YodL and YisK possess shape-modifying activities that are suppressed by mutations in *Bacillus subtilis mreB* and *mbl*

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Abstract

Many bacteria utilize actin-like proteins to direct peptidoglycan (PG) synthesis. MreB and MreB-like proteins are thought to act as scaffolds, guiding the localization and activity of key PG synthesizing proteins during cell elongation. Despite their critical role in viability and cell-shape maintenance, very little is known about how the activity of MreB family proteins is regulated. Using a *Bacillus subtilis* misexpression screen, we identified two genes *yodL* and *yisK*, that when misexpressed lead to loss of cell width control and cell lysis. Expression analysis suggests that *yodL* and *yisK* are previously uncharacterized Spo0A-regulated genes, and consistent with these observations, a Δ*yodL* Δ*yisK* mutant exhibits reduced sporulation efficiency. Suppressors resistant to YodL's killing activity occurred primarily in *mreB* and resulted in amino acid substitutions at the interface between MreB and the highly conserved morphogenic protein RodZ, whereas suppressors resistant to YisK occurred primarily in *mbl*, and mapped to Mbl's predicted ATP-binding pocket. YodL's shape-altering activity appears to require MreB, as a Δ*mreB* mutant is resistant to the effects of YodL but not YisK. Similarly, YisK appears to require Mbl, as a Δ*mbl* mutant is resistant to cell-widening effects of YisK, but not YodL. Collectively, our results suggest that YodL and YisK likely modulate MreB and Mbl activity, possibly during the early stages of sporulation.

Importance

The peptidoglycan (PG) component of cell envelope confers structural rigidity to bacteria and protects them from osmotic pressure. MreB and MreB-like proteins are thought to act as scaffolds for PG synthesis, and are essential in bacteria exhibiting non-polar growth. Despite the critical role of MreB-like proteins, we lack mechanistic insight into how their activities are regulated. Here, we describe the discovery of two *B. subtilis* proteins, YodL and YisK, which modulate MreB and Mbl activity. Our data suggest YodL specifically targets MreB, whereas YisK targets Mbl. The apparent specificity with which YodL and YisK are able to differentially target MreB and Mbl make them potentially powerful tools for probing the mechanics of cytoskeletal function in bacteria.

Introduction

Bacterial cell growth requires that the machineries directing enlargement and division of the bacterial cell envelope be coordinated in both time and space (1). The cell envelope is comprised of membranes and a macromolecular mesh of peptidoglycan (PG) that possesses both rigid and elastic properties (2, 3). PG is highly cross-linked, allowing bacteria to maintain shapes and avoid lysis, even in the presence of several atmospheres of internal turgor pressure. PG rearrangements are required during the inward redirection of growth that occurs at the time of cell division, but are also necessary when cells insert new PG and dynamically modify their morphologies in response to developmental or environmental signals (4, 5). To avoid lysis during PG rearrangements, bacteria must carefully regulate the making and breaking of glycan strands and peptide crosslinks (3). In rod-shaped bacteria, PG enlargement during steady-state growth is constrained in one dimension along the cell's long-axis and can either occur through polar growth, as is the case in *Agrobacterium tumefaciens* and *Streptomyces coelicolor,* or through incorporation of new cell wall material along the length of the cell cylinder, as observed in *Escherichia coli*, *Bacillus subtilis,* and *Caulobacter crescentus* (6).

To control cell diameter and create osmotically stable PG, bacteria that exhibit non-polar growth require the activity of the highly conserved actin-like protein MreB. Biochemical, genetic, and cell biological data suggest that MreB likely directs PG synthesis during cell elongation and in some bacteria, MreB may also function during cell division (7-9). MreB possesses ATPase activity, and polymerizes at sites along the cytoplasmic side of the inner membrane (10). ATP binding and hydrolysis is required for MreB polymerization and activity (11) and two S-benzylisothiourea derivatives, A22 and MP265, target the ATPase domain of MreB in Gram negative organisms, possibly preventing nucleotide hydrolysis and/or release (12-

15). Depletion or inactivation of MreB is lethal except in some conditional backgrounds (16, 17), so organisms sensitive to A22 and/or MP265 lose shape and eventually lyse (12-15).

MreB has been found to interact with several other proteins involved in PG synthesis, including the bitopic membrane protein RodZ (8, 16, 18-20). RodZ interacts directly with MreB through a cytoplasmic helix-turn-helix motif located at its N-terminus (18). A co-crystal structure of RodZ and MreB shows the N-terminus of RodZ extending into a conserved hydrophobic pocket located in subdomain IIA of MreB (18). Depletion of RodZ also leads to loss of cell shape and cell death (21-23). However, in various mutant backgrounds, *rodZ* can be deleted without loss of rod shape or viability, indicating that RodZ is not absolutely required for MreB's function in maintaining shape (24-26). Based on these observations and others, it has been proposed that MreB-RodZ interactions may regulate some aspect of MreB activity (10, 26).

Gram-positives often encode multiple paralogs (27). *B. subtilis* possesses three *mreB* family genes: *mreB*, *mbl*, and *mreBH*. *mreB* is distinguished from *mbl* and *mreBH* by its location within the highly conserved *mreBCD* operon. Although *mreB*, *mbl*, and *mreBH* are essential, it has been reported that each can be deleted under conditions in which cells are provided sufficient magnesium (28-30), or in strain backgrounds lacking *ponA*, the gene encoding penicillin binding protein 1 (PBP1) (20). In addition, all three genes can be deleted in a single background with only minor effects on cell shape if any one of the paralogs is artificially overexpressed in *trans* from an inducible promoter (31). The ability of any one of the paralogs to compensate for the loss of the others, at least under some growth conditions, strongly suggests that MreB, Mbl, and MreBH share significant functional redundancy (31, 32).

At the same time, several lines of evidence suggest that the paralogs possess non-overlapping functions. The genes themselves exhibit different patterns of transcriptional

regulation, suggesting that each likely possesses specialized activities that are important in different growth contexts. For example, *mreB* and *mbl* are maximally expressed at the end of exponential growth, but expression falls off sharply during stationary phase (33), whereas *mreBH* is part of the SigI heat-shock regulon (34). There is also evidence suggesting that each protein may possess specialized activities. For example, MreBH interacts with the lytic transglycosylase LytE, and is required for LytE localization (35), whereas the lytic transglycosylase CwlO, depends on Mbl for wildtype function (35). More recently MreB (but not Mbl or MreBH) was shown to aid in escape from the competent cell state (33).

Aside from RodZ (10, 26), only a handful of proteins targeting MreB activity in vivo have been identified. In *E. coli*, the YeeU-YeeV prophage toxin-antitoxin system is comprised of a negative regulator of MreB polymerization, CbtA (36), and a positive regulator of MreB bundling, CbeA (37). Another *E. coli* prophage toxin, CptA, is also reported to inhibit MreB polymerization (38). The MbiA protein of *C. crescentus* appears to regulate MreB in vivo, however, its physiological role is unknown (39). Given the importance of PG synthesis to cell viability and in cell shape control, it is likely that many undiscovered factors exist that modulate the activity of MreB and its paralogs.

