germination protease Gpr

The inferred *ymfB* gene product exhibits a low level of amino acid sequence similarity to ClpP serine proteases (Bolhuis et al., 1999), being 14% identical and 27% similar to *Escherichia coli* ClpP (Fig S1). This apparent homology raised the possibility that YmfB functions in sporulation but that its function is masked by redundancy with another protease or peptidase present in the forespore. To investigate this hypothesis, we constructed double mutants of *ymfB* and genes encoding proteases or peptidases known to be produced in the forespore. Similar to *ymfB*, the gene coding for the germination protease Gpr is activated by σ^G and repressed by SpoVT (Bagyan *et al.*, 1996, Wang et al., 2006). A second gene *yyaC*, whose gene product exhibits sequence similarity to Gpr, is expressed under the control of the forespore-specific sigma factor σ^F (Wang et al., 2006). We constructed double mutants of *ymfB* and either *gpr* or *yyaC* and measured the number of colony-forming units (CFU)

after heat-treatment of cultures grown in sporulation-inducing medium for 28 hours at 37°C (Fig 1A). Cultures of *ymfB* or *yyaC* single mutants yielded similar CFU after heat-treatment compared to the wild type, whereas the *gpr* mutant showed an approximately two-fold reduction (a–c, e in Fig 1A). Strikingly, CFU for the *ymfB gpr* double mutant were lower than for the *gpr* single mutant (d in Fig 1A). In contrast, a *ymfB yyaC* double mutant produced similar CFU to that of the wild type (f in Fig 1A). In addition to the reduction in CFU, colonies formed from *ymfB gpr* mutant spores did not appear after overnight incubation on solid agar medium, an amount of time that is typically sufficient for the formation of colonies from wild type spores. Instead the *ymfB gpr* mutant spores required an additional day to reach the size of wild type colonies. A similar, but less pronounced phenotype was observed for *gpr* single mutant spores (Fig 1B).

ymfB function depends on an adjacent overlapping gene

The *ymfB* gene is the upstream member of a putative, two-gene operon in which the 3′ end of its open reading frame overlaps by four nucleotides with that of the adjacent, 70-codon gene *ylzJ* (Fig 2). To investigate whether the phenotype of the *ymfB* mutation was due to the absence of YmfB or to a polar effect on the expression of *ylzJ* or both, we carried out a complementation experiment by introducing a copy of *ymfB* with its native promoter (contained on a fragment that spanned nucleotide positions -300 to +780 relative to the *ymfB* translational start site; Fig 2A) into the chromosome at the *sacA* locus. This construct, however, did not restore the CFU of the double mutant to that of the *gpr* single mutant (g in Fig 1A).

Given that *ylzJ* orthologs and the overlapping stop-start operon architecture are conserved among all endospore-forming bacteria that contain *ylzJ* (Fig 2B), we wondered whether the function of *ymfB* might depend on both members of the operon. Interestingly, whereas *ylzJ* is present in all sequenced *Bacillus* species, all but one species of *Clostridium* that have *ymfB* lack a copy of *ylzJ* (Fig S2). Accordingly, we next made a construct carrying the entire *ymfB ylzJ* operon (Fig 2A) and introduced it at *sacA* into the *ymfB gpr* double mutant. This construct did restore the CFU to that of the *gpr* single mutant (h in Fig 1A).

It was conceivable that the phenotype of our *ymfB* mutation was entirely due to a polar effect on *ylzJ* and not to the absence *ymfB*. Indeed, a strain deleted for *gpr* and *ylzJ*, in which the open reading frame of *ymfB* was left unperturbed, showed a similar phenotype to the *ymfB gpr* double mutant (i in Fig 1A). To investigate the possibility that the phenotype was solely due to a polar effect on *ylzJ*, we carried out site-directed mutagenesis of *ymfB*. The proteolytic active site of ClpP serine proteases is characteristically composed of a catalytic triad of Ser-His-Asp (Wang *et al.*, 1997). By aligning the amino acid sequence of YmfB with those of *B. subtilis* and *E. coli* ClpP, we identified three likely active site residues in YmfB (Fig S1). Next, we created three constructs carrying both *ymfB* and *ylzJ*, in which the codon for each putative active site residue was replaced with a codon for alanine (resulting in the amino acid substitutions S124A, H147A, and D200A). When introduced into the *ymfB gpr* double mutant all three complementation constructs with the putative active site codon mutations failed to restore CFU to that of the *gpr* single mutant (j-l in Fig 1A).

