

Induction and regulation of a secreted peptidoglycan hydrolase by a membrane Ser/Thr kinase that detects muropeptides

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Summary

Here, we report that the model Gram-positive organism, *Bacillus subtilis*, expresses and secretes a murelytic enzyme, YocH, in response to cell wall-derived muropeptides derived from growing cells but not lysed cells. This induction is dependent on PrkC, a membrane Ser/Thr kinase that binds to peptidoglycan and that belongs to a broadly conserved family including the essential PknB kinase of *M. tuberculosis*. YocH stimulates its own expression in a PrkC-dependent manner demonstrating the presence of an autoregulatory loop during growth. Cells lacking YocH display a survival defect in stationary phase but enzymes secreted by other cells in the culture rescue this defect. The essential translation factor EF-G is an *in vivo* substrate of PrkC and this phosphorylation occurs in response to muropeptides. Therefore, we hypothesize that YocH is used by the bacterium to digest peptidoglycan released by other bacteria in the milieu and that the presence of these fragments is detected by a membrane kinase that modifies a key regulator of translation as well as to stimulate its own expression.

Introduction

The bacterial cell wall is composed largely of peptidoglycan, a polymer that provides the characteristic cell shape and the ability to withstand osmotic pressure. Peptidoglycan consists of repeated glycan strands composed of $\beta(1,4)$ linkages between N-acetylglucosamine and N-acetylmuramic acid (NAM) sugars that have covalent cross-links between NAM-associated peptides. During growth, bacteria turn over their cell wall material due to the

actions of peptidoglycan hydrolases and/or amidases that process mature peptidoglycan to allow the insertion of new material (Doyle *et al.*, 1988). These fragments serve as signals in a range of host–microbe interactions including *Bordetella pertussis* infection and *Vibrio fischeri*-squid symbiosis (Cloud-hansen *et al.*, 2006) and stimulate the innate immune response (Hasegawa *et al.*, 2006) by binding to host proteins like Nod1/2 (Girardin *et al.*, 2003) and Toll-like receptor 2 (Asong *et al.*, 2009). In addition to this central role in host–bacterial interactions, peptidoglycan fragments act as an inter-bacterial signal that stimulates growth of dormant *Bacillus subtilis* spores (Shah *et al.*, 2008).

Dormant cells of *Micrococcus luteus* are stimulated to divide (resuscitate) by exposure to non-dormant *M. luteus* cells (Votyakova *et al.*, 1994; Mukamolova *et al.*, 1998). This stimulation requires resuscitation-promoting factor (Rpf), a secreted 17 kDa protein (Mukamolova *et al.*, 2002). A homologue of *M. luteus* Rpf from *M. tuberculosis* is structurally similar to lysozyme (Cohen-gonsaud *et al.*, 2005; Ruggiero *et al.*, 2009), a protein known to digest peptidoglycan by breaking the $\beta(1,4)$ linkage between the N-acetyl glucosamine and the N-acetyl muramic acid and Rpf proteins from both *M. luteus* and *M. tuberculosis* are capable of digesting peptidoglycan *in vitro* (Mukamolova *et al.*, 2006). *M. tuberculosis* contains five Rpf proteins, and a mutant strain lacking all five still grows *in vitro*, but is defective in restoration of growth from stationary phase (Kana *et al.*, 2008). While strains lacking multiple Rpf proteins are defective in animal models of tuberculosis (Tufariello *et al.*, 2004; Downing *et al.*, 2005; Russell-goldman *et al.*, 2008), the delayed reactivation observed for the single *rpfB* mutant (Tufariello *et al.*, 2006) indicates that it may be sufficient for exit from dormancy. RpfB interacts with a peptidoglycan endopeptidase, RipA, at cell division sites (Hett *et al.*, 2007; Hett *et al.*, 2008) suggesting that RpfB plays a role in peptidoglycan metabolism. However, despite the clear importance of these proteins in the life cycle of this pathogen, the molecular mechanism underlying their regulation remains mysterious tBLAST analysis of the *B. subtilis* genome with the RpfB sequence reveals a distant homologue, YabE (Ravagnani *et al.*, 2005). While YabE is predicted to be an

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integral membrane protein and therefore, unlike RpfB, is probably not secreted, the YabE paralogue YocH is secreted (Tjalsma *et al.*, 2004). YocH contains two LysM domains that mediate peptidoglycan binding (Buist *et al.*, 2008) and three aspartate residues in its C-terminal 'Stationary phase survival' (Sps) domain (Ravagnani *et al.*, 2005) that are involved in catalysis by the *Escherichia coli* lytic transglycosylase MltA (Blackburn and Clarke, 2001; Van straaten *et al.*, 2007). Thus, YocH could function analogously to Rpf proteins by digesting peptidoglycan present in the extracellular milieu.

The essential two-component YycFG system in *B. subtilis* senses aspects of cell wall metabolism (Dubrac *et al.*, 2008; Winkler and Hoch, 2008). The membrane-bound sensor kinase YycG responds to an as yet unidentified signal at the division septum and phosphorylates the response regulator YycF to coordinate growth and division with cell wall restructuring (Bisicchia *et al.*, 2007; Fukushima *et al.*, 2008). YycF regulates a number of genes that encode proteins with putative cell wall degrading and/or modifying activities including *yocH* (Howell *et al.*, 2003). YycG interacts with the extracellular domains of the membrane-bound proteins YycH and YycI (Szurmant *et al.*, 2005) and a deletion of YycI results in increased levels of expression from P_{yocH} (Szurmant *et al.*, 2007). In addition to these direct effects, the YycFG system downregulates *lseA* (YoeB), an inhibitor of autolysins (Salzberg and Helmann, 2007; Yamamoto *et al.*, 2008). While the molecule that activates this system is unknown, the involvement of the YycFG in cell wall metabolism suggests that it could be a cell wall component such as peptidoglycan.

Digested peptidoglycan and purified muropeptides germinate dormant *B. subtilis* spores via PrkC, the eukaryotic-like membrane Ser/Thr kinase which contains PASTA ('Penicillin and Ser/Thr kinase Associated') repeats in its extracellular domain that bind peptidoglycan (Shah *et al.*, 2008). During spore germination and logarithmic growth, PrkC phosphorylates EF-G (Gaidenko *et al.*, 2002; Shah *et al.*, 2008), the essential ribosomal GTPase involved in mRNA and tRNA translocation as well as ribosome recycling (Savelsbergh *et al.*, 2009). Recently, the ribosome-associated GTPases CpgA and EF-Tu have also been reported to be *in vitro* PrkC substrates (Absalon *et al.*, 2009), further supporting a role for PrkC in regulating translation. PrkC may also play a role in controlling cell division because the PrkC homologues *M. tuberculosis* PknB (Dasgupta *et al.*, 2006; Parikh *et al.*, 2009), *Streptococcus agalactiae* Stk1 (Silvestroni *et al.*, 2009) and *Corynebacterium glutamicum* PknB (Fiuza *et al.*, 2008; Schultz *et al.*, 2009) phosphorylate proteins involved in peptidoglycan synthesis and/or cell division.