In the present work we describe the identification of YodL and YisK, modulators of MreB and Mbl activity that are expressed during early stages of *B. subtilis* sporulation. Misexpression of either *yodL* or *yisK* during vegetative growth results in loss of cell width control and cell death. Genetic evidence indicates that YodL targets and inhibits MreB activity, whereas YisK targets and inhibits Mbl. Our data also show that YisK activity affects cell length control through an Mbl and MreBH-independent pathway.

Materials And Methods

General methods. All *B. subtilis* strains were derived from *B. subtilis* 168. *E. coli* and *B. subtilis* strains utilized in this study are listed in Table S2. Plasmids are listed in Table S3. Oligonucleotide primers are listed in Table S4. Details on plasmid and strain construction can be found in the Supplementary text. *Escherichia coli* DH5α was used for cloning. All *E. coli* strains were grown in LB-Lennox medium supplemented with 100 µg/ml ampicillin. The following concentrations of antibiotics were used for generating *B. subtilis* strains: 100 µg/ml spectinomycin, 7.5 µg/ml chloramphenicol, 0.8 mg/ml phleomycin, 10 µg/ml tetracycline, 10 µg/ml kanamycin. To select for erythromycin resistance, plates were supplemented with 1 µg/ml erythromycin (erm) and 25 µg/ml lincomycin. *B. subtilis* transformations were carried out as described previously (40). When indicated, the LB in the *B. subtilis* microscopy experiments was LB-Lennox broth. Sporulation by resuspension was carried out at 37°C according to the Sterlini-Mandelstam method (41). Penassay broth (PAB) is composed of 5 g peptone, 1.5 g beef extract, 1.5 g yeast extract, 1.0 g D-glucose (dextrose), 3.5 g NaCl, 3.68 g dipotassium phosphate, and 1.32 g monopotassium phosphate per liter of distilled water. To make solid media, the relevant media was supplemented with 1.5% (w/v) bacto-agar.

Microscopy. For microscopy experiments, all strains were grown in the indicated medium in volumes of 25 ml in 250 ml baffled flasks, and placed in a shaking waterbath set at 37°C and 280 rpm. Unless stated otherwise, misexpression was performed by inducing samples with 1.0 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and imaging samples 90 min post-induction. Fluorescence microscopy was performed with a Nikon Ti-E microscope equipped with a CFI Plan Apo lambda DM 100X objective, Prior Scientific Lumen 200 Illumination system, C-FL

159 UV-2E/C DAPI and C-FL GFP HC HISN Zero Shift filter cubes, and a CoolSNAP HQ2
160 monochrome camera. Membranes were stained with TMA-DPH [1-(4-
161 trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate] (0.02 mM) and
162 imaged with exposure times of 1 sec with a neutral density filter in place to reduce cytoplasmic
163 background. All GFP images were captured with a 1 sec exposure time. All images were
164 captured with NIS Elements Advanced Research (version 4.10), and processed with NIS
165 Elements Advanced Research (version 4.10) and ImageJ64 (42). Cells were mounted on glass
166 slides with 1% agarose pads or polylysine-treated coverslips prior to imaging. To quantitate cell
167 lengths for Fig 5, the cell lengths for 500 cells were determined for each population. The
168 statistical significance of cell length differences between populations was determined using an
169 unpaired student's t-test.
170
171 **Plate growth assays.** *B. subtilis* strains were streaked on LB-Lennox plates containing 100
172 µg/ml spectinomycin and 1 mM IPTG. The plates were supplemented with the indicated
173 concentrations of $MgCl_2$ when indicated. Plates were incubated at 37°C overnight and images
174 were captured on a ScanJet G4050 flatbed scanner (Hewlett Packard).
175
176 **Heat Kill.** Spore formation was quantified by growing cells in Difco sporulation medium
177 (DSM)(43). A freshly grown single colony of each strain was inoculated into 2 mL of DSM
178 media and placed in a roller drum at 37°C, 60 rpm for 36 hrs. To determine colony forming
179 units/ml, an aliquot of each culture was serially diluted and plated on DSM agar plates. To
180 enumerate heat resistant spores/ml, the serial diluted cultures were subjected to a 20 min heat
181 treatment at 80°C and plated on DSM agar plates. The plates were incubated at 37°C overnight

and the next day colony counts were determined. The relative sporulation frequency compared to wildtype was determined by calculating the spores/CFU of each experimental and dividing it by the spores/CFU of wildtype. The reported statistical significance was determined using an unpaired student's t-test.

Transcriptional fusions. Transcriptional fusions were constructed by fusing a ~200 bp region up to the start codon of either *yodL* or *yisK* to *gfp* or *lacZ* and integrating the fusions into the *B. subtilis* chromosome at the *amyE* locus (for more details, see strain construction in the supplemental text). Microscopy was conducted on each strain over a timecourse in sporulation by resuspension media (see general methods) or in a nutrient exhaustion timecourse in CH (44). Beta-galactosidase assays were performed as described (45), except all samples were frozen at -80°C before processing. All experiments were performed on at least three independent biological replicates.

Suppressor selections. Single colonies of BYD048 (3X P_{hy}-*yodL*, P_{hy}-*lacZ*) or BYD076 (3X P_{hy}-*yisK*, P_{hy}-*lacZ*) were used to inoculate independent 5 ml LB-Lennox cultures. Six independent cultures were grown for each strain. The cultures were grown for 6 hrs at 37°C and 0.3 µl of each culture was diluted in 100 µl LB and plated on an LB-Lennox agar plate containing 100 µg/ml spectinomycin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After overnight growth, suppressors that arose were patched on both LB-Lennox agar plates supplemented with 100 µg/ml spectinomycin and LB-Lennox agar plates supplemented with 100 µg/ml spectinomycin, 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 µg/ml X-Gal and grown at 37°C overnight. Only blue colonies were selected for further analysis; this screen

205 eliminated mutants unable to derepress P_{hy} in the presence of IPTG. In addition, each P_{hy}-*yodL*
206 or P_{hy}-*yisK* construct was transformed into a wildtype background to ensure that the construct
207 remained fully functional with respect to preventing cell growth on LB-Lennox agar plates
208 supplemented with the relevant antibiotic and 1 mM isopropyl-β-D-thiogalactopyranoside
209 (IPTG).
210
211 **Whole-genome sequencing and analysis.** Genomic DNA was isolated from six YodL-resistant
212 suppressors obtained from independent cultures as well as the parent strain (BYD048) by
213 inoculating a single colony in 6 ml LB-Lennox media and growing at 37°C for 4 hr in a roller
214 drum. Cells were collected by spinning at 21,130 x g for 2 min at room temperature,
215 resuspending the pellets in lysis buffer [20 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8, 100 mM
216 NaCl, and 2 mg/ml lysozyme] and incubing at 37°C for 30 min. Sarkosyl was added to a final
217 concentration of 1% (w/v). Protein was removed by extracting with 600 µl phenol, centrifuging
218 at 21,130 x g for 5 min at room temperature, and transferring the top (aqueous layer) to a new
219 microcentrifuge tube. This was followed by an extraction with 600 µl phenol-saturated
220 chloroform and centrifugation at 21,130 x g for 5 min at room temperature. After transferring
221 the aqueous layer to a new microcentrifuge tube, a final extraction was performed with 100%
222 chloroform, followed by centrifugation at 21,130 x g for 5 min at room temperature. The
223 aqueous layer was transferred to a new microcentrifuge tube, being careful to avoid the
224 interphase material. To precipitate the genomic DNA, a $1/10^{th}$ volume of 3.0 M Na-acetate and 1
225 ml of 100% ethanol was added, and the tube was inverted multiple times. The sample was
226 centrifuged at 21,130 x g for 1 min at room temperature in a microcentrifuge. The pellet was
227 washed with 150 µl 70% ethanol and resuspended in 500 µl TE [10 mM Tris pH 7.5, 1 mM

