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A putative bactoprenol glycosyltransferase, CsbB, in *Bacillus subtilis* activates SigM in the absence of co-transcribed YfhO

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ABSTRACT

Bacteria are equipped with complex cell surface structures, such as cell walls. How they maintain cell surface integrity through cell wall metabolism during growth and adaptation to unfavorable environmental conditions is still elusive. In the Gram-positive soil bacterium *Bacillus subtilis*, one extracytoplasmic function (ECF) sigma factor, SigM, is believed to play a primary role in cell surface integrity. Here, we find that expression of CsbB, which is known to be involved in the extracellular stress response, causes constitutive activation of SigM when YfhO, a membrane protein with unknown function, is lost. CsbB has similarity with the well-characterized bactoprenol glucosyltransferase GtrB found in Gram-negative bacteria. Substitution of a single amino acid residue at the putative catalytic site of CsbB abolishes this constitutive activation, and expression of *Escherichia coli* GtrB in *B. subtilis* causes similar effects as expression of CsbB, suggesting that SigM is activated by the glycosyltransferase activity of CsbB. A comparison with the Gtr system in Gram-negative bacteria suggests that accumulation of glycosylated bactoprenol catalyzed by CsbB reduces the bactoprenol pool in the absence of YfhO. Reduction of bactoprenol synthesis causes similar effects as expression of CsbB. We propose that it is the shortage of available bactoprenol within a cell that induces SigM activity.

1. Introduction

The bacterial cell wall has a mesh-like architecture composed of peptidoglycan (PG), which consists of glycan strands made up of two alternating amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), cross-linked with a pentapeptide [1]. This structure must be rigid enough to maintain cell shape and to protect the cytoplasmic membrane from turgor pressure. On the other hand, it must be ductile enough to allow cell growth and to permit responses to environmental changes. The units composed of GlcNAc and MurNAc with a pentapeptide are assembled on the cytosolic surface of the membrane on a bactoprenol molecule, which has the structure of C55 isoprenoid alcohol [2], via a pyrophosphate linker, resulting in the formation of Lipid II. This molecule translocates to the outside surface of the membrane and is inserted by transpeptidases and transglycosylases into the PG matrix. After disposal of the unit, the bactoprenol phosphate (C55-P) is shuttled back to the cytosolic surface of the membrane to be reused.

In Gram-positive bacteria, PG is further modified with carbohydrate-based anionic polymers, the teichoic acids, that is, lipoteichoic acid (LTA) and wall teichoic acid (WTA), which play an important role in cell envelope integrity. WTAs are usually composed of glycerolphosphate (GroP) or ribitol phosphate repeats and more complex sugars. These components are polymerized with a C55-P on the cytoplasmic surface and transported across the membrane before being covalently linked to the PG. LTAs consist mainly of polymerized GroP, attached via a glycolipid anchor to the membrane. In general, the GroP backbone chain of both WTA and LTA is modified with D-alanine residues and in many bacteria with additional glycosyl groups. Both LTA and WTA are phosphate-rich molecules, which give a net negative charge to the PG layers of the cell surface, and appear to influence various essential functions on the cell surface [3,4]. In addition to PG and WTA synthesis, C55-P and glycosyltransferase are involved in the synthesis of lipopolysaccharide (containing O-antigen), exopolysaccharide, capsules, and glycoprotein [5].

Extra-cytoplasmic-function (ECF) sigma factors are activated in response to changes in environmental conditions and through expression of their regulons perform functions in the outer cell surface and periplasmic space [6]. In *Bacillus subtilis*, a Gram-positive sporulating soil bacterium, seven ECF sigma factors have been identified. One, SigM, is strongly involved in cell envelope integrity. Expression and activity of SigM are elevated under acid, heat, salt, superoxide and cell envelope stresses [7]. SigM dependent genes and operons contribute to cell envelope stress responses [8].

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In addition to its structural role, the bacterial cell wall has various functional roles, which are crucial to growth, survival, and pathogenicity. However, a detailed understanding of modification mechanisms of the cell wall and their regulation has not yet been achieved. In *B. subtilis*, the ECF sigma factor SigM is activated when the cells are exposed to antibiotics that interfere with cell wall synthesis [9]. Recently, it was reported that defects in LTA synthesis induce the transcription of the SigM gene [10]. Consequently, we assumed that the activity of SigM reflects the cell wall integrity status in *B. subtilis*. For this report, in order to investigate unidentified mechanisms that maintain cell wall integrity, we have screened for and analyzed a gene that affected the activity of SigM.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

All bacterial strains, plasmids, and primers used in this study are listed in Supplemental Table 1–3, respectively. Bacterial cultures were grown in liquid LB medium with vigorous shaking or on solid LB plate containing 1.5 % agar at 37 °C. The following antibiotics were used if necessary: For *B. subtilis*, spectinomycin (Sp), 100 μg/ml; neomycin (Nm), 5 μg/ml; chloramphenicol (Cm), 5 μg/ml; erythromycin (Em), 0.5 μg/ml, and for *Escherichia coli*, ampicillin (Ap), 50 μg/ml. Transformation of *B. subtilis* and *E. coli* was performed as described previously [11,12].