To investigate the possibility that amino acid substitutions in the inferred catalytic site were proteolytically destabilizing YmfB rather than functionally inactivating it, we created variants of YmfB bearing a hexahistidine-tag at the C-terminus. Hexahistidine did not block the function of YmfB in that otherwise wild type YmfB bearing the tag (YmfB-His_6) was functional as judged by complementation analysis (data not shown). Immunoblot analysis using anti-hexahistidine antibodies of whole cell lysates made from strains producing YmfB-His_6 and mutant derivatives of the tagged protein bearing the S124A or D200A

amino acid substitutions showed that the mutant proteins accumulated to the same levels as the wild type, tagged protein (Fig 1C).

The results so far support the following conclusions. First, the function of *ymfB* depends on *ylzJ*. Second, the function of the two-gene operon is redundant with *gpr*. Third, the putative catalytic site residues of YmfB are required for YmfB function, which suggests that YmfB is indeed a ClpP-like protease.

ymfB gpr mutant spores are delayed in spore outgrowth

Spores lacking Gpr are delayed in the start of vegetative growth due to a failure to initiate the degradation of SASP during spore germination (Sanchez-Salas *et al.*, 1992, Setlow, 2007). We therefore suspected that YmfB/YlzJ might also function in outgrowth. To assay the efficiency of spore outgrowth of *ymfB* and *gpr* mutant spores, we germinated spores at an OD_{600} of approximately 0.15 in LB medium. None of the mutant strains showed conspicuous defects in spore germination, as judged by the rate of loss of OD_{600} in the first hour after inoculation (Fig S3). Wild type and *ymfB* mutant spores required approximately two hours to resume growing robustly (as judged by the rise in OD_{600} above 0.2), whereas as expected, *gpr* mutant spores required approximately an additional three hours. *ymfB gpr* double mutant spores were, however, further delayed, and did not resume vegetative growth until approximately eight hours after inoculation in LB medium (Fig 3). In agreement with the results for CFU after heat-treatment, a construct carrying *ymfB* and *ylzJ* (but not *ymfB* alone) integrated at the *sacA* locus, restored the efficiency of outgrowth to that of the *gpr* single mutant (Fig 3). Finally, strains carrying constructs specifying putative catalytically inactive YmfB variants failed to complement the outgrowth defect (Fig S4).

Degradation of SASP is impaired in *ymfB gpr* mutant spores

We hypothesized that YmfB/YlzJ functions by contributing (directly or indirectly) to the degradation of SASP during spore outgrowth. To investigate this possibility, we determined the relative levels of SASP-α, -β, and -γ at various times after the initiation of germination. Dormant and germinated spores were dried, disrupted, and acid-extracted to isolate SASP. The levels of the three major SASP were determined from samples subjected to polyacrylamide gel electrophoresis (PAGE) at low pH. SASP were fully degraded within the first hour in wild type and *ymfB* mutant spores (Fig 4), in agreement with previous reports (Setlow, 1988). Consistent with the role of Gpr in degradation of SASP, one and three hours after the initiation of germination, a significant amount of SASP remained in *gpr* mutant spores. In germinated *ymfB gpr* double mutant spores the degradation of SASP was further delayed, with between 1.5 and 2-fold higher levels of the three major SASP in the *ymfB gpr* double mutant compared to the *gpr* single mutant at one and three hours after germination had been initiated (Fig 4). We conclude that YmfB acts in parallel to Gpr in the degradation of SASP during spore germination and outgrowth.