EDTA, pH 8.0]. To eliminate potential RNA contamination, RNase was added to a final concentration of 200 µg/ml and the sample was incubated at 55°C for 1 hr. To remove the RNase, the genomic DNA was re-purified by phenol-chloroform extraction and ethanol precipitation as described above. The final pellet was resuspended in 100 µl TE. Bar-coded libraries were prepared from each genomic DNA sample using a TruSeq DNA kit according to manufacture specifications (Illumina), and the samples were subjected to Illumina-based whole-genome sequencing using a MiSeq 250 paired-end run (Illumina). CLC Genomics Workbench (Qiagen) was used to map the sequence reads against the Bs168 reference genome and to identify single nucleotide polymorphisms, insertions, and deletions. Mutations associated with the P_{hy} integration contructs and those in which less than 40% of the reads differed from the reference genome were excluded as candidate changes responsible for suppression in our initial analysis (Table S1). The remaining suppressors mutations were identified by PCR amplifying *mreB* (using primer set OAS044 and OAS045) and *mbl* (using primer set OAS046 and OAS047), and sequencing with the same primers. To determine if the candidate suppressors alleles identified were sufficient to confer resistance to the original selective pressure, each was linked to a kanamycin resistance cassette and moved by transformation into a clean genetic background (see supplemental Strain Construction).

Results

YodL and YisK affect cell width

To identify novel factors involved in cellular morphogenesis, we created an ordered gene misexpression library comprising over 800 previously uncharacterized genes from *B. subtilis*. Each gene was placed under the control of an IPTG-inducible promoter (P_{hy}) and integrated in

single copy (1X) at *amyE*, a non-essential locus in the *B. subtilis* chromosome. The library (called the BEIGEL for *Bacillus* Ectopic Inducible Gene Expression Library), was screened for misexpression phenotypes that perturbed growth on solid media, and also resulted in obvious defects in nucleoid morphology, changes in cell division frequency, and/or perturbations in overall cell shape in liquid cultures. Two strains, one harboring P_{hy}-*yodL* and one harboring P_{hy}-*yisK*, were unable to form colonies on plates containing inducer (Fig 1A) and also produced wide, irregular cells with slightly tapered poles following misexpression in LB liquid media (Fig 1B). Cell lysis and aberrant cell divisions were also observed. Introducing a second copy (2X) of each P_{hy} misexpression construct into the chromosome did not appreciably enhance cell widening at the 90 min post-induction timepoint, although cell lysis was more readily observed (Fig 1B). P_{hy}-*yisK* (2X) misexpression also led to a drop in optical density over time (Fig S1A), consistent with the cell lysis observed microscopically. We conclude that the activities of *yodL* and *yisK* target one or more processes integral to width control during cell elongation.

The *yodL* and *yisK* misexpression phenotypes are similar to those observed when proteins involved in cell elongation are perturbed in *B. subtilis* (20, 31, 46). Since the addition of magnesium was previously reported to suppress the lethality and/or morphological phenotypes associated with depletion or deletion of some proteins important for cell elongation in *B. subtilis* (16, 20, 29, 31, 47), we assessed if the P_{hy}-*yodL* and P_{hy}-*yisK* misexpression phenotypes could be rescued by growing cells with media supplemented with two different concentrations of $MgCl_2$. The YodL-producing cells failed to grow on any LB media containing inducer, regardless of $MgCl_2$ concentration (Fig 1A). In contrast, LB supplemented with 25 mM $MgCl_2$ restored viability to the strain producing YisK (Fig 1A). Interestingly, even 25 mM $MgCl_2$ was not sufficient to suppress the cell-widening effect associated with YodL and YisK misexpression

(Fig 1B), although these cells did not lyse (Fig S1C). Since PAB medium was often used in the prior studies showing $MgCl_2$ supplementation rescued cell shape (16, 20, 29, 31, 47), we also assayed for growth on PAB following YodL and YisK expression. PAB supplemented with 25 mM $MgCl_2$ rescued growth on plates (Fig S2A), but still did not rescue morphology in liquid culture (Fig S2B).

yodL and *yisK* expression

To better understand the possible physiological functions of the *yodL* and *yisK* gene products, we analyzed the genes and their genetic contexts bioinformatically. *yodL* is predicted to encode a 12.5 kDa hypothetical protein which, based on amino acid similarity, is conserved in the *Bacillus* genus. In data from a global microarray study analyzing conditional gene expression in *B. subtilis*, *yodL* is expressed as a monocistronic mRNA, exhibiting peak expression ~2 hrs after entry into sporulation (48). *yodL* expression is most strongly correlated with expression of *racA* and *refZ* (*yttP*) (48), genes directly regulated by Spo0A (49). *yodL* was not previously identified as a member of the Spo0A regulon controlling early sporulation gene expression (49, 50), however a more recent study found that *yodL* expression during sporulation is reduced in a Δ*spo0A* mutant (51). Consistent with this observation, we identified a putative Spo0A box approximately ~75 bp upstream of the annotated *yodL* start codon (Fig 2A). *yisK* is predicted to encode a 33 kDa protein and is annotated as a putative catabolic enzyme based on its similarity to proteins involved in the degradation of aromatic amino acids (52). *yisK* was previously identified as a member of the SigH regulon, and possesses a SigH -35/-10 motif (50)(Fig 2B). Expression of *yisK* peaks ~2 hrs after entry into sporulation (39) and is most strongly correlated with expression of *kinA* (48), a gene regulated by both SigH (the stationary

phase sigma factor)(50, 53-55) and Spo0A (49, 56). As with *yodL*, we identified a putative Spo0A box in the regulatory region upstream of the *yisK* start codon (Fig 2B).

To independently test if *yodL* and *yisK* expression are consistent with Spo0A-dependent regulation, we fused the putative regulatory regions upstream of each gene to a *gfp* reporter gene, and integrated the fusions into the *amyE* locus. We then followed expression from the promoter fusions over a timecourse in CH liquid broth, a rich medium in which the cells first grow exponentially, transition to stationary phase, and finally gradually enter sporulation (Fig 3A-3C). In this timecourse, GFP signal from P_{yisK}-*gfp* increased dramatically from time 0 (OD_{600} ~0.6) to time 1 hr (OD_{600} ~1.6)(Fig 3C), consistent with *yisK*'s prior characterization as a SigH-regulated gene (50). In contrast, GFP fluorescence from P_{yodL}-*gfp* became evident at a later timepoint (120 min) and was more heterogeneous (Fig 3C), consistent with expression patterns previously observed for other Spo0A-P regulated genes (57, 58).