The germination of spores in response to peptidoglycan fragments and the presence of a pathway respon-

sive to cell wall metabolism suggested that exposure of growing *B. subtilis* cells to peptidoglycan fragments could induce specific genes. Here we show that incubation of growing cells with small soluble peptidoglycan fragments results in the induction of *yocH*, which we further demonstrate encodes a secreted peptidoglycan hydrolase. This induction of *yocH* is dependent on PrkC and YocH autoregulates its own expression via the PrkC pathway.

Results

Peptidoglycan-derived muropeptides induce yocH

Bacteria release significant amounts of peptidoglycan into the extracellular milieu during growth (Doyle *et al.*, 1988). To identify *B. subtilis* genes induced by this exogenous peptidoglycan, we incubated *B. subtilis* cells with cell-free supernatants derived from growing *B. subtilis* cultures that contain large quantities of peptidoglycan fragments (Mauck *et al.*, 1971). In an initial microarray-based screen, we identified several genes induced by this treatment and chose to focus on *yocH*. This gene encodes a protein with similarity in its C-terminus to the catalytic domain of the *E. coli* lytic transglycosylase MltA (Ravagnani *et al.*, 2005), and it is regulated by the cell wall-sensing YycFG system (Howell *et al.*, 2003). The induction of *yocH* by cell-free supernatants was dependent on the growth phase of the culture they were derived from because *yocH* levels were higher following incubation with supernatants isolated from growing cells as compared with those isolated from non-growing cells (Fig. 1A).

Consistent with the known presence of peptidoglycan fragments in culture supernatants, *yocH* was induced ~5-fold when growing *B. subtilis* cells were incubated with mutanolysin-digested *B. subtilis* peptidoglycan (Fig. 1B). Incubation of lysostaphin-digested *Staphylococcus aureus* peptidoglycan that contains an L-lys with log-phase *B. subtilis* cells did not induce *yocH*, indicating that an *m*-Dpm residue in the peptidoglycan stem peptide was likely necessary (Fig. 1B). The specific inducing molecule was identified using synthetic muropeptides. As with mutanolysin-digested *B. subtilis* peptidoglycan, *m*-Dpm containing muropeptides activated *yocH* (Fig. 1C), indicating that a muropeptide was sufficient for this induction. The inability of synthetic L-lys-containing muropeptides to induce *yocH* was consistent with the specificity of induction observed with peptidoglycan fragments (Fig. 1C).

YocH is a secreted protein with peptidoglycan binding and muralytic properties

YocH is a member of the extracellular proteome of *B. subtilis* (Tjalsma *et al.*, 2004) and consistent with this

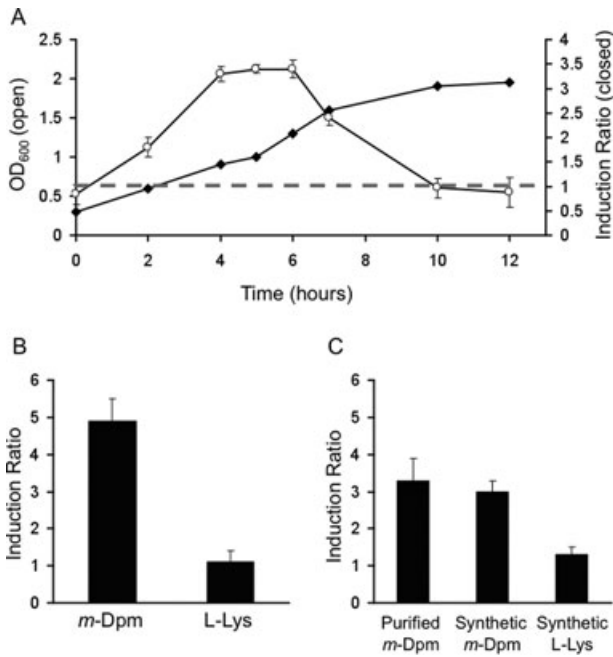


Fig. 1. *m*-Dpm type peptidoglycan induces *yocH* expression. A. Cell-free supernatants from *B. subtilis* isolated at different OD₆₀₀ values (♦) were added to log phase cultures of *B. subtilis* at an OD₆₀₀ ~0.3 for 30 min. *yocH* induction ratios (○) were measured for each sample. Dashed line indicates no induction. B. Mutanolysin-digested *B. subtilis* peptidoglycan (*m*-Dpm) and lysostaphin-digested *S. aureus* peptidoglycan (L-lys) were added to log phase cultures of *B. subtilis* at an OD₆₀₀ ~0.3 for 30 min. C. 25 μM *m*-Dpm-containing disaccharide tripeptide (P3; 'purified *m*-Dpm'), 20 μM *m*-Dpm-containing synthetic muropeptide (DHI-100; 'synthetic *m*-Dpm'), and 20 μM L-lys-containing synthetic muropeptide (DHI-138, 'synthetic L-lys') were added to log phase cultures of *B. subtilis* at an OD₆₀₀ ~0.3 for 30 min. RT-PCR using *yocH* specific primers was performed with DNase I-treated RNA isolated from both cultures and untreated controls. *yocH* band intensities were measured using ImageJ (NIH) and induction ratios determined. All experiments were performed in triplicate. The induction ratios are listed in Table S1.

observation, YocH-FLAG is found in the secreted fraction of log-phase *B. subtilis* cells (Fig. 2A). YocH contains two LysM peptidoglycan-binding domains (Steen *et al.*, 2003) and His₆-YocH purified from *E. coli* remained bound (~40%) to the insoluble peptidoglycan pellet (Fig. S1). This binding is similar to that observed with the extracellular domain of PrkC (Shah *et al.*, 2008). To test its putative muralytic activity, purified YocH-His₆ was subjected to zymogram analysis and a clearance band was seen at the appropriate molecular weight (Fig. 2B). As the catalytic activity of MltA absolutely requires residue D308 (Van straaten *et al.*, 2005), a mutant form of the His-tagged protein (His₆-YocH_{D264A}) that has an alanine in the equivalent position was purified. The absence of a clearance band associated with this protein in a zymogram (Fig. 2B) indicates that YocH likely uses a similar catalytic mechanism to digest peptidoglycan.