Discussion

Here we have identified a function for YmfB, a distant relative of ClpP proteases that is highly conserved among endospore-forming bacteria. We have shown that YmfB acts during spore outgrowth but that its role in the transition to vegetative growth is masked by redundancy with the germination protease Gpr. Our data are consistent with the idea that YmfB, like Gpr, targets and degrades SASP. Gpr is an atypical aspartic acid protease that initiates SASP degradation by cleaving these DNA-binding proteins at one or two sites (Setlow, 1988, Carroll & Setlow, 2005). An appealing hypothesis is that YmfB acts on fragments of SASP generated by Gpr and converts these peptides to single amino acids to be used for *de novo* protein synthesis during the resumption of vegetative growth. If so,

however, two inferences can be drawn from the observed redundancy between YmfB and Gpr. First, given that YmfB is dispensable in the presence of Gpr under the tested conditions, we presume that an additional proteolytic system(s) contributes to breaking down Gpr-generated SASP fragments. Second, because a *ymfB gpr* double mutant exhibits a more severe phenotype than a *gpr* single mutant, YmfB must be capable of degrading SASP when Gpr is absent, albeit with low efficiency.

Interestingly, YmfB function depends on the 70-amino acid polypeptide YlzJ, encoded by the gene directly downstream of, and overlapping with, the *ymfB* open-reading frame. YlzJ has no clear similarity to other proteins, and further studies will be needed to determine at what step of SASP degradation (if our hypothesis that YmfB degrades Gpr-generated SASP fragments is correct) YlzJ influences YmfB function. Because YlzJ lacks any obvious nucleotide-binding motifs, we infer YlzJ is not an energy-dependent unfoldase that unfolds substrates for YmfB. Indeed, given the small size and unstable nature (see below) of SASP and Gpr-generated SASP fragments, unfoldase activity might be unnecessary for YmfB. Alternative possibilities are that YlzJ is important for YmfB activity or oligomerization or that YlzJ acts as an adaptor for YmfB substrates. Several substrates of ClpP protease complexes require adaptor proteins to deliver substrates for degradation. For example, SspB delivers ssrA-tagged proteins to the ClpXP protease for degradation (Levchenko *et al.*, 2000, Flynn *et al.*, 2004), and MecA targets the competence regulator ComK for degradation by the ClpCP protease in *B. subtilis* (Turgay *et al.*, 1997, Turgay *et al.*, 1998). It is interesting to note that whereas the *ymfB ylzJ* operon is conserved among *Bacillus* species, *ylzJ* is missing from all *Clostridium* species that encode YmfB, with the exception of *C. thermocellum*, the YmfB ortholog of which appears to share a more recent common ancestor with *Bacillus* orthologs then other clostridial orthologs do (Fig S2). If our hypothesis that YlzJ is an adaptor is correct, then it is mysterious why it is not needed for the function of YmfB in *Clostridium*.

Despite the low sequence similarity of YmfB to ClpP, three potential active site residues are highly conserved among YmfB orthologs. Consistent with the idea that YmfB is a ClpP protease, substitution of these residues with alanine abolished YmfB function. Additional structural and biochemical experiments will be needed to determine whether YmfB is indeed a ClpP-like protease. ClpP by itself degrades small peptides but has no significant activity against native or denatured proteins (Thompson & Maurizi, 1994, Thompson et al., 1994). Instead, two stacked heptameric rings of ClpP associate with hexameric rings of AAA+ unfoldases (such as ClpC and ClpX) to create proteolytically-competent complexes. These unfoldases recognize, denature, and translocate substrates (Striebel et al., 2009). Access to the axial pore of ClpP protease is gated by four highly conserved charged residues in the channel loop of the pore (EQTSRGER in *E. coli* ClpP, Fig S1) (Lee *et al.*, 2010). Association of the unfoldases with ClpP induces a conformational rearrangement of the protease that allows unfolded polypeptides to diffuse through the axial pore (Alexopoulos *et al.*, 2012, Baker & Sauer, 2012). The highly conserved channel loop residues (including the charged residues) are not well conserved in YmfB (Fig S1), raising the possibility that its pore is not strictly gated and may accommodate at least partially unfolded substrates. Interestingly, previous work has suggested that SASP likely become inherently unstable after germination. SASP-α and -β are protected from degradation when bound to DNA. In the absence of DNA, these proteins are rapidly cleaved by Gpr in vitro, as well as by the serine proteases trypsin and chymotrypsin (Setlow & Setlow, 1995). It is conceivable that YmfB forms a complex with an unfoldase typically associated with ClpP in order to degrade its substrates in an ATP-dependent manner. An alternative hypothesis, however, is that SASP become unstable upon dissociation from DNA and Gpr-mediated cleavage after germination, and are then readily accommodated in the YmfB pore for degradation, alone or in a complex with YlzJ, independent of AAA+ unfoldases.