To quantitate expression from the promoters, we generated P_{yodL}-*lacZ* and P_{yisK}-*lacZ* reporter strains and collected samples over a CH timecourse beginning with early exponential (OD_{600} = 0.2). Expression from P_{yodL}-*lacZ* rose steadily beginning about 2 hrs after exit from exponential growth, and continued to rise at least until the final timepoint taken (Fig 3D). In contrast, expression from P_{yisK}-*lacZ* rose as cells transitioned from early to late exponential growth, reached peak levels shortly after exit from exponential growth, and remained steady for the remainder of the timepoints (Fig 3E). Wildtype expression from both P_{yodL}-*lacZ* and P_{yisK}-*lacZ* required both SigH and Spo0A, and was largely eliminated in the absence of both regulators (Fig 3D and 3E). We did not attempt to draw further conclusions from this data, since Spo0A and SigH each require the other for wildtype levels of expression (see discussion).

We then followed expression from the promoter fusions over a timecourse following the sporulation by resuspension method, which generates a more synchronous entry into sporulation (59). At time 0, neither the strain harboring P_{yodL}-*gfp*, nor the strain harboring P_{yisK}-*gfp* showed appreciable levels of fluorescence (Fig 4A), appearing similar to a negative control harboring *gfp* without a promoter (Fig S3). Between 0 and 40 min, both strains showed detectable increases in fluorescence. At 60 min, when the first polar divisions characteristic of sporulation begin to manifest, both strains were more strongly fluorescent (Fig 4A). GFP fluorescence from P_{yodL} was qualitatively more intense than fluorescence produced from P_{yisK} (all images were captured and scaled with identical parameters to allow for direct comparison). Moreover, the GFP signal continued to accumulate in the strain harboring P_{yodL}-*gfp* for at least two hrs (Fig 4A) and was heterogenous, consistent with activation by Spo0A. In contrast, the fluorescence signal produced from P_{yisK}-*gfp* was similar across the population and appeared similar at the 60 and 120 min timepoints (Fig 4A), consistent with SigH regulation.

To quantitate expression from the promoters during a sporulation by resuspension timecourse, we collected timepoints from strains harboring either the P_{yodL}-*lacZ* or P_{yisK}-*lacZ* reporter constructs and performed beta-galactosidase assays. Expression from P_{yodL}-*lacZ* rose rapidly between the 40 min and 100 min timepoints, and steadily declined thereafter (Fig 4B). The decline in signal was not observed for the GFP reporter, likely because the GFP is stable once synthesized (60). In contrast, expression from P_{yisK}-*lacZ* was highest at the time of resuspension (T0) and declined up until the final timepoint (Fig 4C).

Collectively, the patterns expression we observe for *yodL* are consistent with those observed for genes activated by high-threshold levels of Spo0A during sporulation, including *racA*, *spoIIG*, and *spoIIA* (61). In contrast, *yisK*'s expression pattern is similar to that observed

for *kinA* (48, 54, 62), with expression increasing in late exponential and stationary phase and early sporulation in a SigH-dependent manner (Fig 3), but decreasing during a sporulation by resuspension timecourse (Fig 4). We do not exclude the possibility that YodL and YisK might also function in other growth contexts.

A Δ*yodL* Δ*yisK* mutant is defective in sporulation

Since *yodL* and *yisK* expression correlates with other early sporulation genes, we next investigated if the gene products influenced the production of heat-resistant spores. To determine the number of heat-resistant spores in a sporulation culture, we quantified the number of colony forming units (CFU) present in cultures before (total CFU) and after (heat-resistant CFU) a heat treatment that kills vegetative cells. These values were normalized to display the sporulation efficiency of the mutants relative to wildtype. Single mutants in which either *yodL* or *yisK* were deleted displayed only mild (97% and 94%, respectively) reductions in relative sporulation efficiency (Table 1). Although the single mutants always sporulated less efficiently than wildtype in each experimental replicate, the differences were not statistically significant with only six experimental replicates. In contrast, the Δ*yodL* Δ*yisK* double mutant produced ~20% less heat-resistant spores than wildtype ($P<0.0006$)(Table 1). No decrease in total CFU was observed for any of the mutants compared to wildtype, indicating that the reduction in heat-resistant spores in the Δ*yodL* Δ*yisK* mutant was not due to reduced cell viability before heat treatment (Table 1). The gene downstream of *yisK*, *yisL*, is transcribed in the same direction as *yisK*. To determine if the reduction in sporulation we observed might be partially attributable to polar effects of the *yisK* deletion on *yisL* expression, we introduced P_{yisK}-*yisK* at an ectopic locus (*amyE*) in the Δ*yodL* Δ*yisK* mutant and repeated the heat-kill assay. The ectopic copy of P_{yisK}-

yisK restored sporulation in the Δ*yodL* Δ*yisK* to levels statistically indistinguishable from the Δ*yodL* single mutant (Table 1). These results lend support to the idea that YodL and YisK function during early sporulation and possess activities that, directly or indirectly, affect the production of viable spores. We do not exclude the possibility that YodL and YisK might also function outside the context of sporulation.

Given that *yisK* and *yodL* expression during vegetative growth leads to cell widening, we hypothesized that *yisK* and *yodL* mutants might produce thinner cells or spores during sporulation. However, no qualitative differences in cell or spore width were observed for the Δ*yodL*, Δ*yisK*, or Δ*yodL* Δ*yisK* mutant populations compared to wildtype during a sporulation timecourse (Fig S4). We also observed no qualitative differences in the shapes of germinating cells (data not shown). Thus, although YodL and YisK contribute to the production of heat-resistant spores, they do not appear to be required to generate any of the major morphological changes required for spore production.

MreB and Mbl are genetic targets of YodL and YisK activity

To identify genetic targets associated with YodL and YisK activity, we took advantage of the fact that misexpression of the proteins during vegetative growth prevents colony formation on plates and performed suppressor selection analysis. Strains harboring three copies of each misexpression cassette were utilized to reduce the chances of obtaining trivial suppressors in the misexpression cassette itself. In addition, P_{hy}-*lacZ* was used as a reporter to eliminate suppressors unable to release LacI repression following addition of inducer. In total, we obtained 14 suppressors resistant to YodL expression and 13 suppressors resistant to YisK expression. Six of the suppressors resistant to YodL were subjected to whole-genome

391 sequencing. The results of the sequencing are shown in Table S1. All of the suppressors
392 possessed mutations in either *mreB* or *mbl*, genes previously shown to be important in regulating
393 cell width (Table S1). Using targeted sequencing, we determined that the remaining suppressor
394 strains resistant to YodL also harbored mutations in *mreB* or *mbl*. Since the phenotypes of YodL
395 and YisK expression were similar, we also performed targeted sequencing of the *mreB* and *mbl*
396 chromosomal regions in the YisK-resistant suppressors. All but one of the YisK-resistant
397 suppressors possessed mutations in *mbl*; the remaining suppressor harbored a mutation in *mreB*.
398 To determine if the point mutations we identified were sufficient to confer resistance to
399 YodL or YisK misexpression, we generated the mutant alleles in clean genetic backgrounds (see
400 Supplementary text) and assayed for resistance to three copies (3X) of each misexpression
401 construct (Table 2). In all cases but one, the engineered strains were resistant to the same
402 selective pressure applied in the original selections (either 3X *yodL* or 3X *yisK*)(Table 2),
403 indicating that the *mreB* or *mbl* mutations identified through sequencing were sufficient to confer
404 resistance. When we attempted to engineer a strain harboring only $MreB_{S154R}$, all but one of the
405 strains also possessed a second substitution, $MreB_{R230C}$. Although the remaining strain possessed
406 only the $MreB_{S154R}$ substitution in MreB, unlike the original suppressor identified by whole
407 genome sequencing (Table S1), the $MreB_{S154R}$ harboring strain was also sensitive to YodL
408 expression. Based on these data, we suspect that the strain harboring $MreB_{S154R}$ might be
409 unstable, and possibly predisposed to the accumulation of second-site mutations.
410 The YodL-resistant strains generally possessed mutations resulting in amino acid
411 substitutions with charge changes (Table 2). When mapped to the *T. maritima* MreB structure,
412 5/7 of the unique suppressor strains possessed amino acid substitutions in a region important for
413 mediating the interaction between MreB and the bitopic membrane protein RodZ ($MreB_{G143A}$,

414 $MreB_{N145D}$, $MreB_{P147R}$, $MreB_{S154R}$, and $MreB_{R282S}$)(Table 2 and Fig S5)(18, 63); three of these
415 substitutions occur in residues that make up the RodZ-MreB binding surface ($MreB_{N140}$,
416 $MreB_{P142}$, and $MreB_{R279}$ in *T. maritima*)(18).

