

YycH Regulates the Activity of the Essential YycFG Two-Component System in *Bacillus subtilis*

Hendrik Szurmant, Kristine Nelson, Eun-Ja Kim, Marta Perego, and James A. Hoch*

The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, California 92037

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Of the numerous two-component signal transduction systems found in bacteria, only a very few have proven to be essential for cell viability. Among these is the YycF (response regulator)-YycG (histidine kinase) system, which is highly conserved in and specific to the low-G+C content gram-positive bacteria. Given the pathogenic nature of several members of this class of bacteria, the YycF-YycG system has been suggested as a prime antimicrobial target. In an attempt to identify genes involved in regulation of this two-component system, a transposon mutagenesis study was designed to identify suppressors of a temperature-sensitive YycF mutant in *Bacillus subtilis*. Suppressors could be identified, and the prime target was the *yycH* gene located adjacent to *yycG* and within the same operon. A *lacZ* reporter assay revealed that YycF-regulated gene expression was elevated in a *yycH* strain, whereas disruption of any of the three downstream genes within the operon, *yycI*, *yycJ*, and *yycK*, showed no such effect. The concentrations of both YycG and YycF, assayed immunologically, remained unchanged between the wild-type and the *yycH* strain as determined by immunoassay. Alkaline phosphatase fusion studies showed that YycH is located external to the cell membrane, suggesting that it acts in the regulation of the sensor domain of the YycG sensor histidine kinase. The *yycH* strain showed a characteristic cell wall defect consistent with the previously suggested notion that the YycF-YycG system is involved in regulating cell wall homeostasis and indicating that either up- or down-regulation of YycF activity affects this homeostatic mechanism.

A living organism that is able to sense environmental factors and respond accordingly has a great evolutionary advantage, and all organisms have evolved signal transduction mechanisms that allow for such processes. Most of these signal transduction processes in bacteria and archaea are carried out by the so-called two-component systems (TCS) (11). Although less common in eukaryotes, they are found as phosphorelays in organisms ranging from yeast to higher plants but not in animals (27). In bacteria, TCS regulate various processes such as motility, sporulation, cell division, and virulence, to name a few (10, 14, 23–25). The general TCS is comprised of a sensor histidine kinase—with a carboxy-terminal histidine kinase activity and an N-terminal sensing domain—and a response regulator protein which is activated after accepting a phosphoryl group from the histidine kinase to which it is paired. The most common output for the response regulator is the up- or down-regulation of the expression of specific target genes.

Of the numerous TCS, very few have proven essential for the survival of bacteria, at least under laboratory conditions (24). Among the essential ones is the highly conserved YycF (response regulator)-YycG (histidine kinase) TCS of low-G+C content gram-positive bacteria (5). Members of this group include important pathogens such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis* (1, 8, 14). Not surprisingly, this system has gained significant interest since its first discovery. It has been suggested to serve as a prime target for the development of novel antibiotics (28).

Additionally, phenotypic data for strains with lowered *yycF* expression levels show defects in cell division and cell wall homeostasis, i.e., several cell developmental processes (5, 12, 14). Therefore, this TCS appears to affect several genetic networks of gram-positive bacteria. A noteworthy exception to the rule are orthologous systems in *Lactococcus lactis* and *Streptococcus mutans* that have proven not to be essential (13, 19).

Most studies to date have focused on identifying the genes controlled by this TCS. A consensus sequence for YycF-dependent gene expression was identified in *B. subtilis* and revealed that most YycF-controlled genes are involved in cell wall homeostasis (12). Among those are the essential *tagAB* and *tagDEF* operons, which code for components of teichoic acid biosynthesis. Additionally, the *yocH* gene, which codes for a putative autolysin, and the *ykvT* gene, which codes for a putative cell wall hydrolase, were shown to be under direct YycF control (12). Others include the essential *ftsAZ* operon which is involved in cell division (7). However, this operon has multiple promoters, and the YycF-dependent promoter is not essential. In *S. pneumoniae*, expression of a single gene, *pcsB*, was identified as responsible for the essentiality of the *yycFG* system in that organism (16, 17). Placing this gene under a constitutive promoter overcame the essential nature of the YycFG system.

Despite the high conservation of the YycFG TCS in low-G+C content gram-positive bacteria, two operon subcategories have been observed (18) (Fig. 1). In the apparently more common one (class I), YycG has two transmembrane helices and an extracellular domain. In the less common one (class II), YycG is not essential and has only one transmembrane helix and no extracellular domain as seen in *S. pneumoniae*. In the class I system, four downstream genes, *yycHIJK*, are located

* Corresponding author. Mailing address: Division of Cellular Biology, Mail code MEM-116, Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. Phone: (858) 784-7905. Fax: (858) 784-7966. E-mail: hoch@scripps.edu.