In sum, we hypothesize that YmfB is an orphan member of the ClpP family of proteases that likely functions without a cognate AAA+ unfoldase and is dedicated to the degradation of a specialized family of small DNA-binding proteins during the process of spore outgrowth. A test of our hypothesis requires a determination of the structure of YmfB and YlzJ and reconstitution of their activity biochemically.

Experimental Procedures

General methods

E. coli strain DH5α was used for propagating plasmids, and was grown and transformed using standard procedures (Sambrook *et al.*, 1989). Site-directed mutagenesis on *ymfB* was performed using QuikChange (Agilent Technologies), according to the suppliers recommendations. *B. subtilis* strains used in this work are listed in Table 1. Transformation of *Bacillus* was done as previously described (Wilson & Bott, 1968).

Determination of colony-forming units after heat treatment

Strains were inoculated from single colonies in 5 ml Difco sporulation (DS) medium and incubated for approximately 28 hours at 37°C. Cultures were heated at 80°C for 20 minutes, and dilutions were plated on DS medium agar plates. Colonies were counted after approximately 40 hours of incubation at 37°C, as explained in the Results section.

Immunoblot analysis

Strains expressing wild type, S124A, and D200A C-terminal hexahistidine-tagged YmfB variants from constructs integrated at the *sacA* locus were induced to sporulate by resuspension as previously described (Nicholson & Setlow, 1990). Samples were harvested and pelleted 4.5 hours after the induction of sporulation, corresponding to the maximum activity of the *ymfB* promoter (Traag *et al.*, 2013). Cell pellets were resuspended in SDS sample buffer and subjected to SDS-PAGE. Proteins were transferred to Immobilon-P PVDF membrane (Millipore), and probed with anti-hexahistidine and anti-σ^A antibodies as primary antibodies.

Spore preparation, purification and germination

Spores of various strains were prepared at 37°C on 2x Schaeffer's-glucose agar plates, harvested, purified and stored as described previously (Nicholson & Setlow, 1990). All spores used in this work were free (>98%) of growing or sporulating cells, germinated spores and cell debris as determined by phase contrast microscopy. Spores at an OD_{600} of 5–10 were heat-treated at 80°C for 20 min, cooled on ice for at least 15 min, and germinated at an OD_{600} of 0.15 in LB medium at 37°C. Changes in OD_{600} were monitored in a Synergy 2 plate reader (BioTek) for several hours.

Analysis of SASP levels

For the analysis of SASP levels, dried dormant and germinated spores were disrupted and SASP were extracted as previously described (Paidhungat *et al.*, 2000, Nicholson & Setlow, 1990). Briefly, spores were prepared as described above, heat-treated to activate spores for germination, and germinated at 37°C and an OD_{600} of 5 in 50 ml of LB medium containing 10 mM L-alanine. 10 ml samples taken at appropriate time points were pelleted by centrifugation, flash-frozen in liquid nitrogen, and lyophilized. Dry pellets were disrupted by shaking in a capsule of a Wig-L-Bug Shaker dental amalgamator with 70 mg of glass beads and a 0.25 inch diameter steel ball for ten one min periods with one min of cooling in between. Disrupted spores were extracted twice with 700 μl 3% acetic acid and 30 mM HCl, and the supernatants were combined, dialyzed overnight in Spectrapor 3 tubing (MW cutoff,