417 A majority of the YisK-resistant Mbl variants clustered in regions of Mbl that are
418 predicted to make up the ATP-binding pocket (Table 2 and Fig S6). Moreover, seven of the
419 substitutions occurred in amino acids previously associated with resistance to the MreB inhibitor
420 A22 in *C. crescentus* and *Vibrio cholerae* (Fig S6)(12, 64, 65).

421 $MreB_{R117G}$ and Mbl_{E250K} were independently isolated in both the YodL and YisK
422 suppressor selections, raising the possibility that at least some of the other MreB and Mbl
423 variants might exhibit cross-resistance to YodL and YisK misexpression. To test for cross-
424 resistance, we generated the mutant alleles in clean genetic backgrounds, and then introduced 3X
425 copies of P_{hy}-*yisK* into the YodL-resistant suppressors, and 3X copies P_{hy}-*yodL* into the YisK-
426 resistant suppressors. We then assayed for the ability of the misexpression strains to grow on
427 media in the presence of inducer. The results, summarized in Table 2, show that several of the
428 variants exhibited resistance to both YodL and YisK. Three MreB variants, $MreB_{N145D,}$
429 $MreB_{P147R}$ and $MreB_{R282S}$, exhibited specificity in their resistance to YodL compared to YisK.
430 Three Mbl variants, Mbl_{R63C}, $Mbl_{\Delta S251}$, and Mbl_{P309L}, showed specificity in their resistance to
431 YisK over YodL. These results suggest that the alleles exhibiting cross-resistance to both YisK
432 and YodL are likely to be general, possibly conferring gain-of-function to either MreB or Mbl
433 activity.

434

435 **YodL and YisK's cell-widening activities require MreB and Mbl, respectively**
436

437 The phenotypic consequences of YodL and YisK misexpression are similar but not
438 identical (Fig 1B), suggesting that YodL and YisK might have distinct targets. Consistent with
439 this idea, YodL and YisK coexpression resulted in phenotypes distinct from misexpression of
440 either YodL or YisK alone. More specifically, cells co-expressing YodL and YisK did not grow
441 on plates, regardless of media or $MgCl_2$ concentration (Fig 5A and Fig S2A) and growth without
442 lysis in liquid media required the presence of $MgCl_2$ (Fig S1, Fig S2B, and Fig 5B). Importantly,
443 the co-expressing cells displayed a round morphology that strongly contrasted with strains
444 expressing either YodL or YisK alone (Fig 5B and Fig S2B). The round morphology was
445 unlikely due to higher expression of gene products (1X P_{hy}-*yodL* plus 1X P_{hy}-*yisK*), since cells
446 harboring two copies (2X) of either P_{hy}-*yodL* or P_{hy}-*yisK* did not become round (Fig 1B and Fig
447 S2B).

448 Based on the observation that YodL and YisK coexpression yields distinct phenotypes,
449 and the fact that all of the YodL-specific suppressor mutations occurred in *mreB* ($MreB_{N145D}$,
450 $MreB_{P147R}$ and $MreB_{R282S}$), while all of the YisK-specific suppressor mutations occurred in *mbl*
451 (Mbl_{R63C}, $Mbl_{\Delta S251}$, and Mbl_{P309L}), we hypothesized that YodL targets MreB, whereas YisK
452 targets Mbl. To test these hypotheses, we assessed if MreB and Mbl are specifically required for
453 YodL and YisK function by taking advantage of the fact that *mreB* and *mbl* can be deleted in a
454 Δ*ponA* background with only minor changes in cell shape (20, 31). The Δ*ponA* strain, which
455 does not make PBP1, produces slightly longer and thinner cells than the parent strain, and
456 requires $MgCl_2$ supplementation for normal growth (66, 67). We generated Δ*ponA* Δ*mreB* and
457 Δ*ponA* Δ*mbl* strains and then introduced either two copies of P_{hy}-*yodL* or two copies of P_{hy}-*yisK*
458 into each background. We reasoned that 2X expression would provide a more stringent test for
459 specificity than 1X expression, as off-target effects (if any), would be easier to detect. To assess

460 the requirement of either *mreB* or *mbl* for YodL and YisK activity, cells were grown to
461 exponential phase in LB media supplemented with 10 mM $MgCl_2$, back-diluted to a low optical
462 density, and induced for 90 min before images were captured for microscopy. Uninduced
463 controls all appeared as regular rods, although Δ*ponA* deletion strains were noticeably thinner
464 than wildtype parents (Fig 6). The Δ*ponA* cells became wider following YodL expression,
465 indicating that PBP1 is not required for YodL activity. We also observed that the poles of the
466 Δ*ponA* mutant were less elongated and tapered than the wild-type control following YodL
467 expression, suggesting that this particular effect of YodL expression is PBP1-dependent (Fig
468 6A). A Δ*ponA* Δ*mbl* mutant phenocopied the Δ*ponA* parent following YodL expression (Fig
469 6A), indicating that Mbl is not required for YodL's activity. In contrast, the Δ*ponA* Δ*mreB* strain
470 did not show morphological changes following YodL expression, and instead appeared similar to
471 the uninduced control. We conclude that YodL requires MreB for its cell-widening activity.
472 We performed a similar series of experiments for YisK misexpression. The Δ*ponA*
473 mutant was sensitive to YisK expression, indicating that PBP1 is not required for YisK-
474 dependent cell-widening. Similarly, expression of YisK in a Δ*ponA* Δ*mreB* mutant also resulted
475 in loss of cell width control (Fig 6B), indicating that MreB is not required for YisK activity;
476 however, unlike YisK expression in a wildtype or Δ*ponA* background, the cells became round
477 (Fig 6B), more similar to the YodL and YisK co-expressing cells (Fig 5 and Fig S2B). In
478 contrast, a Δ*ponA* Δ*mbl* mutant did not lose control over cell width following YisK expression
479 (Fig 6B), indicating that YisK activity requires Mbl for its cell-widening activity. We conclude
480 that YodL requires MreB, but not Mbl for its cell-widening activity, whereas YisK requires Mbl,
481 but not MreB.
482

YisK possesses at least one additional target

Although YisK expression in a Δ*ponA* Δ*mbl* mutant did not result in cell-widening, we observed that the induced cells appeared qualitatively shorter than the uninduced control, suggesting that YisK might possess a second activity (Fig 6B). Quantitation of cell lengths in a Δ*ponA* Δ*mbl* mutant following YisK expression revealed that the YisK-induced cells were ~20% shorter than the uninduced cells (Fig 7A). In contrast, YodL expression did not result in a change in cell length in a Δ*ponA* Δ*mreB* mutant (Fig 7B), suggesting the the cell shortening effect is specific to YisK. We hypothesized that MreBH, the third and final *B. subtilis* MreB family member, might be YisK's additional target. We hypothesized that if MreBH is the additional target, then the cell shortening observed upon YisK expression in a Δ*ponA* Δ*mbl* mutant strain should be lost in a Δ*ponA* Δ*mbl* Δ*mreBH* mutant background. However, we found that even when *mreBH* was additionally deleted, YisK expression still resulted in cell shortening (Fig 7C). We conclude that YisK likely has at least one additional target that is not MreB or Mbl dependent, and that this additional target regulates some aspect of cell length.