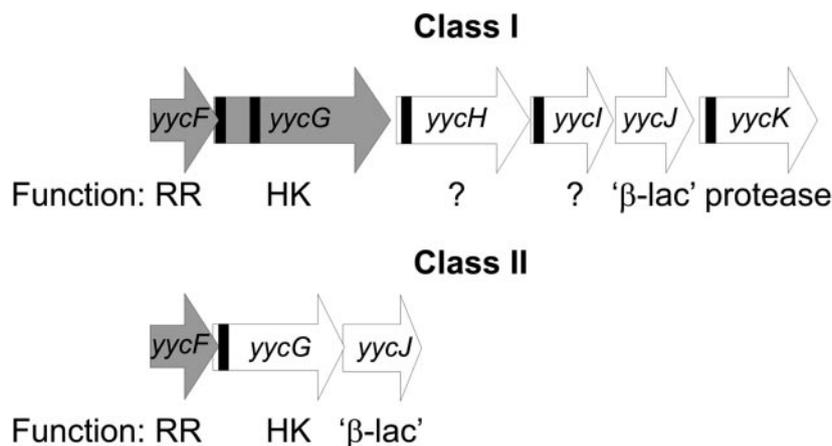


FIG. 1. The essential *yycFG* two-component system is organized in either of two different operons. Essential genes are in gray. Sequences encoding for putative transmembrane regions are in black. RR, response regulator; HK, histidine kinase; ?, unknown; 'β-lac', homologous to enzyme family which includes β-lactamases; protease, serine protease.

within the same operon. In the class II system, only *yycJ* is organized within the same operon, whereas no homologs to *YycH* and *YycI* can be found encoded anywhere else on the genome of the organisms. An exception to the rule is *L. lactis*, whose *yycFG* system appears to belong to class II; however, its *YycG* protein has two transmembrane domains (18).

Here we demonstrate that *Bacillus subtilis* *YycH* but not any of the downstream genes in the *yyc* operon has a *YycF*-inhibitory function. We also show that *YycH* is secreted and is therefore likely to act on the cytoplasmic *YycF* protein indirectly through inhibition of *YycG*. Lack of this inhibitory function by deleting *yycH* causes a cell wall and growth defect.

MATERIALS AND METHODS

Strain construction and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in Luria Bertani (LB) medium in the presence of appropriate antibiotics (for *Escherichia coli*, kanamycin at 30 μg/ml, ampicillin at 100 μg/ml, and spectinomycin at 100 μg/ml; for *B. subtilis*, chloramphenicol at 5 μg/ml, erythromycin at 5 μg/ml, kanamycin at 3 μg/ml, and spectinomycin at 100 μg/ml).

To place the temperature-sensitive *yycF*(*H215P*) mutation into a JH642 genetic background, JH642 was transformed with JH17041 (5) chromosomal DNA selecting for *trp*⁺ and screening for temperature sensitivity, generating strain JH17038.

To construct a *lacZ* reporter strain, the genomic region coding for the putative *yocH* promoter (*B. subtilis*, 168 coordinates from 2092948 to 2093171) was amplified by PCR and cloned into *EcoRI* and *BamHI* sites of the vector pJM115, creating pYOCHP. This plasmid was transformed into JH642, selecting for *Kan*^r and screening for amylase deficiency, thereby creating strain JH25001. JH25001 was transformed with plasmids pJC11, pJC12, pJC13, and pJC14 to create strains JH25002 (*yycH*), JH25003 (*yycI*), JH25004 (*yycJ*), and JH25005 (*yycK*), respectively.

To create a nonpolar *yycH* strain, the *HindIII* *yycH* fragment of pJC11 was subcloned into pJM117 to create pJS03. Correct orientation was verified by restriction analysis and DNA sequencing. To remove the *lacZ* coding region from this vector, pJS03 was digested with *Bsu36I* and then partially digested with *BlnI*. A 6,800-bp fragment was isolated and religated, resulting in pJS04. This vector was transformed into JH25001, selecting for *Cm*^r. Colonies were screened by PCR for correct insertion into the chromosome, creating strain JH25011. This strain carries the *yycH* mutation, while all the downstream genes are placed under the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *P*_{Spac} promoter.

To make a *yycH*'-'*phoA* reporter strain, the chromosomal region including ~120 bp upstream and the first ~360 bp of the *yycH* coding sequence were

amplified by PCR introducing a 5' *BamHI* site and a 3' *EcoRV* site (*B. subtilis*, 168 coordinates from 4150528 to 4151102). The fragment was cloned into the respective sites of pJV211, creating pJS01. This plasmid was digested with *HindIII*, and the fragment that codes for *yycH*'-'*phoA* was cloned into the *HindIII* site of the vector pMA5, creating pJS02. Correct orientation was verified by restriction analysis. This vector was digested with *SstI* and religated. This process removes a fragment containing the *E. coli* origin and places the cloned gene under the control of the strong *HptII* promoter. The resulting vector was transformed into MH3402 to create JH25014.