3500 kda) against 500 ml 1% acetic acid, and lyophilized. The dried residue was dissolved in 30 μl 8M urea, and aliquots were subjected to PAGE at low pH, and gels were stained with Coomassie blue. Stained gels were quantified using the ImageQuant software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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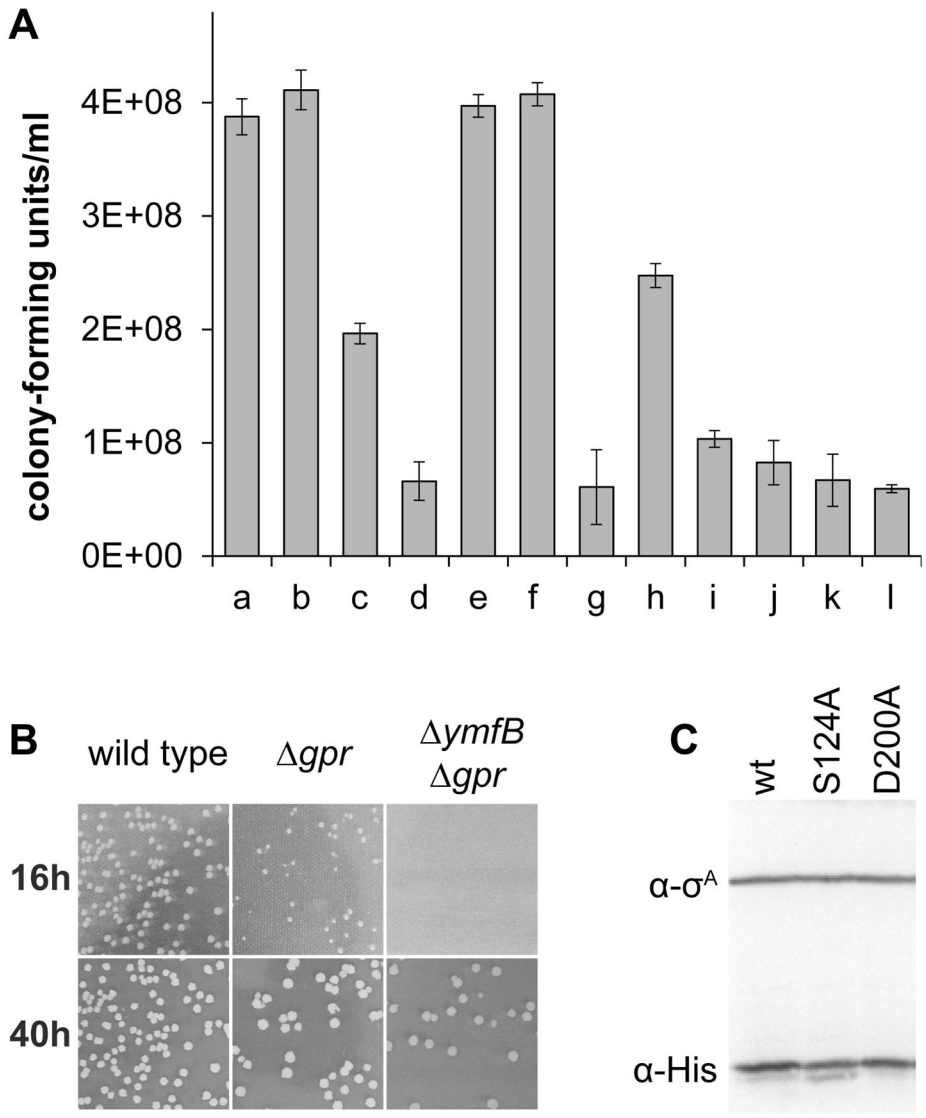

Fig. 1. Synergy between *ymfB* and *gpr*

A. Colony-forming units (CFU) after heat-treatment of sporulating cultures of wild type (a), Δ*ymfB* (b), Δ*gpr* (c), Δ*ymfB* Δ*gpr* (d), Δ*yyaC* (e), Δ*ymfB* Δ*yyaC* (f), and Δ*ymfB* Δ*gpr* mutants carrying *ymfB* (g), or *ymfB ylzJ* (h) at the *sacA* locus. Also shown are CFU for Δ*ylzJ* Δ*gpr* (i) and Δ*ymfB* Δ*gpr* mutants carrying $ymfB^{S124A}$ *ylzJ* (j), $ymfB^{H147A}$ *ylzJ* (k), or $ymfB^{D200A}$ *ylzJ* (l) at the *sacA* locus. Colonies were counted from dilutions plated on DS medium and incubated at 37°C for approximately 40 hours. CFU were determined from at least three biological replicates for each strain. Error bars indicate standard errors (SEM).