PrkC is required for muropeptide-mediated *yocH* induction

The *B. subtilis* membrane Ser/Thr kinase PrkC is required for germination of spores in response to peptidoglycan and to muropeptides (Shah *et al.*, 2008). To examine whether PrkC was also required for peptidoglycan-mediated *yocH* induction, RNA was isolated from wild-type and $\Delta prkC$ cultures that were either untreated or treated with mutanolysin-digested peptidoglycan. As before, *yocH* was induced ~5-fold in wild-type cultures, but treatment of $\Delta prkC$ cultures with peptidoglycan fragments did not lead to *yocH* induction (Fig. 3A). This requirement for PrkC was not observed for other genes induced by peptidoglycan such as *wprA* that encodes an extracellular protease (Fig. S2). The kinase activity of PrkC was necessary because peptidoglycan fragments did not induce *yocH* (Fig. 3A) in a strain expressing a PrkC mutant allele (PrkC_{K40A}) that eliminates PrkC kinase activity *in vitro* (Madec *et al.*, 2002). Additionally, overexpression of PrpC, the phosphatase belonging to the phylogenetically diverse PPM family that is co-expressed with PrkC and dephosphorylates PrkC (Obuchowski *et al.*, 2000), blocked induction of *yocH* by peptidoglycan (Fig. 3B). By contrast, overexpression of a mutant PrpC (PrpC_{D36N}) that carries an inactivating mutation in a conserved active site residue (D36) (Obuchowski *et al.*, 2000) did not inhibit *yocH* induction.

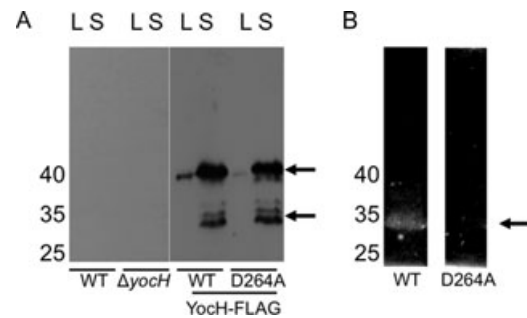


Fig. 2. YocH is a secreted peptidoglycan binding protein with muralytic activity.

A. Cellular lysates (L) and concentrated supernatants (S) from growing wild-type (PY79), WT; $\Delta yocH$ (JDB1881), $\Delta yocH$; $\Delta yocH$ P_{yocH-gfp} P_{spac-yocH-FLAG} (JDB2090), WT YocH-FLAG; and $\Delta yocH$ P_{yocH-gfp} P_{spac-yocH_{D264A}-FLAG} (JDB2091), D264A YocH-FLAG cultures were subjected to 8% SDS-PAGE and immunoblotted with α -FLAG antibodies. The upper band (top arrow) presumably is the unprocessed form whereas the lower band (bottom arrow) is the processed form.

B. YocH-his₆ and YocH_{D264A}-his₆ purified from supernatants generated by growing $\Delta yocH$ P_{yocH-gfp} P_{spac-yocH-his₆} (JDB2016) and $\Delta yocH$ P_{yocH-gfp} P_{spac-yocH_{D264A}-his₆} (JDB2092) cultures were subjected to zymogram analysis.

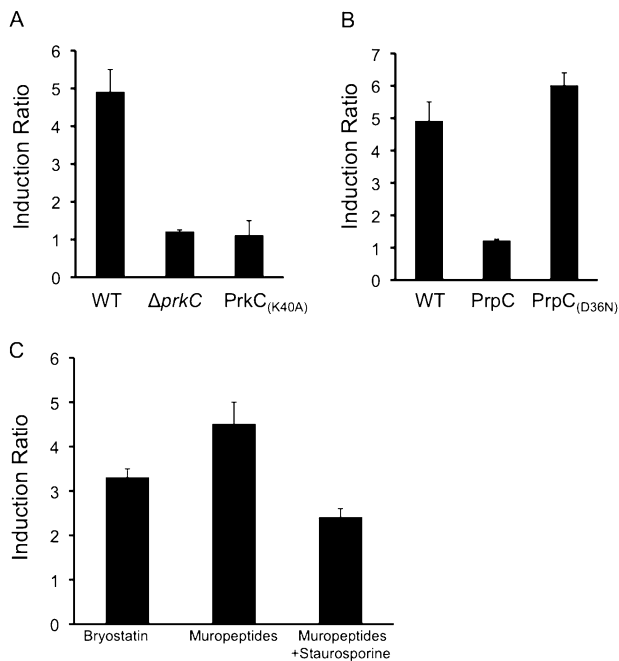


Fig. 3. Induction of *YochH* is dependent on PrkC kinase. A. RNA was isolated from wild-type (PB2), $\Delta prkC$ (PB705) and $\Delta prkC$ P_{spac-prkC_{K40A}-FLAG} (JDB2227) cultures following incubation with or without mutanolysin-digested *B. subtilis* peptidoglycan. B. RNA was isolated from wild-type (PB2), $\Delta prpC$ P_{spac-prpC} (JDB2094) and $\Delta prpC$ P_{spac-prpC_{D36N}} (JDB2097). C. RNA was isolated from log-phase cultures of wild-type *B. subtilis* (PY79) that were subjected to either no treatment or treatment with either 10 μ M bryostatin (bryostatin) or with mutanolysin-digested peptidoglycan (muropeptides) or with mutanolysin-digested peptidoglycan along with 100 pM staurosporine (muropeptides + staurosporine) for 30 min. RT-PCR was conducted with *yochH*-specific primers. The ratios of treated cells compared with the non-treated cells for three separate samples were calculated. The induction ratios are listed in Table S1.

Kinase stimulatory or inhibitory molecules affect *yochH* induction

The similarity of the kinase domain of the PrkC homologue *M. tuberculosis* PknB with eukaryotic Ser/Thr kinases (Ortiz-lombardia *et al.*, 2003; Young *et al.*, 2003) suggested that small molecules which either inhibit or activate these kinases would similarly affect PrkC. Bryostatin, a natural product synthesized by a marine bacterium, potently activates eukaryotic Ser/Thr kinases through direct binding to the phorbol ester binding site (Hale *et al.*, 2002) and stimulates germination of *B. subtilis* spores in a PrkC-dependent fashion (Shah *et al.*, 2008). Exposure of growing cultures to bryostatin caused ~3-fold induction of *yochH* (Fig. 3C) and this induction was dependent on the presence of PrkC because it was not observed in a $\Delta prkC$ strain (data not shown). Staurosporine, a small molecule ATP mimic produced by some strains of *Streptomyces*, inhibits eukaryotic Ser/Thr kinases by blocking ATP binding (Ruegg and Burgess,

1989). Growing cultures of *B. subtilis* incubated with peptidoglycan fragments in the presence of staurosporine reduce *yochH* induction as compared with in its absence (Fig. 3C), consistent with the loss-of-function phenotype of the PrkC K40A active site mutant (Fig. 3A).