To create a *yocH* insertion strain, an internal *yocH* fragment (*B. subtilis*, 168 coordinates from 2092572 to 2092889) was amplified by PCR and cloned into *EcoRI* and *BamHI* sites of pJM134, creating pJS05. This plasmid was transformed into JH25001, selecting for *Sp*^c and creating strain JH25012. JH25002 genomic DNA was transformed into JH25012, selecting for *Cm*^r and creating JH25013.

Transposon mutagenesis. Strain JH17038, harboring the temperature-sensitive *yycF*(*H215P*) mutation, was transformed with the pIC133 transposon delivery vector (22) selecting for erythromycin resistance at a growth temperature of 30°C. One transformant was grown at 30°C for 16 h and then subcultured and grown at 37°C for 16 h in LB medium in the presence of spectinomycin to select for transposition of the mini-*Tn10* transposon. The culture was plated on LB medium in the presence of spectinomycin and selected for the ability to grow at the restrictive temperature (47°C). Transformants were backcrossed into the parent strain JH17038 to assure linkage of spectinomycin resistance and suppression of temperature sensitivity. The location of the transposon insertion was confirmed by DNA sequencing. The mini-*Tn10* plasmid and adjacent chromosomal region were recovered from each suppressor strain by digesting the chromosomal DNA with *EcoRI*, which was self-ligated prior to transformation into *E. coli* DH5α. Plasmid was recovered from the colonies obtained and analyzed by restriction mapping and DNA sequencing.

β-Galactosidase assay. Cells were grown in LB medium at 37°C in the presence of the appropriate antibiotics. Samples were taken at indicated times. β-Galactosidase activity was determined as previously described, and the activity is reported in Miller units (6, 15).

Analysis of cell wall defects. Cell wall defects were detected by growing the indicated *B. subtilis* strains at 37°C until early stationary phase in LB medium. Cells were collected by centrifugation and washed twice in protoplast buffer (25 mM potassium phosphate, pH 7, 10 mM magnesium chloride, 0.1 mM EDTA, 20% sucrose, and 30 mM sodium lactate) supplemented with 250 μg/ml chloramphenicol. Strains were resuspended to an *A*_{525 nm} of 1.0 in this buffer and supplemented with 0 or 4 mg/ml lysozyme. Cultures were incubated at 37°C for 30 min. The resulting protoplasts (or cells) were collected by centrifugation at 13,000 × *g* for 20 min, resuspended in 100 μl of 1× sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min, and 10 μl were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were analyzed visually following Coomassie brilliant blue staining.

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description or genotype	Source or reference
Plasmids		
pIC133	mini- <i>Tn10</i> -OS delivery vector, <i>Erm</i> ^r , <i>Spc</i> ^r	22
pJM103	<i>Amp</i> ^r , <i>Cm</i> ^r	20
pJM115	<i>amyE</i> integration vector for <i>lacZ</i> fusions, <i>Amp</i> ^r <i>Kan</i> ^r	2
pJM117	pMUTIN 4 derivative, <i>Amp</i> ^r <i>Cm</i> ^r	M. Perego, unpublished
pJM134	<i>Spc</i> ^r gene in pBluescript (Stratagene) <i>Amp</i> ^r <i>Spc</i> ^r	M. Perego, unpublished
pMA5	<i>B. subtilis</i> / <i>E. coli</i> shuttle vector	3
pJV211	pJM103-' <i>phoA</i>	3
pJV217	pMA5- <i>kapB</i> _{C26P} '-' <i>phoA</i>	3
pJC11	pJM103-' <i>yycH</i> '	5
pJC12	pJM103-' <i>yycI</i> '	5
pJC13	pJM103-' <i>yycJ</i> '	5
pJC14	pJM103-' <i>yycK</i> '	5
pYOCHP	pJM115- <i>P</i> _{<i>yocH</i>} - <i>lacZ</i>	This work
pJS01	pJM103- <i>yycH</i> '-' <i>phoA</i>	This work
pJS02	pMA5- <i>yycH</i> '-' <i>phoA</i>	This work
pJS03	pJM117-' <i>yycH</i> '	This work
pJS04	pJM117-' <i>yycH</i> ' Δ <i>lacZ</i>	This work
pJS05	pJM134-' <i>yocH</i> '	This work
Strains		
<i>E. coli</i>		
DH5 α	Cloning host	Lab stock
TG1	Cloning host	Lab stock
<i>B. subtilis</i>		
MH3402	<i>pheA1 trpC2 phoA::cat phoB::mTn10</i>	F. M. Hulett
JH642	<i>pheA1 trpC2</i>	Lab stock
JH17038	<i>pheA1 yycF</i> _{H215P}	This work
JH17041	<i>leu-8 met-5 yycF</i> _{H215P}	5
JH25001	<i>amyE::(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25002	<i>yycH::pJC11 amyE(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25003	<i>yycI::pJC12 amyE(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25004	<i>yycJ::pJC13 amyE(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25005	<i>yycK::pJC14 amyE(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25011	<i>yycH::pJS03 amyE(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25012	<i>yocH::pJM134 amyE(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25013	<i>yocH::pJM134 yycH::pJC11 amyE(P</i> _{<i>yocH</i>} - <i>lacZ aph3-A)</i>	This work
JH25014	<i>phoA::cat phoB::mTn10 pJS04</i>	This work