B. Delayed appearance of colonies formed from Δ*ymfB* Δ*gpr* mutant spores. Colony formation after 16 and 40 hours on DS medium from heat-treated sporulating cultures of the wild type and of Δ*gpr* and Δ*ymfB* Δ*gpr* mutants

C. Strains producing C-terminal hexahistidine-tagged wild type (wt) YmfB or $YmfB^{S124A}$ (S124A) and $YmfB^{D200A}$ (D200A) variants from constructs integrated at the *sacA* locus were induced to sporulate by resuspension and samples were harvested 4.5 hours after the induction of sporulation. Whole cell lysates were subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was subsequently probed with anti-hexahistidine (α-

His) antibody, which corresponded to the tagged YmfB variants, and anti-σ^A (α-σ^A) antibody, as a loading control.

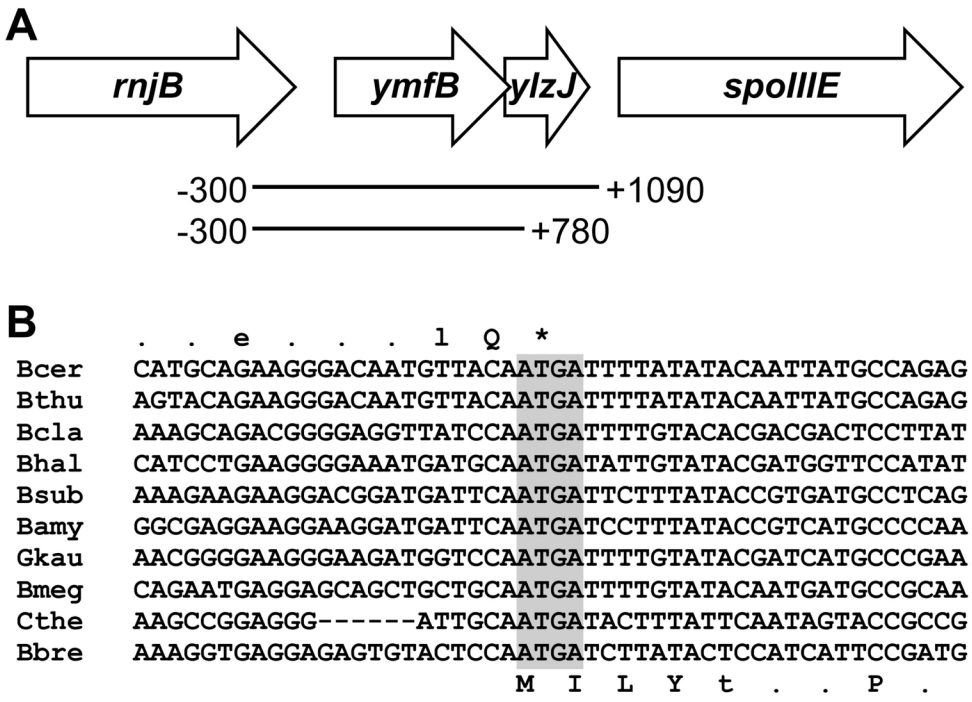

Fig. 2. The *ymfB ylzJ* operon

A. The *ymfB* ORF is immediately followed by, and overlaps with, the *ylzJ* ORF. The two-gene *ymfB ylzJ* operon is flanked upstream by *rnjB*, coding for a ribonuclease, and downstream by *spoIIIE*, coding for the DNA translocase essential for sporulation. Fragments contained in constructs pBAT439 (nucleotides −300/+780 relative to the first nucleotide of the *ymfB* translational start site) or pBAT440 (nucleotides −300/+1090) are indicated below the schematic. B. Alignment of the *ymfB* 3′ end and *ylzJ* 5′ end from *B. cereus* (Bcer), *B. thuringiensis* (Bthu), *B. clausii* (Bcla), *B. halodurans* (Bhal), *B. subtilis* (Bsub), *B. amyloliquefaciens* (Bamy), *Geobacillus kaustophilus* (Gkau), *Bacillus megaterium* (Bmeg), *C. thermocellum* (Cthe), *Brevisbacillus brevis* (Bbre). Consensus sequences of the C-terminal residues of YmfB orthologes and N-terminal residues of YlzJ orthologs are given above and below the sequence, respectively. The four nucleotide overlap (shaded in grey) between the ORFs of *ymfB* and *ylzJ* is conserved among all species that possess *ylzJ* orthologs.