Discussion

YodL and YisK's functional targets

Misexpression of YodL during vegetative growth results in cell-widening and lysis, and spontaneous suppressor mutations conferring resistance to YodL occur primarily in *mreB*. MreB is also required for YodL's cell-widening activity, whereas Mbl is not. By comparison, expression of YisK during vegetative growth also results in cell-widening and lysis, however, spontaneous suppressor mutations conferring resistance to YisK occur primarily in *mbl*. YisK's cell-widening activity requires Mbl, but not MreB. The simplest interpretation of these results is

that YodL targets MreB function, while YisK targets Mbl function. Alternatively, YodL and YisK could target other factors that affect cell shape and simply require MreB and Mbl for their respective functions.

MreB variants specifically resistant to YodL activity, $MreB_{N145D,}$ $MreB_{P147R}$ and $MreB_{R282S,}$ all result in charge change substitutions in residues previously shown to constitute the RodZ-MreB interaction surface (equivalent *T. maritima* residues are: $MreB_{N140,}$ $MreB_{N142}$ and $MreB_{R279}$)(18). $MreB_{G143A,}$ which exhibits cross-resistance to YisK, also maps near the RodZ-MreB interaction interface. The two remaining YodL-resistant MreB variants occur in ($MreB_{R117G}$) or near ($MreB_{G323E}$) residues previously associated with bypass of RodZ essentiality in *E. coli* (Fig S5)(25). A simple model explaining both the nature of the MreB variants we obtained in the suppressor selections, and YodL's MreB-dependent effect on cell shape, is that YodL acts by disrupting the interaction between RodZ and MreB. In this model, MreB's RodZ-independent activities would remain functional, and several observations are consistent with this idea. If YodL were to completely inactivate MreB function, then we would expect that expressing YodL in a Δ*ponA* Δ*mbl* Δ*mreBH* background would generate round cells, similar to the phenotype observed when MreB is depleted in a Δ*mbl* Δ*mreBH* mutant background (31), or when *mreB, mbl,* and *mreBH* are deleted in backgrounds with upregulated *sigI* expression (the triple mutant is otherwise lethal)(30). However, we observe that cells expressing YodL in a Δ*ponA* Δ*mbl* Δ*mreBH* mutant instead form wide rods (Fig 6A). If YodL does specifically target MreB activity, then these results suggest that MreB likely retains at least some of its width-maintenance function. Morgenstein et al. recently found that the interaction between RodZ and MreB in *E. coli* is required for MreB rotation, but that MreB rotation was not required for rod

shape or cell viability under standard laboratory conditions (26). This study is consistent with prior findings indicating that RodZ is not absolutely required for maintenance of rod shape (25).

We hypothesize that the substitutions obtained in residues near the RodZ-MreB interface either enhance RodZ-MreB interaction, or decrease the ability of YodL to disrupt the RodZ-MreB interface. Although we did not identify YodL-resistant suppressor mutations in *rodZ*, it is possible that the requisite *rodZ* mutations are rare or lethal for the cell, thus we cannot rule out the possibility that YodL could target RodZ function. Similarly, although we found that MreB is required for YodL activity, we can envision a scenario in which a YodL-MreB interaction may be necessary to localize YodL to a cellular location where it can be effective against RodZ or some other cellular component. We think this possibility is less likely, as cells expressing YodL have a distinct phenotype from RodZ depletion in *B. subtilis*. More specifically, YodL expression results in cell widening and tapered poles (Fig 1B), whereas RodZ-depleted cells generate wide rods (22), similar to the phenotype we observed following YodL expression in a Δ*ponA* Δ*mbl* Δ*mreBH* mutant (Fig 6A). These results argue against the idea that YodL could work by inactivating RodZ function completely. Future work aimed at characterizing the nature of the YodL resistant suppressors and the effect of YodL on MreB function will shed light on the mechanism underlying YodL's observed activity.

Only three Mbl variants, Mbl_{R63C}, $Mbl_{\Delta S251}$, and Mbl_{P309L}, showed specificity in resistance to YisK over YodL. Mbl_{R63C}, Mbl_{D153N}, Mbl_{G156D}, Mbl_{T158A}, Mbl_{E204G}, $MreB_{P309L}$ and Mbl_{A314T} occur in residues that form Mbl's predicted ATP-binding pocket (Fig S6), and substitutions in all seven of these residues have been previously implicated in A22 resistance (Fig S6)(12, 64, 65). We speculate that most, if not all of the substitutions in Mbl's ATP-binding pocket are gain-of-function with respect to Mbl polymerization, a hypothesis that can ultimately

be tested in vitro. Similarly, we hypothesize that the Mbl_{M51I} substitution, located at the MreB head-tail polymerization interface (63), may overcome YisK activity by promoting Mbl polymerization. $MreB_{E262}$ of *C. crescentus*, equivalent to *B. subtilis* Mbl_{E250} (Fig S6), is located at the interaction interface of antiparallel MreB protofilament bundles (68). If *B. subtilis* Mbl_{E250} is also present at this interface (this has not been tested to our knowledge), then Mbl_{E250K} could promote resistance to YodL and YisK by enhancing Mbl bundling. How might YisK exert its activity? One idea is that YisK disrupts Mbl bundling, possibly by competing for sites required for protofilament formation. An alternative possibility is that YisK somehow prevents Mbl from effectively binding or hydrolyzing ATP. It is also possible that Mbl is simply required for YisK to target one or more other factors involved in cell-width control.

In addition to Mbl-dependent cell widening, YisK expression resulted in cell shortening, an effect that only became apparent in a Δ*ponA* Δ*mbl* mutant background (Fig 6B and 7A). Given the similarities of MreB, Mbl, and MreBH to each other, we initially hypothesized that YisK-dependent effects on MreB and/or MreBH might be responsible for the decrease in cell length we observed; however, we found that *mreBH* was not required for cell shortening (Fig 6B and Fig 7C). Since YisK expression results in a dramatic loss of cell shape in Δ*mreB* mutant backgrounds (Fig 6A), we were unable to confidently assess cell length changes to determine if there is a requirement for MreB in the cell-shortening phenotype. It is unlikely that YisK's additional activity affects MreB's role in maintaining cell width (at least not without Mbl), as YisK-expressing cells retain rod shape when *mbl* and *mreBH* are both deleted (Fig 6B). An exciting alternative possibility is that YisK activity affects another factor involved in cell length control. One attractive candidate is the cell wall hydrolase CwlO, a known modulator of cell length in *B. subtilis* (69) which recent genetic data also suggests depends at least in part on Mbl

(35). Future experiments aimed at determining the identity and function of YisK's additional target should shed light on how cells regulate both cell length and cell width.