Antisera to YycG and YycF. The gene coding for the full-length YycF protein was cloned in pET28 generating a six-His tag fusion. The protein was expressed in *E. coli* BL21(DE3) pLysS and purified to homogeneity.

The gene coding the cytoplasmic portion of YycG was cloned in pET16 generating a 10-His tag fusion. The protein was expressed in *E. coli* BL21(DE3) pLysS and purified to homogeneity.

Antisera were generated in New Zealand White rabbits using standard protocols of this institution.

Immunoblot analysis. Strains were grown in LB medium, and samples were taken at indicated time points. Cells were collected and lysozyme treated as described above. Cell extracts were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and visualized immunologically, using anti-YycG or anti-YycF antibodies (1:1,000), horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:7,500), and the ECL Plus system from Amersham. A Storm 840 from Molecular Dynamics was utilized for fluorescent detection of the ECL substrate, and bands were analyzed using ImageQuant software.

Alkaline phosphatase assay. Strains were scored for a Pho⁺ phenotype by streaking them on LB plates supplemented with 50 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate and the appropriate antibiotics. Phenotypes were analyzed visually after a 14-h incubation at 37°C.

RESULTS

YycH affects the YycFG two-component system. In an attempt to identify genes that are involved in down-regulation of the essential YycFG TCS, a transposon mutagenesis study was

performed on the previously described temperature sensitive YycF(H215P) strain (5), selecting for the rescue of the temperature-sensitive phenotype. Thirty-two transformants with the ability to grow at the nonpermissive temperature of 47°C were isolated. Backcross transformation into the temperature-sensitive strain JH17038 to confirm linkage between temperature resistance and the transposon antibiotic resistance marker was successful for only 4 of the 32 isolates, based on a very low apparent competence of the temperature-sensitive strain. Sequence analysis of the region adjacent to the transposon insertion revealed that these four had the transposon located within the *yycH* gene, which is found immediately downstream of the histidine kinase gene *yycG* (data not shown). Two of the four insertions were in distinctly different areas of the gene, corresponding to base pair position -1 and position 932, while the others were siblings of the two. PCR performed on the remaining 28 original transformants using oligonucleotides annealing to the upstream and downstream regions of *yycH* revealed an additional four mutants with an insertion in *yycH* (not shown). However, these were not further analyzed and could have been siblings of the others. These results suggested that disruption of *yycH* or down-regulation of one of the three genes located downstream within the same operon was responsible for the

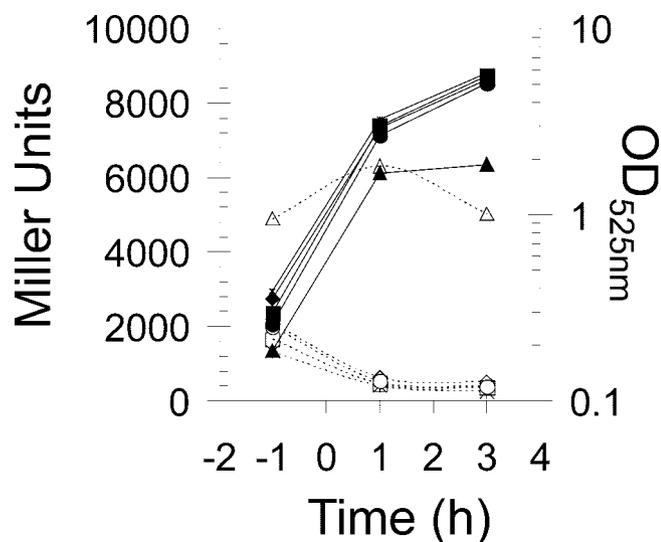


FIG. 2. YycF-dependent expression in *yycH*, *yycI*, *yycJ*, and *yycK* mutant strains. Shown are growth in optical density (OD) units (solid lines and solid symbols) and β -galactosidase activity in Miller units (broken lines and open symbols) of strains expressing *lacZ* from the *amyE* locus under control of the YycF-dependent *yocH* promoter. Strains are wild type (squares), *yycH* (triangles), *yycI* (circles), *yycJ* (diamonds), and *yycK* (stars).

rescue of the temperature-sensitive phenotype. Transcriptional up-regulation of the *yycF* gene or increased YycF activity could conceivably elevate the residual activity of YycF(H215P) at the nonpermissive temperature.