PrkC phosphorylates EF-G in response to peptidoglycan fragments

The ribosomal GTPase EF-G is an *in vivo* and *in vitro* substrate of PrkC (Gaidenko *et al.*, 2002). Because EF-G is phosphorylated by PrkC during spore germination in response to muropeptides (Shah *et al.*, 2008), we examined whether PrkC also phosphorylated EF-G in log-phase cells in response to peptidoglycan fragments. Immunoprecipitated EF-G from log-phase cells that had been incubated with mutanolysin-digested peptidoglycan was probed with an anti-phosphothreonine antibody (Fig. 4). Although equivalent amounts of EF-G were immunoprecipitated from wild-type and $\Delta prkC$ cells treated with muropeptides, the greater phosphorylation seen in wild-type cells indicated that PrkC phosphorylates EF-G in the presence of muropeptides (Fig. 4, Fig. S3). EF-Tu was reported to be a substrate of PrkC *in vitro* (Absalon *et al.*, 2009); however, probing immunoprecipitated EF-Tu with phosphothreonine antibodies did not reveal a signal corresponding to EF-Tu (Fig. S4). Threonine phosphorylation of proteins that migrated at ~100–130 kDa was observed, but the identity of those proteins remains unknown.

YochH regulates its own expression

As expression of *yochH* is induced by mutanolysin-digested peptidoglycan (Fig. 1B) and *YochH* is itself a peptidoglycan hydrolase (Fig. 2B), *YochH* could regulate its own expression. That is, would the digestion of peptidoglycan by *YochH* generate fragments that induce P_{*yochH*}? To examine this possibility, *yochH* was placed under inducible control in a strain lacking endogenous *yochH* and

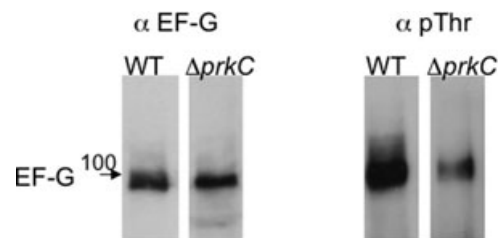


Fig. 4. PrkC phosphorylates EF-G in response to peptidoglycan fragments. Protein lysates were generated from wild-type *B. subtilis* (PB2) or from a $\Delta prkC$ (PB705) strains incubated with peptidoglycan isolated from log-phase *B. subtilis* cells for 60 min. Lysates were immunoprecipitated with α -EF-G and subjected to Western blotting with α -EF-G and α -phosphothreonine antibodies.

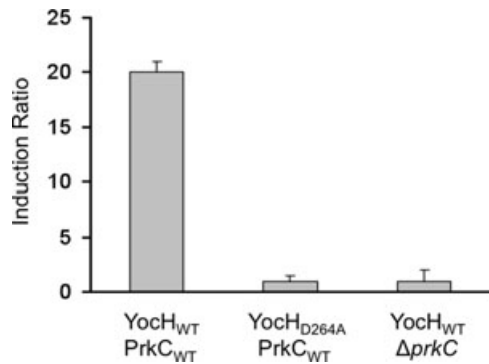


Fig. 5. YocH autoregulation requires functional YocH and PrkC. Early exponential phase cultures ($OD_{600} \sim 0.2$) of strains carrying inducible $\Delta yocH P_{spac}\text{-}yocH\text{-FLAG } P_{yocH}\text{-gfp}$ ('YocH_{wt} PrkC_{wt}'; JDB2090), $\Delta yocH P_{spac}\text{-}yocH_{D264A}\text{-FLAG } P_{yocH}\text{-gfp}$ ('YocH_{D264A} PrkC_{wt}'; JDB2091) and $\Delta prkC \Delta yocH P_{spac}\text{-}yocH\text{-FLAG } P_{yocH}\text{-gfp}$ strain ('YocH_{wt} $\Delta prkC$ '; JDB2296) were treated with IPTG (1 mM) for 2 h at 37°C. RNA was isolated from treated and untreated cultures and RT-PCR was performed with *gfp*-specific primers. Following agarose electrophoresis, the ratios of the signals from treated and untreated sample were obtained. They are listed in Table S1.

expression of a P_{yocH} reporter was measured so if *yocH* were subject to autoregulation, expression of YocH would activate P_{yocH} . In support of this model, addition of inducer (IPTG) to a strain where *yocH* was under the control of P_{spac} increased the activity of a P_{yocH} reporter ~20-fold as compared with the same strain grown in the absence of inducer (Fig. 5). As expression of YocH_{D264A}, a mutant form of YocH that is unable to digest peptidoglycan (Fig. 2B) had no effect on P_{yocH} reporter RNA levels (Fig. 5), this autoregulation depends on the muralytic activity of YocH. The requirement of PrkC for induction of *yocH* in response to peptidoglycan (Fig. 3A) suggested that PrkC mediates the observed *yocH* autoregulation. We examined this possibility by introducing a $\Delta prkC$ mutation into the strain carrying $P_{spac}\text{-}yocH$ and the P_{yocH} reporter. This strain no longer increased P_{yocH} reporter expression in response to *yocH* induction (Fig. 5). Thus, YocH appears to autoregulate its own expression by generating mucopeptides that are in turn sensed by the membrane kinase PrkC.

Deletion of *yocH* results in a post-exponential phase survival defect

YocH contains an Sps domain in its C-terminus. Proteins containing this domain have been proposed to facilitate survival in stationary phase by analogy with the Rpf proteins of *M. tuberculosis* and *M. luteus* (Ravagnani *et al.*, 2005). Consistent with this possibility, a $\Delta yocH$ strain (Fig. 6A, dashed line) exhibits a post-exponential phase survival defect as compared with the wild-type parent (Fig. 6A, solid line) using buffered Luria–Bertani (LB)

medium (Gaidenko *et al.*, 2002). This defect is rescued by the expression of YocH (Fig. 6B, solid line). The muralytic activity of YocH was necessary for this complementation because expression of the YocH_{D264A} mutant that lacks muralytic activity (Fig. 2A) did not restore wild-type levels of post-exponential survival (Fig. 6B, dashed line).