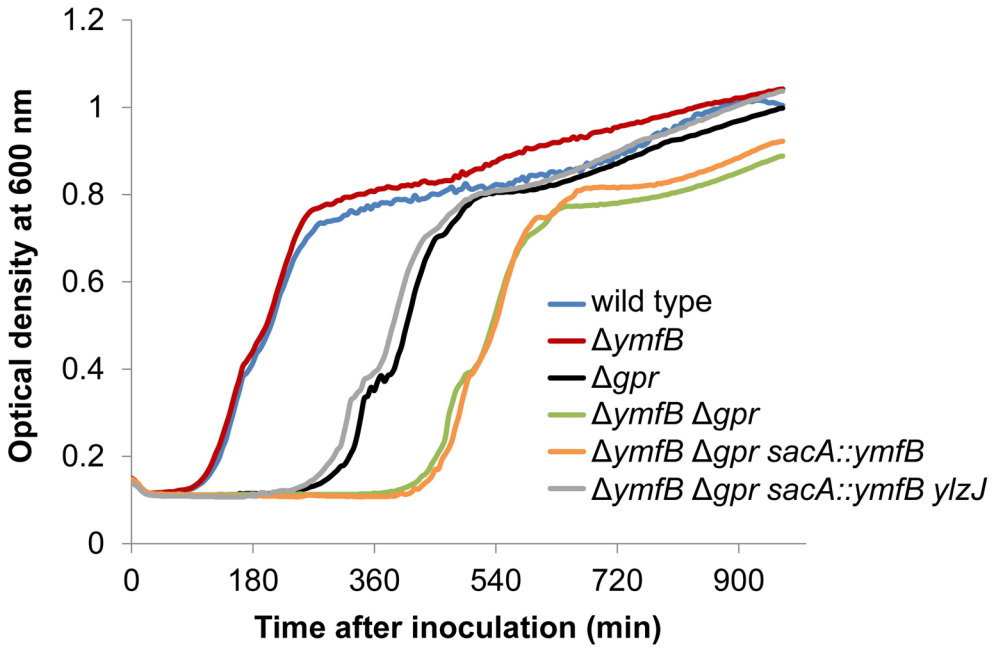

Fig. 3. Outgrowth of wild type and mutant spores

Purified spores from wild type (blue), *ymfB* mutant (red), *gpr* mutant (black), *ymfB* and *gpr* double mutant (green) and double mutant strains carrying *ymfB* (orange) or *ymfB ylzJ* (grey) at the *sacA* locus were heat-treatedand germinated at an OD_{600} of 0.15 in LB medium at 37°C. Spore outgrowth was followed by monitoring changes in OD_{600} of the germinating and outgrowing culture. Experiments were repeated starting from independent spore preparations. The data shown are from a single representative experiment.