YycH, but not YycI, YycJ, or YycK, down-regulates YycF-dependent gene expression. In order to determine if the rescue of the temperature-sensitive phenotype of the *yycF*(H215P) mutant was due to up-regulation of YycF activity, a YycF-dependent reporter strain was developed. The *yocH* gene, coding for a putative autolysin, was previously identified to be induced by the YycFG TCS (12). The promoter of *yocH* was cloned in front of the *lacZ* reporter gene and inserted in the *amyE* locus in *B. subtilis* as a single copy using the pJM115 vector (2), giving rise to strain JH25001.

Since a transposon in *yycH* would inactivate it and also disrupt the transcription of all genes downstream of it in the operon, the *yycH*, *yycI*, *yycJ*, and *yycK* genes were individually disrupted in strain JH25001, resulting in strains JH25005, JH25003, JH25004, and JH25005, respectively. β -Galactosidase activities in the different strains were determined (Fig. 2), and we observed at least 10-fold higher activity in the *yycH* strain than in the wild type. All other strains exhibited β -galactosidase activities comparable to that of the wild type. To ensure that *yycH* alone and not a polarity effect on the downstream genes was responsible for the observed phenotype, a *yycH* mutant was constructed (JH25011), which placed the downstream genes under the control of the IPTG inducible P_{Spac} promoter by using the pMUTIN derivative pJM117 (26) (M. Perego, unpublished). As for the previous *yycH* strain, β -galactosidase activity was induced about 10-fold over wild type, both in the absence or presence of IPTG (Fig. 3). This indicated that *yycH* inactivation alone is responsible for the elevated expression of the YycF-dependent gene *yocH*.

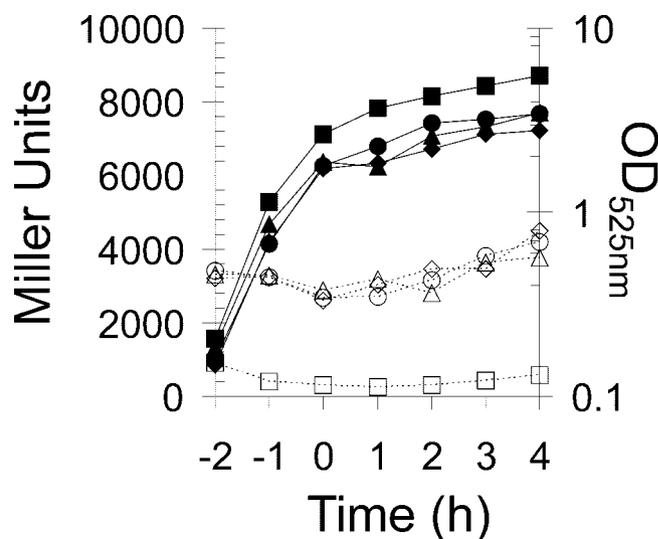


FIG. 3. YycF-dependent expression in JH25002 (*yycH::pJM103*) versus JH25011 (*yycH::pJM117*) strains. In strain JH25011, the genes downstream of *yycH* within the same operon are placed under control of the IPTG inducible P_{Spac} promoter. Shown are growth in optical density (OD) units (solid lines and solid symbols) and β -galactosidase activity in Miller units (broken lines and open symbols) of strains expressing *lacZ* from the *amyE* locus under control of the YycF-dependent *yocH* promoter. Strains are wild type (squares), JH25002 (triangles), and JH25011 in the presence of 1 mM IPTG (circles) or in its absence (diamonds).

***yycH* strains have a characteristic stationary phase growth defect.** The two different *yycH* strains JH25002 and JH25011, the latter in the absence or presence of IPTG, have growth rates indistinguishable from the wild-type strain during the exponential growth phase. However, growth of the *yycH* strains slows significantly upon reaching stationary phase (Fig. 2 and 3), and the final cell density is about 50% of the wild-type strains. Disruption of any of the three downstream genes, *yycI*, *yycJ*, or *yycK*, does not affect *B. subtilis* growth or final cell density.