A strain carrying a $\Delta prkC$ mutation also exhibits a post-exponential phase survival defect (Gaidenko *et al.*, 2002). As PrkC is necessary for induction of *yocH* in response to peptidoglycan fragments (Fig. 3A), we examined whether the $\Delta yocH$ phenotype involved the PrkC pathway by comparing the post-exponential phase survival defects of strains carrying single $\Delta yocH$ and $\Delta prkC$ mutations and a strain carrying both mutations. As the defect of strains carrying either single mutation was the same as that of a

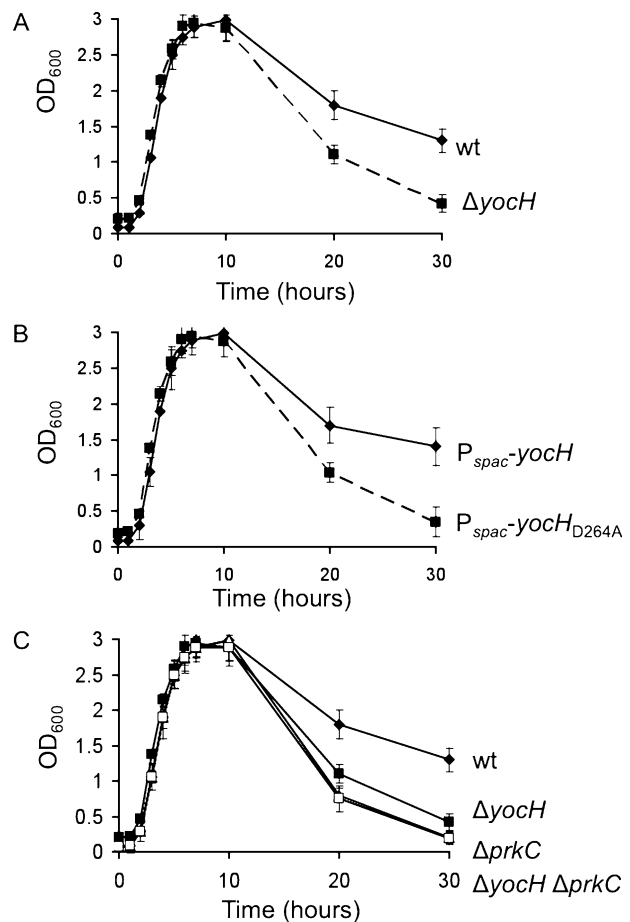


Fig. 6. Absence of YocH decreases post-exponential phase survival.

A. Exponential and post-exponential phase survival of wild-type (♦, solid line; JDB3) and $\Delta yocH$ (■, dashed line; JDB1881) strains. B. Exponential and post-exponential phase survival of $\Delta yocH P_{spac}\text{-}yocH$ (♦, solid line; JDB2090) and $\Delta yocH P_{spac}\text{-}yocH_{D264A}$ (■, dashed line; JDB2091) strains. C. Exponential and post-exponential phase survival of wild-type (♦; PB2), $\Delta yocH$ (■; JDB2338), $\Delta prkC$ (Δ; PB705) and $\Delta yocH \Delta prkC$ (□; JDB2340) strains. Cultures were grown in buffered LB at 37°C and OD_{600} was measured at designated intervals.

Table 1. Cell autonomous activity of YocH.

Strains	cfu ml ⁻¹ (T ₀)	cfu ml ⁻¹ (T ₂₄)	% T ₀
Wild type	5.6 × 10 ⁸	3.08 × 10 ⁸	55 ± 5
—	—	—	—
Δ <i>yocH</i>	5.5 × 10 ⁸	1.37 × 10 ⁸	25 ± 4
—	—	—	—
Wild type	2.4 × 10 ⁸	1.15 × 10 ⁸	48 ± 2
Δ <i>yocH</i>	2.2 × 10 ⁸	1.2 × 10 ⁸	55 ± 8
Δ <i>yocH</i> P _{spac} - <i>yocH</i>	5.16 × 10 ⁸	3.1 × 10 ⁸	60 ± 5
—	—	—	—
Δ <i>yocH</i> P _{spac} - <i>yocH</i>	2.2 × 10 ⁸	1.2 × 10 ⁸	54 ± 4
Δ <i>yocH</i>	2.45 × 10 ⁸	1.1 × 10 ⁸	45 ± 3
Δ <i>yocH</i> P _{spac} - <i>yocH</i> _{D264A}	5.5 × 10 ⁸	1.08 × 10 ⁸	20 ± 5
—	—	—	—
Δ <i>yocH</i> P _{spac} - <i>yocH</i> _{D264A}	2.39 × 10 ⁸	0.55 × 10 ⁸	23 ± 6
Δ <i>yocH</i>	2.18 × 10 ⁸	0.48 × 10 ⁸	22 ± 8

Single cultures of a wild-type strain (JDB1760; cmR), a Δ*yocH* strain (JDB1881; ermR), a Δ*yocH* P_{spac}-*yocH* (JDB2090; specR) and Δ*yocH* P_{spac}-*yocH*_{D264A} (JDB2091; specR) were grown in buffered LB medium up to OD₆₀₀ ~2.0. Then single or mixed cultures containing approximately equal cfus of each strain (T₀) were assayed for cfus following growth in buffered LB medium for 24 h at 37°C (T₂₄). To determine cfus, aliquots were plated on appropriate antibiotic plates. The cfus obtained for each strain at T₀ were normalized to 100% to determine the fraction of survivors at T₂₄.

strain carrying both mutations (Fig. 6C), YocH and PrkC likely function in the same pathway.

YocH acts in a cell-autonomous fashion

YocH is secreted and found in the extracellular milieu (Fig. 2A; Tjalsma *et al.*, 2004), suggesting that it could function cell autonomously. That is, in a culture containing both wild-type and Δ*yocH* cells, does YocH secreted from wild-type cells increase the survival of cells unable to produce YocH? The survival of a Δ*yocH* strain was increased in the presence of a wild-type strain, suggesting that YocH was able to rescue the mutation (Table 1). The muralytic activity of YocH was necessary for this rescue because a strain expressing a mutant YocH that does not hydrolyse peptidoglycan (YocH_{D264A}) failed to rescue the survival defect of the Δ*yocH* strain (Table 1).

Discussion

Growing bacteria turn over their cell wall and consequently release peptidoglycan fragments into the environment. These molecules therefore would serve as an inter-bacterial signal for the presence of growth-permissive conditions and their detection would allow bacteria to monitor the hospitability of the environment for continued growth. Muropeptides are recognized by dormant bacterial spores of a number of species and induce germination of these spores (Shah *et al.*, 2008). Here we have demonstrated that these molecules are also recognized by growing *B. subtilis* and induce the

expression of a secreted peptidoglycan hydrolase. The enzymatic activity of this secreted protein is necessary for this induction and indicates the presence of an auto-regulatory loop.

Induction of yocH

Supernatants derived from cultures of growing cells triggered *yocH* activation (Fig. 1A). Consistent with the presence of peptidoglycan fragments in these supernatants, *m*-Dpm-containing peptidoglycan isolated from growing *B. subtilis* cells and treated with mutanolysin was also effective at inducing *yocH* (Fig. 1B). As *m*-Dpm-containing muropeptides or a synthetic *m*-Dpm disaccharide tripeptide induced *yocH* (Fig. 1C), muropeptides were likely the inducing molecule. The inability of a synthetic L-lys-containing muropeptide (Fig. 1B) or digested *S. aureus* peptidoglycan to induce *yocH* (Fig. 1C) indicates the presence of an L-lys residue in the stem peptide was sufficient to prevent induction. This specificity was similar to that observed during *B. subtilis* spore germination where the presence of a *m*-Dpm residue was necessary to promote induction (Shah *et al.*, 2008) and, interestingly, is similar to that observed with eukaryotic proteins that interact with muropeptides (Girardin *et al.*, 2003; Boneca, 2005; Guan and Mariuzza, 2007).