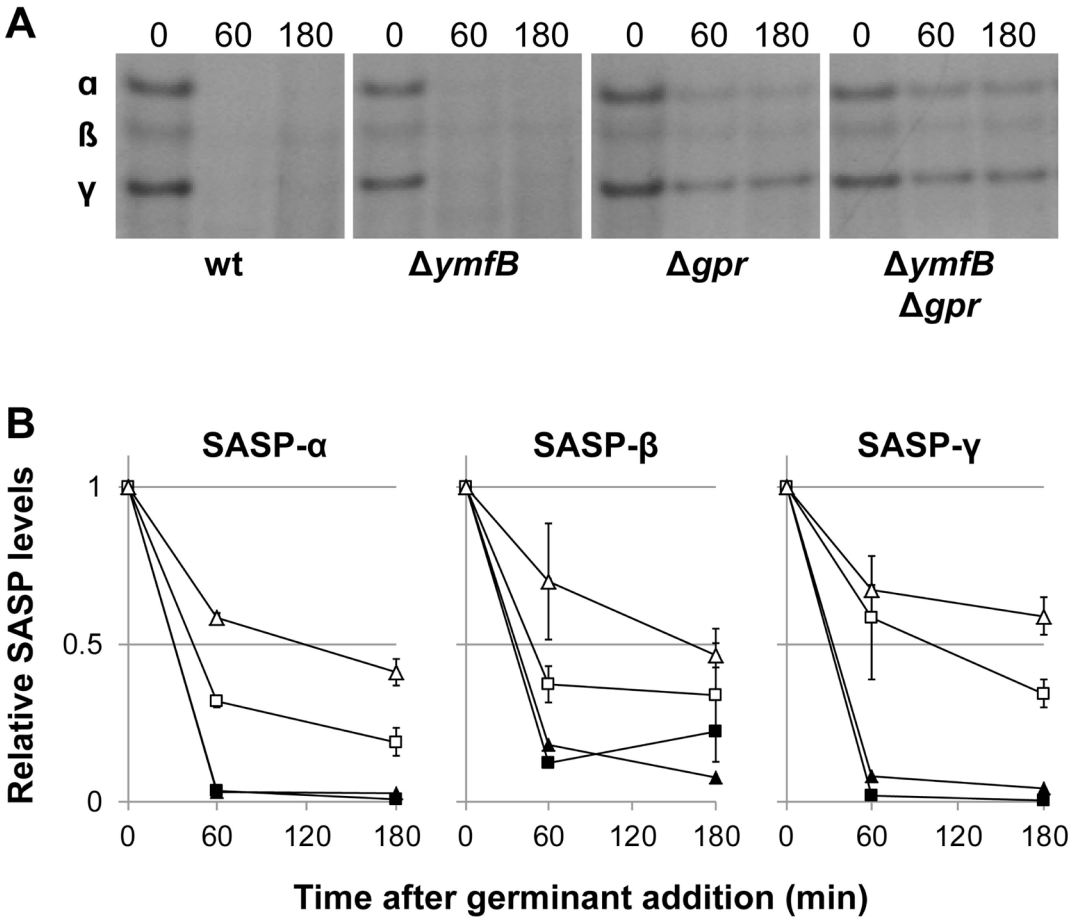

Fig. 4. Levels of SASP-α, -β, and -γ during spore germination and outgrowth

A. Purified spores from wild type, *ymfB* mutant, *gpr* mutant, *ymfB* and *gpr* double mutant were heat-treated and germinated at an OD_{600} of 5 in LB medium containing 10 mM L-alanine at 37 °C. 10 ml samples were harvested at time points indicated above the gels (time in min), frozen in liquid nitrogen, and lyophilized. SASP were extracted from dry samples, dialyzed, lyophilized, and redissolved. 6 μl aliquots were subjected to polyacrylamide gel electrophoresis at low pH, and the gel was stained with Coomassie Blue. Experiments were repeated starting from independent spore preparations. The gels presented are representative pictures. B. Bands on Coomassie Blue stained gels corresponding to SASP-α, -β, and -γ from wild type (closed squares), *ymfB* mutant (closed triangles), *gpr* mutant (open squares), *ymfB* and *gpr* double mutant (open triangles) spores were quantified using ImageQuant software. Values are averages from two independent experiments. The average signal at time zero for each SASP band was set to one, and signals at other time points are given relative to the intensity at T0. Error bars indicate standard errors (SEM).

Table 1

B. subtilis strains used in this study.

Strain[a]	Genotype	Source or reference
PY79	Prototrophic derivative of *B. subtilis subsp. subtilis* 168	(Youngman *et al.*, 1984)
RL5369	*ymfB::spc*	(Traag et al., 2013)
RL5496	*gpr::erm*	This study
RL5497	*ymfB::spc gpr::erm*	This study
RL3892	*yyaC::tet*	(Wang et al., 2006)
RL5498	*ymfB::spc yyaC::tet*	This study
RL5499	*ymfB::spc gpr::erm sacA::ymfB*	This study
RL5500	*ymfB::spc gpr::erm sacA::ymfB ylzJ*	This study
RL5501	*ylzJ::spc gpr::mls*	This study
RL5502	*ymfB::spc gpr::erm sacA::ymfB*^{S124A} *ylzJ*	This study
RL5503	*ymfB::spc gpr::erm sacA::ymfB*^{H147A} *ylzJ*	This study
RL5504	*ymfB::spc gpr::erm sacA::ymfB*^{D200A} *ylzJ*	This study
RL5505	*sacA::ymfB*^{His6} *cat*	This study
RL5506	*sacA::ymfB*$^{S124A\text{-}His6}$ *cat*	This study
RL5506	*sacA::ymfB*$^{D200A\text{-}His6}$ *cat*	This study