The *yycH* strain has a YocH-independent cell wall defect. Since some YycF-dependent genes are involved in cell wall homeostasis (12), the *yycH* strain was examined for a potential cell wall defect in a simple experiment. Wild-type and *yycH* mutant strains were subjected to lysozyme treatment in an appropriate buffer. Strains were resuspended and incubated in the presence or absence of lysozyme; the cells/protoplasts were then collected by centrifugation, boiled in SDS sample buffer, and subjected to SDS-PAGE. As expected, cellular proteins of the wild-type strain were detected only when the cells were previously treated with lysozyme. The *yycH* strain, however, lysed whether treated or not with lysozyme, possibly indicating a weakened cell wall or unrestrained autolytic activity in the mutant strain (Fig. 4).

YocH is a putative autolysin, and its expression is greatly induced by YycF in a *yycH* mutant. It is possible that *yocH* overexpression might be responsible for the observed cell wall defect in the *yycH* strain. To test this notion, *yocH* was disrupted by single crossover integration of an internal fragment cloned in the spectinomycin-carrying plasmid pJM134 (M. Perego, unpublished) in both wild-type and *yycH* genetic back-

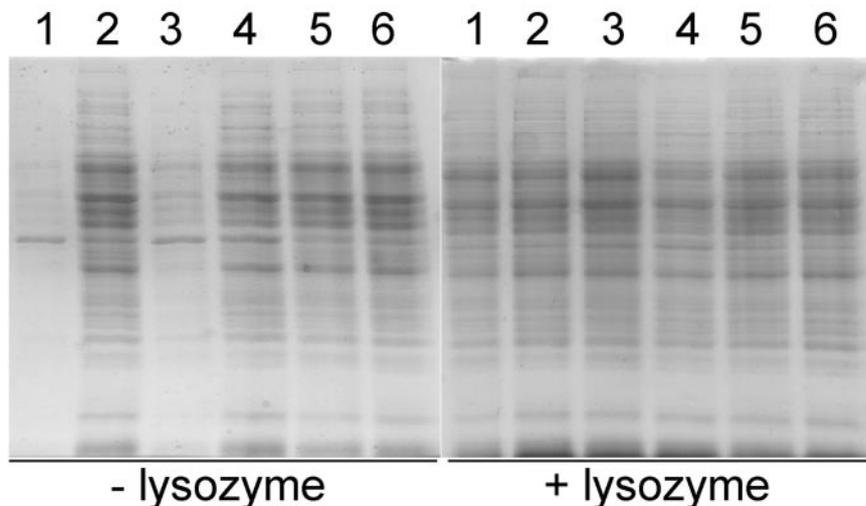


FIG. 4. Cell wall defect in *yycH* strains is independent of *yocH* expression. Whole-cell protein extract after treatment with or without lysozyme, separated by SDS-PAGE and visualized by Coomassie staining. Extract was from wild type (lane 1), JH25002 (*yycH*) (lane 2), JH25012 (*yocH*) (lane 3), JH25013 (*yycH yocH*) (lane 4), and JH25011 (*yycH:pJMI17*) in the presence (lane 5) or absence (lane 6) of 1 mM IPTG.

grounds, yielding strains JH25012 and JH25013, respectively. If YocH was responsible for the apparent cell wall defect in the *yycH* strain, then the disruption of *yocH* should revert the observed phenotype. However, the strain with the *yocH* mutation in the *yycH* mutant background still lysed in the absence of lysozyme. Additionally, the *yocH* strains grew identically to their genetic parental strains (data not shown). Therefore, elevated expression of YocH is neither responsible for the observed cell wall defect in the *yycH* strain nor for its growth defect.

***yycF* and *yycG* protein levels remain unchanged in a *yycH* strain.** One possible explanation for the increased YycF-dependent gene expression in the *yycH* strain is that the levels of YycF and/or YycG are elevated over wild-type levels. To examine this possibility, wild-type and *yycH* liquid cultures were grown, and cell aliquots were taken at indicated time points,

where time zero corresponds to the onset of stationary phase. Cell density was normalized, the cells were lysed, and protein extracts were separated by SDS-PAGE. YycF and YycG were detected immunologically with anti-YycF-antibody and anti-YycG-antibody, respectively (Fig. 5). Both YycF and YycG concentrations appeared to be constant throughout the strains' growth, indicating that they are constitutively expressed. Additionally, YycF and YycG levels remained unchanged in the *yycH* strain. Therefore, YycH does not likely affect the expression of *yycFG*, nor does it affect the stability of the corresponding gene products.