Identification of genes involved in cellular organization through a novel gene discovery pipeline

To systematically identify and characterize novel genes involved in cellular organization, we developed a gene discovery pipeline that combines known regulatory information (48), phenotypes obtained from misexpression screening, and suppressor selection analysis. The ability to identify genetic targets associated with the unknown genes provides a key parameter beyond phenotype from which to formulate testable hypotheses regarding each gene's possible function. The misexpression constructs we generated are inducible and present in single copy on the chromosome. We have found that to obtain phenotypes, our strategy works best when the unknown genes are expressed outside of their native regulatory context. Thus far, we have restricted our gene function discovery pipeline to *B. subtilis*; however, the general approach should be broadly applicable to other organisms and diverse screening strategies.

In this work, we describe the use of the pipeline to identify and characterize two *B. subtilis* genes, *yodL* and *yisK*, that produce proteins capable of targeting activities intrinsic to cell width control. Although *yodL* and *yisK* were not previously recognized as members of the Spo0A regulon, both genes have putative Spo0A boxes and possess promoters that exhibit expression patterns consistent with other Spo0A-regulated genes (Fig 2-4). YisK is also a member of the SigH regulon (50), and our expression analysis is also consistent with expression of *yisK* during stationary phase (Fig 3). If the putative Spo0A box we identified is utilized in vivo, then we would predict based on our expression profiling that *yisK* is transcribed during exponential and early stationary phase via SigH, and then repressed as Spo0A-P accumulates

599 during early sporulation. Such a pattern is similar to the regulation that has been proposed for
600 *kinA* (56, 61). We also observe expression from P_{yodL} and P_{yisK} is reduced in the absence of
601 Spo0A and SigH (Fig 3D-E). The specific contributions of these global regulators to *yodL* and
602 *yisK* regulation cannot be determined by analyzing the expression profiles of the *sigH* and *spo0A*
603 deletion strains alone, since *spo0A* depends on SigH for upregulation during the early stages of
604 sporulation (53, 56). Moreover, since Spo0A inhibits expression of the *sigH* repressor AbrB (70-
605 73), a *spo0A* mutant is also down for *sigH* expression.

606 A Δ*yodL* Δ*yisK* double mutant reproducibly produces ~20% less heat-stable spores than
607 wildtype, suggesting that the YodL and YisK have functions that affect spore development
608 (either directly or indirectly). Most studies on sporulation genes are biased toward factors that
609 reduce sporulation efficiency by an order of magnitude or more in a standard heat-kill assay.
610 However, even small differences in fitness (if reproducible) can contribute significantly to the
611 ability of an organism to persist, especially in competitive environments (74). The 20%
612 reduction in heat-resistant spores we observe in cells lacking YisK and YodL would likely result
613 in a substantial fitness disadvantage to cells in the environment. We do not currently understand
614 how YodL and YisK might function in spore development, but the identification of MreB and
615 Mbl as genetic targets suggests the proteins likely regulate some aspect of PG synthesis. Future
616 studies will be aimed at understanding the molecular and biochemical basis of YodL and YisK
617 activity.

618 In this study, the morphological phenotypes associated with YodL and YisK occurred
619 when the genes were expressed during vegetative growth. Consequently, it is formally possible
620 (although we think unlikely), that the targeting of MreB and Mbl is simply a coincidence that is
621 unrelated to the potential functions of the proteins during stationary phase or sporulation.

Regardless of what YodL and YisK's physiological roles turn out to be, we have already been able to utilize misexpression of the proteins to obtain interesting variants of both MreB and Mbl that can now be used to generate testable predictions regarding how MreB and Mbl function in *B. subtilis*. Moreover, the apparent specificity with which YodL and YisK appear to target MreB and Mbl, respectively, make them potentially powerful tools to differentially target the activities of these two highly similar paralogs in vivo. Of course, more studies will be required to determine if YodL and YisK interact directly or indirectly to modulate MreB and Mbl activity. In the meantime, it is exciting to speculate that many undiscovered modulators of MreB and MreB-like proteins exist, and that we have only just begun to scratch the surface regarding regulation of this important class of proteins. The identification and characterization of such modulators could go a long way toward addressing the significant gaps in our knowledge that exist regarding the regulation of PG synthesis in bacteria.

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Table 1

Strain	Strain #	Total cfu	Heat-resistant cfu	Sporulation efficiency	Relative sporulation efficiency
wildtype	*B. subtilis* 168	2.8×10^8 ($\pm4.7\times10^7$)	1.9×10^8 ($\pm4.5\times10^7$)	66.9% (±5)	100%
Δ*yodL*	BYD276	2.6×10^8 ($\pm3.9\times10^7$)	1.7×10^8 ($\pm2.8\times10^7$)	65.2% (±7)	97%
Δ*yisK*	BYD278	2.7×10^8 ($\pm4.6\times10^7$)	2.4×10^8 ($\pm2.7\times10^7$)	63.1% (±6)	94%
Δ*yodL* Δ*yisK*	BYD279	3.1×10^8 ($\pm6.5\times10^7$)	1.7×10^8 ($\pm4.1\times10^7$)	54.1% (±4)	81%
Δ*yodL* Δ*yisK* P_{yisK}-*yisK*	BYD510	3.4×10^8 ($\pm3.3\times10^7$)	2.3×10^8 ($\pm4.1\times10^7$)	66.2% (±7)	99%

Table 1. Sporulation efficiency of *yodL* and *yisK* mutants. Sporulation efficiency is the number of spores/ml divided by the total cfu/ml × 100%. Relative sporulation efficiency is sporulation efficiency normalized to wildtype × 100%. The data shown is the average of six independent biological replicates. The difference in sporulation efficiency between wildtype and the Δ*yodL* Δ*yisK* double mutant is statistically significant (P<0.0006).

692
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698
699
700 Table 2

Variants obtained following **YodL** misexpression			
mreB	**MreB Variants**	**+YodL**	**+YisK**
CGC→GGC	R117G[1]	R	R
GGA→GCA	G143A	R	R
AAT→GAT*	N145D[2]	R	S
CCA→CGA*	P147R[1,2]	R	S
AGC→AGA	S154R[1,2]	--	--
AGC→AGA	S154R[1]	R	R
CGC→TGC	R230C		
AGA→AGT*	R282S[1,2]	R	S
GGG→GAG	G323E[1]	R	R
mbl	**Mbl Variants**	**+YodL**	**+YisK**
ACG→ATG	T158M	R	R
GAA→AAA*	E250K[1]	R	R
ACA→ATA	T317I	R	R
Variants obtained following **YisK** misexpression			
mreB	**MreB Variants**	**+YisK**	**+YodL**
CGC→TGC	R117G	R	R
mbl	**Mbl Variants**	**+YisK**	**+YodL**
ATG→ATA	M51I[3]	R	R
CGC→TGC	R63C[3]	R	S
GAC→AAC*	D153N[3]	R	R
GGC→GAC	G156D[3]	R	R
ACG→GCG*	T158A[3]	R	R
GAG→GGG	E204G[3]	R	R
GAA→AAA	E250K	R	R
TCT→ΔΔΔ	ΔS251	R	S
CCT→CTT	P309L[3]	R	S
GCC→ACC	A314T[3]	R	R