Supernatants derived from cultures of growing cells triggered *yocH* activation better than those derived from cells in stationary phase (Fig. 1A). This result indicates that the muropeptides contained in these supernatants report not only cell density, but also the growth phase of the culture by detecting peptidoglycan fragments released as the products of growth but not those released during cell lysis. The ability of a cell to differentiate between these molecules would allow it to respond only to conditions conducive to growth. While the molecular basis of these differences is unknown, one possibility is that hydrolysis of the glycosidic linkage between MurNAc and the GlcNAc residues by a lytic transglycosylase results in the formation of a 1,6-anhydroMurNAc containing disaccharide peptide whereas digestion by a lysozyme results in a terminal reducing MurNAc (Vollmer *et al.*, 2008). Thus, if lysozymes and lytic transglycosylases were differentially active during exponential and post-exponential phases, then the presence of the 1,6-anhydro ring containing molecules would indicate the growth phase of the culture. As lytic transglycosylases and penicillin binding proteins (Vollmer *et al.*, 1999) form complexes involved in cell elongation, the presence of anhydro-containing muropeptides would signal that conditions were permissive for growth.

Regulation of YocH

Expression of *yocH* has been previously shown to be regulated by the essential YycFG two-component system

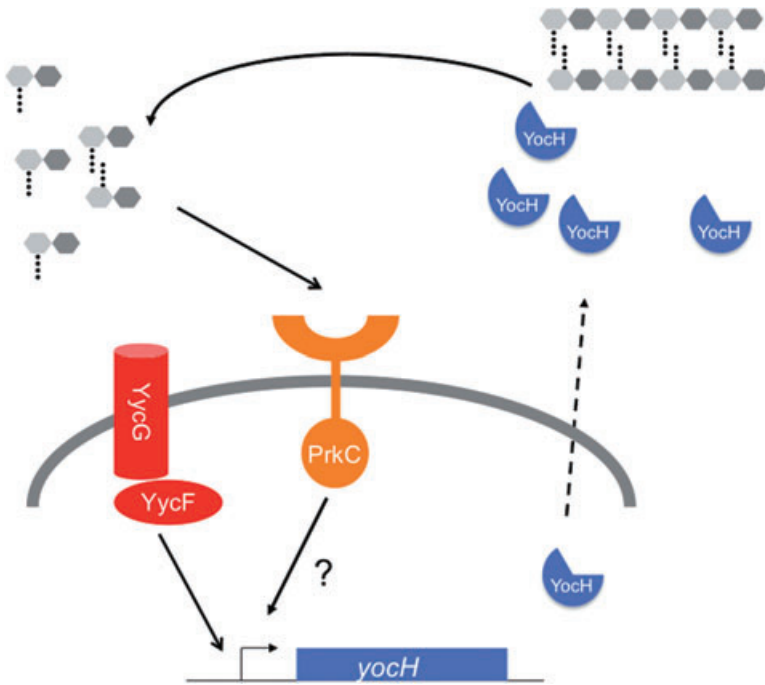


Fig. 7. Model of YocH function. YocH (blue circles) is secreted into the extracellular milieu where it digests peptidoglycan that derives from either the same cell or other cells in the culture. The digested muropeptides bind to the PrkC kinase (orange) in the membrane. This binding induces *yocH* through an unknown pathway (?) that possibly acts in parallel with the two-component YycFG system (red) and results in the production of additional YocH molecules.

in *B. subtilis* (Howell *et al.*, 2003). Here we demonstrate that the kinase activity of PrkC is necessary for *yocH* induction in response to muropeptides because strains lacking PrkC or expressing a mutant PrkC carrying a point mutation in its active site do not respond (Fig. 3A). The ability of the extracellular domain of PrkC to bind insoluble peptidoglycan (Shah *et al.*, 2008) suggests that PrkC directly binds these muropeptides. However, given that PrkC is a Ser/Thr kinase, it is unlikely that it directly activates P_{yocH} . In two-component systems such as YycFG, the membrane kinase phosphorylates a DNA binding protein on an aspartic acid residue, thereby activating transcription of a particular set of genes. Thus, PrkC could phosphorylate YycF on a Ser or Thr residue, thereby changing the ability of YycF to activate transcription. Alternatively, PrkC could phosphorylate proteins involved in cell division as is observed with its homologues *M. tuberculosis* PknB (Kang *et al.*, 2005; Parikh *et al.*, 2009) and *S. agalactiae* Stk1 (Silvestroni *et al.*, 2009). These modifications would result in the activation of P_{yocH} through the YycFG system that detects changes in cell wall metabolism (Dubrac *et al.*, 2008). A third possibility is that PrkC phosphorylates an unknown protein that directly regulates P_{yocH} expression. The gene encoding this protein could be detected in a genetic screen using a P_{yocH} -*lacZ* reporter if constitutively active PrkC mutants were known but as yet these have not been identified.

When *yocH* is placed under IPTG-inducible control, treatment with IPTG increases the expression of a P_{yocH} reporter. Importantly, only YocH capable of digesting pep-

tidoglycan can regulate its own expression because the enzymatically inactive YocH_{D264A} does not similarly affect expression of the reporter (Fig. 5). This autoregulatory loop (Fig. 7) also depends on PrkC (Fig. 5) as was observed with *yocH* induction in response to exogenous muropeptides. This is consistent with the demonstrated muralytic activity of YocH as well as its presence in the supernatant (Fig. 2A and B). We do not know if YycFG is necessary for this loop because these proteins are essential, although their role in sensing cell wall perturbations suggests that they are involved.

Physiological role of YocH

Absence of YocH reduces post-exponential phase survival in liquid culture (Fig. 6A). This phenotype can be attributed to YocH function because inducible expression of wild-type YocH but not a catalytically inactive mutant (YocH_{D264A}) complements this survival defect in liquid cultures (Fig. 6B). As YocH can function in a cell-autonomous manner (Table 1), it is not clear from these complementation experiments whether the source of this peptidoglycan is from the same cell or another cell in the culture. Thus, the inability to digest extracellular peptidoglycan into fragments is detrimental to the cell's survival in post-exponential phase. While peptidoglycan could serve as a nutritional source given that it is composed of disaccharide peptides, the inability of L-lys-containing peptidoglycan fragments to induce *yocH* (Fig. 1B) suggests that this is not likely.