YycH is exported. To further explore the possible function of YycH, this protein was analyzed with various bioinformatic tools (Pfam, SignalP, BLAST, and TMHMM) which predicted it to be an exported protein. A putative transmembrane domain is predicted to range from amino acids 9 to 28. Therefore,

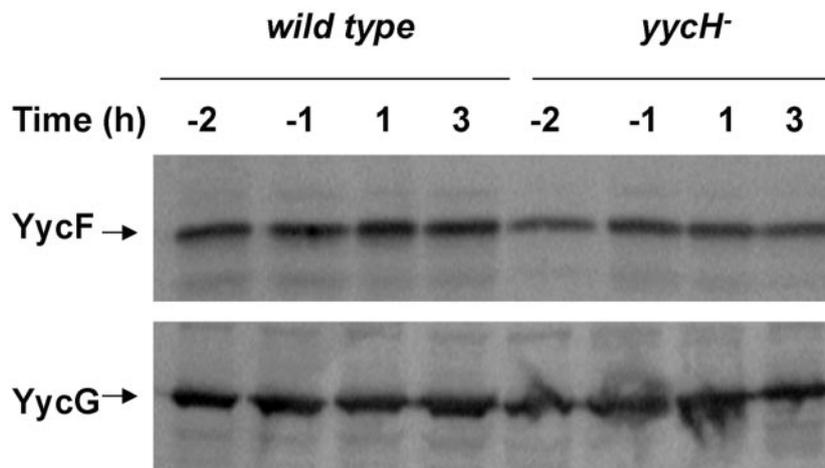


FIG. 5. Time-dependent expression of YycF and YycG in wild-type and *yycH* strains. Shown are immunoblots visualizing expression levels of YycF and YycG in wild-type and *yycH* liquid cultures at indicated times before or after the onset of stationary phase.



*kapB*_{C32P}'-*phoA* *yycH*'-*phoA*

FIG. 6. YycH gets exported to the extracellular space. MH3402 (*phoA phoB*) harboring pMA5-*yycH*'-*phoA* or pMA5'-*phoA* were streaked over a plate containing 50 μ g/ml of the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate. Blueish-green indicates a Pho⁺ phenotype.

either the 8 amino acids N-terminal to this transmembrane domain or the 426 amino acids C-terminal to this transmembrane domain would be localized outside of the cell. To determine whether YycH is indeed exported, a translational alkaline phosphatase fusion was constructed. The first 360 bp of *yycH* were fused in frame to a truncated *phoA* gene which is missing its N-terminal signal peptide. The construct was expressed from the multicopy plasmid pMA5 (3) in the *phoA phoB* strain MH3402 and grown on an LB plate supplemented with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate. Strains expressing the *yycH-phoA* fusion construct turned blue, whereas strains expressing the *kapB(C26P)-phoA* fusion, previously described not to be exported, did not (3) (Fig. 6). Therefore, YycH is either secreted into the medium, or the N-terminal region is embedded in the membrane. In either case, it is located and likely functions outside the cell membrane.

DISCUSSION

The aim of the current investigation was to identify genes or gene products that regulate the YycF-YycG two-component signal transduction system. A transposon mutagenesis study identified insertions in *yycH*, the gene immediately downstream of *yycG*, as a suppressor of a temperature-sensitive *yycF* mutation. This indicated that the function of YycH is either to regulate the transcription of the YycF-YycG TCS or to affect its activity.

Disruption of *yycH* but not of any downstream genes within the *yyc* operon caused a 10-fold increase of YycF-dependent gene expression, as determined by a *lacZ* reporter construct to

the *yycH* promoter. Moreover, immunological experiments indicated that the *yycH* disruption did not increase the cellular level of either YycG or YycF. Thus, the inactivation of *yycH* likely leads to increased activity (i.e., phosphorylation) of YycF.

Alkaline phosphatase gene fusions demonstrated that YycH is a secreted or membrane-embedded protein, consistent with bioinformatic predictions. Therefore, it is likely that YycH affects the levels of YycF phosphorylation indirectly by modulating YycG activity. Several possibilities could account for this inhibitory action. YycH may interact directly with the extracellular domain of YycG or process information for YycG. Some possible scenarios are outlined below.

Increase of YycF-dependent gene expression in the *yycH* strain caused a cell wall and growth defect. These phenotypes, however, were independent of *yycH*, a potential autolysin. This gene, in particular, was investigated because its expression appeared most dependent on YycF levels in a previous study (12). Other possible candidates responsible for the observed phenotypes are the *tagAB* and *tagDEF* operons or the putative cell wall hydrolase gene *yvfK*. While the *pcsB* gene is the crucial YycF target in *S. pneumoniae* (17), it is possible that no single gene product is responsible for the observed phenotypes in *B. subtilis*. Rather, a whole apparatus of cell wall metabolic proteins seems to be controlled by the YycFG system. Interestingly, both a hyperactive YycFG system (as observed in a *yycH* strain) and reduced activity YycFG system [as observed for the temperature sensitive YycF(H215P) mutant] severely compromise a strain's ability to grow. It appears that the YycFG system performs a balancing act of expressing the proper genes at the right time at the right levels to guarantee optimal growth (Fig. 7). Quite possibly, this is only true for organisms that express what we designate the type I YycFG system that is characterized by *yycH* and *yycI* genes. In the organisms (e.g., *Streptococci*) that express a type II YycFG system, the YycG protein does not appear to be essential and has no extracellular putative sensor domain, and the YycH and YycI proteins are not present. However, the dispensability of YycG may only reflect the phosphorylation of YycF by small molecule donors, e.g., acetyl-PO₄, and a low-level requirement for YycF in *pcsB* expression. This reduced complexity of type II systems might mirror the fact that these systems do not control the same set of genes and might not perform a similar balancing act.