701
702 **Table 2. Analysis of suppressor strains resistant to YodL and/or YisK.**

703 The suppressor selections are described in detail in materials and methods. Candidate mutations
704 were introduced into clean genetic backgrounds harboring three copies of P_{hy}-*yodL* or three
705 copies of P_{hy}-*yisK*, and the resultant strains were assessed for resistance (R) or sensitivity (S) to
706 either *yodL* or *yisK* expression as judged by ability to grow on LB plates supplemented with 1

mM IPTG and 100 µg/ml spectinomycin. [1]Originally identified using whole-genome sequencing (Table S1). [2]Residues previously implicated in the RodZ-MreB interaction (18). [3]Residues previously implicated in resistance to A22 (64, 65, 75). The (*) indicates that two suppressors possessing the same nucleotide change were obtained in original selection. The underlined residues displayed specificity in resistance to YodL over YisK (top) or YisK over YodL (bottom).

Figure Legends

Figure 1. Misexpression of YodL and YisK prevents cell growth on solid media and causes loss of cell shape in liquid media.

(A) Cells harboring one (1X) or two (2X) copies of P_{hy}-*yodL* (BAS040 and BAS191) or P_{hy}-*yisK* (BAS041 and BYD074)(B) were streaked on an LB plate supplemented with 100 µg/ml spectinomycin and, when indicated, 1 mM IPTG or 1 mM IPTG and the denoted concentration of $MgCl_2$. Plates were incubated for ~16 hrs at 37°C before image capture (top). (B) The strains described above were grown in LB-Lennox media at 37°C to mid-exponential and back-diluted to an OD_{600} of ~0.02. When indicated, 1 mM IPTG or 1 mM IPTG and the denoted concentration of $MgCl_2$ was added. Cells were grown for 1.5 hrs at 37°C before image capture. Membranes were stained with TMA-DPH. All images were scaled identically.

Figure 2. DNA sequence upstream of *yodL* and *yisK*.

(A) Putative Spo0A box (underlined) upstream of the *yodL* start codon. (B) SigH binding motifs (double underline) and putative Spo0A box (underlined) upstream of *yisK* start codon.

730

731 **Figure 3. Expression from *yodL* and *yisK* promoters during a CH timecourse.**

732 Expression from the putative *yodL* and *yisK* promoter regions was monitored in CH medium at

733 37°C over a timecourse. The OD_{600} (A and B) and production of either GFP (C) or beta-

734 galactosidase (D and E) was monitored at 30 min intervals. Membranes were stained with TMA-

735 DPH. All GFP channel images were captured with 1 sec exposures and scaled identically to

736 allow for direct comparison. In this media, time 0 represents the last exponential timepoint, not

737 the initiation of sporulation.

738

739 **Figure 4. Expression from *yodL* and *yisK* promoters following sporulation by**

740 **resuspension.** Expression from the putative *yodL* and *yisK* promoter regions was monitored in

741 resuspension medium. The production of either GFP (A) or beta-galactosidase (B and C) was

742 monitored at 20 min intervals. Membranes were stained with TMA-DPH. All GFP channel

743 images were captured with 1 sec exposures and scaled identically to allow for direct

744 comparison.

745

746 **Figure 5. YodL and YisK co-misexpression causes cell lysis.**

747 (A) BYD361 (P_{hy}-*yodL*, P_{hy}-*yisK*) and BYD281 (2X P_{hy}-*yodL*, 2X P_{hy}-*yisK*) were streaked on an

748 LB plate with 100 µg/ml spectinomycin and, when indicated 1 mM IPTG or 1 mM IPTG and

749 the denoted concentration of $MgCl_2$. (B) Cells were grown in LB-Lennox media at 37°C to mid-

750 exponential and back-diluted to an OD_{600} of ~0.02. When indicated 1 mM IPTG or 1 mM IPTG

751 and the denoted concentration of $MgCl_2$ were added. Cells were then grown for 1.5 hrs at 37°C

752 before image capture. Membranes are stained with TMA-DPH. All images are shown at the
753 same magnification.
754
755 **Figure 6. YodL and YisK's cell-widening activities require MreB and Mbl, respectively.**
756 (A) Cells harboring 2X copies of P_{hy}-*yodL* in a wildtype (BAS191), Δ*ponA* (BYD176), Δ*ponA*
757 Δ*mreB* (BYD263), Δ*ponA* Δ*mbl* (BYD259) or Δ*ponA* Δ*mbl* Δ*mreBH* (BAS249) background
758 were grown at 37°C in LB supplemented with 10 mM $MgCl_2$ to mid-exponential. To induce
759 *yodL* expression, cells were back-diluted to an OD_{600} of ~0.02 in LB with 10 mM $MgCl_2$, and
760 IPTG (1 mM) was added. Cells were grown for 1.5 hrs at 37°C before image capture.
761 Membranes are stained with TMA-DPH. All images are shown at the same magnification. (B)
762 Cells harboring 2X copies of P_{hy}-*yisK* in a wildtype (BYD074), Δ*ponA* (BYD175), Δ*ponA*
763 Δ*mreB* (BYD262), Δ*ponA* Δ*mbl* (BYD258) or Δ*ponA* Δ*mbl* Δ*mreBH* (BAS248) background
764 were grown at 37°C in LB supplemented with 10 mM $MgCl_2$ to mid-exponential. To induce
765 *yisK* expression, cells were back-diluted to an OD_{600} of ~0.02 in LB with 10 mM $MgCl_2$, and
766 IPTG (1 mM) was added. Cells were grown for 1.5 hrs at 37°C before image capture.
767 Membranes are stained with TMA-DPH. All images are shown at the same magnification.
768
769 **Figure 7. YisK expression results in cell shortening.**
770 (A) Cells harboring 2X copies of P_{hy}-*yisK* in a Δ*ponA* Δ*mbl* background (BYD262) were grown
771 at 37°C in LB supplemented with 10 mM $MgCl_2$ to mid-exponential. To induce *yisK* expression,
772 cells were back-diluted to an OD_{600} of ~0.02 in LB with 10 mM $MgCl_2$ and IPTG (1 mM) was
773 added. Cells were grown for 1.5 hrs at 37°C before image capture. Membranes are stained with
774 TMA-DPH. Cell lengths (n=500/condition) were measured before and after *yisK* expression and

rank-ordered from smallest to largest along the x-axis so the entire population could be visualized without binning. The uninduced population (black) is juxtaposed behind the induced population (semi-transparent, gray). The difference in average cell length before and after P_{hy}-*yisK* induction were statistically significant (P<0.0001). (B) Cells harboring 2X copies of P_{hy}-*yodL* in a Δ*ponA* Δ*mreB* background (BYD263) were grown, quantitated, and plotted as described above. The difference in average cell length before and after P_{hy}-*yodL* induction were not statistically significant. (C) Cells harboring 2X copies of P_{hy}-*yisK* in a Δ*ponA* Δ*mbl* Δ*mreBH* background (BAS248) were grown, quantitated, and plotted as described above. The difference in average cell length before and after P_{hy}-*yisK* induction were statistically significant (P<0.0001).

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