Alternatively, the decrease in post-exponential phase survival of the $\Delta yocH$ strain could be due to a lack of stimulation of PrkC. Absence of PrkC results in a similar reduction in survival and because a $\Delta prkC \Delta yocH$ strain is as defective as a $\Delta prkC$ strain (Fig. 6C), the genes probably act in the same pathway. In contrast to PrkC, absence of PrpC, the partner phosphatase to PrkC, increases post-exponential phase survival as compared with wild type (Gaidenko *et al.*, 2002). These opposite phenotypes suggest that increased phosphorylation of a common substrate of PrkC and PrpC is responsible for this increased survival. As the one identified common substrate of PrkC and PrpC is EF-G, its phosphorylation state (see below) may play a role in post-exponential phase survival. In addition, PrkC homologues play central roles in the physiology of a number of species in situations where post-exponential phase is important including *Streptococcus pneumoniae* StpK in competence (Echenique *et al.*, 2004) and *Enterococcus faecalis* PrkC in persistence (Kristich *et al.*, 2007).

The preferential induction of *yocH* by supernatants from growing cells as compared with cells in stationary phase (Fig. 1A) may indicate that this induction signals the presence of growing cells. Like YocH, *S. aureus* IsaA is a putative soluble lytic transglycosylase (Sakata *et al.*, 2005; Stapleton *et al.*, 2007). Production of IsaA is regulated by the *S. aureus* YycG and YycF homologues (Dubrac and Msadek, 2004) and expression of *isaA* was stimulated during exponential growth and repressed during post-exponential phase (Sakata and Mukai, 2007). As *B. subtilis yocH* is activated in a similar growth phase-dependent fashion, this phenomenon may be common to wide range of bacteria.

Role of EF-G phosphorylation

EF-G is an abundant cellular protein with several activities in regulating translation (Savelsbergh *et al.*, 2009). First, EF-G plays an essential role in the translocation of mRNAs and tRNAs in the ribosome during translation (Shoji *et al.*, 2009). Second, EF-G participates along with ribosome recycling factor in the process of ribosome recycling where the 70S ribosomes are split into subunits and the mRNA and tRNA are released (Hirokawa *et al.*, 2006). The activity of eEF-2, its eukaryotic homologue, is regulated by phosphorylation (Ryazanov *et al.*, 1988) and amino acid withdrawal leads to increases in EF-2 phosphorylation in yeast (Wang *et al.*, 1998). While it is not known whether EF-G is similarly regulated, EF-G is phosphorylated in growing cells in response to muropeptides (Fig. 4). Thus, binding of muropeptides to PrkC would induce EF-G phosphorylation and thereby change the translational capacity of the cell as has been suggested previously for proteins containing an Sps domain (Ravagnani *et al.*, 2005).

In addition to the phosphorylation of EF-G, PrkC is necessary for the signal transduction cascade that results in the induction of *yocH*. A possible connection between these two effects arises in the recent characterization of genes whose expression changed following treatment of *S. aureus* cells with fusidic acid, a molecule that interferes with release of EF-G following translocation. Six out of nine peptidoglycan hydrolase genes under control of YycFG including the secreted peptidoglycan hydrolase IsaA were upregulated by fusidic acid (Delgado *et al.*, 2008). The interaction between the PrkC homologue *S. agalactiae* Stk1 and the two-component regulator CovR in cytotoxin expression (Rajagopal *et al.*, 2006) suggests that a fruitful direction for future research will be examining the functional overlap between the PrkC and YycFG pathways.

Role of muralytic enzymes in exit from dormancy

The human pathogen *M. tuberculosis* can persist in the host for decades following infection before initiating disease. While the transition between latency and reactivation is thought to be essential for the bacterium to cause disease, the mechanism underlying this transition is unclear. Rpf proteins secreted by *M. tuberculosis* are important for this transition because an *rpfB* mutant of *M. tuberculosis* Erdman displayed delayed kinetics of reactivation in a mouse model of dormancy (Tufariello *et al.*, 2006) and mutants of *M. tuberculosis* H37Rv lacking various combinations of *rpf*-like genes were attenuated for growth in mice (Downing *et al.*, 2005). The muralytic activity of *M. tuberculosis* Rpf proteins and the homologous Rpf from *M. luteus* suggests that they facilitate reactivation through cell wall remodelling (Mukamolova *et al.*, 2006; Telkov *et al.*, 2006).

Although *B. subtilis* YocH is not by sequence similarity an Rpf homologue, the work described here demonstrates that it is functionally analogous given that it is secreted (Fig. 2A), muralytic (Fig. 2B) and that its absence results in a post-exponential phase survival defect (Table 1). Our data also demonstrate that *yocH* induction in response to muropeptides is dependent on the kinase activity of PrkC (Fig. 3). *M. tuberculosis* PknB is a homologue of PrkC that is essential and is therefore a high-priority drug target (Fernandez *et al.*, 2006). Our work suggests that, by analogy, PknB may respond to muropeptides that are generated as a result of the muralytic activity of the Rpf proteins. A plausible connection between PknB activation and exit from dormancy is the ability of PrkC to phosphorylate EF-G (Gaidenko *et al.*, 2002; Shah *et al.*, 2008), a protein involved in ribosome recycling. As this process is necessary for the transition from stationary phase to growth in *E. coli* (Janosi *et al.*, 1998), phosphorylation of EF-G could be a trigger for *M. tuberculosis* reactivation.

Table 2. *B. subtilis* strains.

Strain	Genotype	Source
PY79	Wild type	Lab collection
PB2	<i>trpC2</i>	Gaidenko <i>et al.</i> (2002)
PB705	<i>trpC2 prkCΔ1</i>	Gaidenko <i>et al.</i> (2002)
JDB1881	$\Delta yocH::erm$	This study
JDB2014	$\Delta yocH::erm sacA::P_{yocH^-}gfp cm$	This study
JDB2016	$\Delta yocH::erm sacA::P_{yocH^-}gfp cm amyE::P_{spac^-}yocH-his_6 spec$	This study
JDB2090	$\Delta yocH::erm sacA::P_{yocH^-}gfp cm amyE::P_{spac^-}yocH-FLAG spec$	This study
JDB2091	$\Delta yocH::erm sacA::P_{yocH^-}gfp cm amyE::P_{spac^-}yocH_{D264A}-FLAG spec$	This study
JDB2092	$\Delta yocH sacA::P_{yocH^-}gfp cm amyE::P_{spac^-}yocH_{D264A}-his_6$	This study
JDB2094	<i>trpC2 ΔprpCΔ1 amyE::P_{spac⁻}prpC spec</i>	This study
JDB2097	<i>trpC2 ΔprpCΔ1 amyE::P_{spac⁻}prpC_{D36N} spec</i>	This study
JDB2227	<i>trpC2 ΔprkCΔ1 amyE::P_{spac⁻}FLAG-prkC_{K40A} spec</i>	Shah <i>et al.</i> (2008)
JDB2296	<i>trpC2 ΔprkCΔ1 ΔyocH::erm sacA::P_{yocH^-}gfp cm amyE::P_{spac^-}yocH-FLAG spec}}</i>	This study
JDB2338	<i>trpC2 ΔyocH::erm</i>	This study
JDB2340	<i>trpC2 ΔprkCΔ1 ΔyocH::erm</i>	This study