Both, YycF and YycG protein levels appeared to stay constant throughout a culture's growth cycle. This is in contrast to previous reports that suggested that expression of the *yycFGHIJK* operon stops at the onset of stationary phase and that YycF-dependent expression stops as well (5, 12). The former experiment was performed in sporulation medium, while the experiments presented here were performed in the richer LB medium. Potentially, the onset of sporulation inhibits the expression of the *yyc* operon. Alternatively, the YycFG proteins do not turn over rapidly, even though expression stops once cells reach stationary phase.

Both, class I and class II YycG histidine kinases have a characteristic intracellular PAS domain (4). PAS domains are commonly found in two-component histidine kinases and are generally involved in sensing cytoplasmic signals, such as redox potential, and commonly bind small molecules such as ATP, FAD, or heme (21). The absence of an extracellular domain in

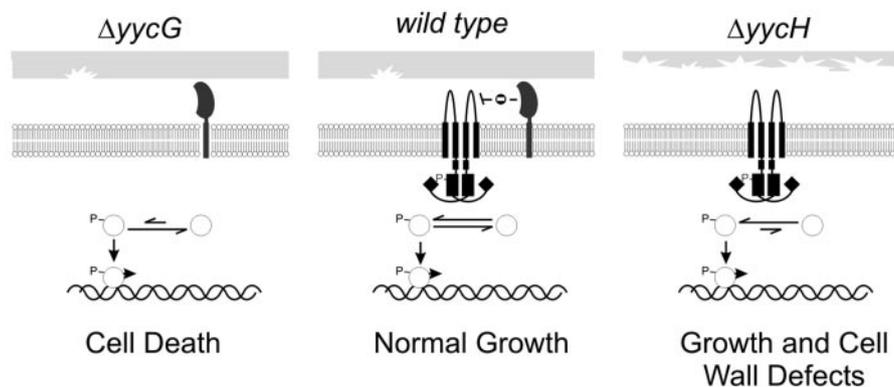


FIG. 7. Model of the YycG-dependent balancing act. YycH (dark gray) gets exported to the extracellular space, where it down-regulates YycG (black) activity in order to keep growth and cell wall levels at the optimum (middle). In the absence of YycG, cells die because of a shift of the equilibrium toward the unphosphorylated form of the response regulator YycF (white) and, therefore, reduced expression of the YycF regulon (left). Cells show cell wall and growth defects in the absence of YycH, likely due to a shift in the equilibrium toward the phosphorylated form of YycF and, therefore, overexpression of the YycF regulon (right).

class II YycG proteins suggests that cytoplasmic or membrane-embedded signals are the only ones detected. Conversely, class I YycG proteins are likely to sense ligands through their extracellular domain in addition to sensing cytoplasmic signals. YycH appears to be involved in this extracellular sensing process. Future work will have to aim at understanding how YycH confers its activity. It might interact with a signaling molecule and then modulate YycG activity through direct interaction, similar to the maltose binding protein in *E. coli* chemotaxis (9, 29). W.-L. Ng and M. Winkler (18) found an apparent link between an organism's class of YycFG system and the capacity for electron transport. They suggested that YycH and YycI might be involved in sensing the redox state of the electron transport. Alternatively, YycH could itself be an enzyme that processes a substrate to generate a ligand for YycG. These are only some possibilities to explain the observed effects. It should be noted that YycI is predicted to be a secreted protein as well, and Ng and Winkler speculated that it, too, might be involved in the sensing process. The present data were unable to account for such an activity, as YycF-dependent expression remained unchanged in a *yycI* strain. However, it is possible that an activity might be apparent under different conditions than those present in a standard laboratory culture.

In conclusion this study suggests that YycH is an inhibitor of the essential YycG protein in *B. subtilis*. The functions of the other proteins organized in the *yyc* operon, YycI, YycJ, and YycK, remain elusive. They do not, however, seem to be involved in YycF-dependent gene expression, at least under laboratory conditions.

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