Experimental procedures

Bacillus subtilis strains used in this study are listed in Table 2 and details of their construction are described in *Supporting information*. *B. subtilis* strains were grown in LB except for experiments involving survival in post-exponential phase where the cultures were grown in buffered LB (Gaidenko *et al.*, 2002). For experiments where growing cells were treated with cell free supernatants, 3 ml of cultures was grown in TSS medium [per litre: 10 ml of 50% glucose, 10 ml of 1.2% of MgSO₄, 10 ml of 0.4% FeCl₃-citrate (1:1), and 100 ml of 10× TSS salts (20 g of NH₄Cl, 3.6 g of K₂HPO₄, 60 g of Tris base) and the pH was adjusted to 7.5 with HCl]. The cells were grown to an OD₆₀₀ = 0.3, harvested and resuspended in 3 ml of cell-free supernatants isolated from cells grown to OD₆₀₀ of 0.3, 0.6, 0.9, 1.0, 1.3, 1.6 and 1.9, shaken for 30 min and harvested for RNA isolation. For experiments where growing cells were treated with PG, 3 ml of cultures was grown to an OD₆₀₀ = 0.3 and treated with either of the following agents: 5 mg of digested or undigested *m*-Dpm or lys-type peptidoglycan, 25 μM purified disaccharide-tripeptide P3 (*m*-Dpm), 20 μM of synthetic disaccharide-dimer DHI-100 (*m*-Dpm) or 20 μM of synthetic disaccharide dimer DHI-138 (L-lys) for 30 min at 37°C prior to harvesting and RNA isolation. P3 was a gift from Dr David Popham (Virginia Tech) and DHI-100 and DHI-138 were gifts from Dr Shahriar Mobashery (Notre Dame) and their synthesis is described in Lee *et al.* (unpublished data). Lysostaphin and mutanolysin were obtained from Sigma.

Peptidoglycan isolation

Peptidoglycan was isolated from growing *B. subtilis* or *S. aureus* cells as described (Shah *et al.*, 2008). Resuspended *B. subtilis* peptidoglycan (50 mg ml⁻¹) was digested with mutanolysin (10 μg ml⁻¹) overnight at 37°C prior to inactivation of mutanolysin at 80°C for 20 min. *S. aureus* peptidoglycan (50 mg ml⁻¹) was digested with lysostaphin (0.5 mg ml⁻¹) overnight at 37°C prior to inactivation of the enzyme at 80°C for 20 min.

RNA isolation and RT-PCR analysis

RNA was isolated from cell pellets using RNeasy kit (Qiagen) according to the manufacturer's instructions. Prior to treatment with lysozyme, RNA was stabilized with Bacterioproctect (Qiagen). Isolated RNA was treated with DNase I (37°C, 50 min) followed by heat inactivation (65°C, 10 min). RT-PCR reactions were carried out in 25 μl volumes with 100 ng of RNA for each sample using SSIII RT/Platinum Taq enzyme (Invitrogen) according to the manufacturer's instructions. Briefly, reverse transcription was carried out at 50°C for 30 min followed by PCR [Denaturation: 94°C (15 s), PCR: 30 cycles of 94°C (15 s), 55°C (30 s) and 68°C (1 min kb⁻¹)]. Total sample volumes were loaded on 0.8% agarose gels followed by electrophoresis and staining with SYBR gold (Invitrogen). ImageJ (NIH) was used for gel quantification.

Analysis of secreted proteins

Cells were grown in 3 ml LB medium until OD₆₀₀ = ~0.1. For expression of proteins from P_{spac}, the cultures were induced with 1 mM IPTG for 2 h. The cultures were centrifuged (5000 g, 5 min) and the supernatant added to ice-cold ethanol (5× volume, 15 min). Following centrifugation (3000 g, 10 min), the precipitated pellets were air-dried and directly resuspended in 80 μl 2× SDS-loading dye. For cellular protein extracts, the pellets were resuspended in 500 μl 10 mM Tris, pH 8.0, 10% sucrose and treated with 1 mg ml⁻¹ lysozyme for 15 min prior to resuspension in 80 μl 2× SDS-loading dye. Resuspended pellets were subjected to SDS-PAGE followed by immunoblotting with α-FLAG antibodies (Sigma) and by detection by ECL (Amersham).

Purification of secreted proteins

Bacillus subtilis strains were grown in 30 ml LB medium until OD₆₀₀ = ~0.1. For expression of his-tagged proteins from P_{spac}, 30 ml of cultures was induced with 1 mM IPTG for 2 h prior to removal of cells by centrifugation. The supernatants

were concentrated (Vivaspin columns, MWCO = 10 kDa) to a final volume of 0.5 ml and purified using Ni²⁺ affinity chromatography as described (Shah *et al.*, 2008).

Zymogram analysis

Zymogram analysis was carried out as described (Piuri and Hatfull, 2006). Briefly, gels were cast with 0.01% SDS and 30 μ l (~5 mg) *B. subtilis* peptidoglycan resuspension. Following electrophoresis, the gel was incubated at 37°C for 16 h in 1% Triton X-100, 25 mM Tris-HCl pH 8.5, washed once in water and stained for 3 h with 0.5% methylene blue in 0.01% KOH. Clearance bands were visualized following destaining (5 \times water wash).

Immunoprecipitation and Western analysis

For detection of YochH in secreted fractions, α -FLAG antibodies (Sigma) were used (1:3000). For immunoprecipitation of EF-G, 3 ml of cultures of *B. subtilis* was harvested with or without treatment with *B. subtilis* peptidoglycan (10 mg) for 60 min, incubated with lysozyme (1 mg ml⁻¹) in Tris-EDTA buffer (pH 8.0) for 15 min in a total volume of 400 μ l and sonicated (45 s, 2 pulses). The samples were centrifuged and the resulting supernatants were added to 10 μ l EF-G-Protein A Dynabead (Invitrogen) complex as described (Shah *et al.*, 2008). Following immunoprecipitation for 1 h at 4°C, the beads were washed 3 \times with PBS and directly resuspended in 50 μ l of 2 \times SDS-loading dye and boiled for 30 min. Fifteen microlitres of samples was loaded onto duplicate 8% gels for probing with α -EF-G antibodies (1:3000 dilution) and α -phosphothreonine antibodies (1:250 dilution, Invitrogen).

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