2.2. β-Galactosidase assay and immunoblot analysis

Strains harboring a *lacZ*-fusion at the *amyE* locus were grown in LB medium with shaking at 37 °C overnight. The culture was resuspended in fresh medium. After further incubation, samples of the culture were withdrawn for β-galactosidase assay as described previously [12]. One unit of enzymatic activity was defined as $1000\times$ ABS_{420}/$O.D._{600}$/ml/min.

Immunoblot analysis was performed as described previously [12]. Immunodetection was carried out with anti-FLAG antibody (Sigma–Aldrich) as the primary antibody. Detection was performed using horseradish peroxide-labeled anti-rabbit IgG antibody (Bio-Rad) as a second antibody with an ECL plus detection kit (GE Healthcare) following the instruction manual.

2.3. Construction of mini-Tn10 libraries

Random mini-Tn*10* libraries of *B. subtilis* strains were constructed using the plasmid pIC333 [13]. Strain BSU41 (*amyE*::P*sigM′-lacZ*) was transformed with pIC333 and spread on LB plates containing Em. Plates were incubated overnight at 28 °C and about 70 single colonies were inoculated into liquid LB medium containing Em. After 3 h incubation at 28 °C, they were shifted to 37 °C and grown for 3 h. Cultures were diluted to 10^{-6} and spread onto LB plates containing Sp, 0.7 M NaCl and 0.005 % 5-bromo-4-chloro-3-indoryl-β-D-galactopyranoside (X-gal).

2.4. Determination of the mini-Tn10 insertion site

Genomic DNA was extracted from candidate strains, digested with *Hin*dIII, self-ligated, and used to transform *E. coli*, excising the ColE1 replication origin and β-lactamase gene in mini-Tn*10*, including flanking *B. subtilis* genomic DNA. Alternatively, genomic DNA of candidate strains was digested with *Pst*I and self-ligated, followed by inverse PCR using primers Tn inverse-F and Tn inverse-R. The mini-Tn*10* insertion site was determined by DNA sequencing of these plasmids.

2.5. Construction of deletion mutants

In-frame and marker-free deletion of the *csbB*, *yfhO* gene and *csbB–yfhO* regions was performed by the method described previously [14]. In each case, three DNA fragments were amplified by PCR using *B. subtilis* genomic DNA as the template: (A) upstream region (primer pairs csbB_DF1 and csbB_DR1, yfhO_DF1 and yfhO_DR1, csbB_DF1 and csbByfhO_DR1 were used), (B) downstream region (primer pairs csbB_DF2 and csbB_DR2, yfhO_DF2 and yfhO_DR2, yfhO_DF2 and yfhO_DR2 were used), (C) intragenic region (primer pairs csbB_1F and csbB_1R, yfhO_1F and yfhO_1R, yfhO_1F and yfhO_1R were used). The DNA fragment of the MazF-encoding cassette was amplified by PCR using genomic DNA of strain TMO310 and primers pAPNC-F and chpA-R. These PCR products, (A), (B), MazF fragment, and (C), were fused by a second step of recombinant PCR. The resulting DNA fragment was used to transform *B. subtilis* 168. Transformants were selected on LB plates containing Sp, followed by evaluation of Isopropyl β-D-1-thiogalactopyranoside (IPTG)-sensitive growth. In the obtained colonies, the internal region of each gene with MazF-encoding gene was excised due to intramolecular homologous recombination in genomic DNA. Deletion of each gene was confirmed by PCR.

2.6. Construction of plasmids for overexpression of csbB and gtrB

A DNA fragment containing the *csbB* gene with flanking SD sequence and the *gtrB* gene was amplified by PCR using *B. subtilis* 168 and *E. coli* C600 genomic DNA as template, respectively and primers csbB-SD-F and csbB-R, and gtrB-F and gtrB-R, respectively. This fragment was cloned into the *Bam*HI-*Sma*I site of pHCMC05 [15], yielding pHCMC05-SD-*csbB* and pHCMC05-*gtrB*, respectively.

To generate pHCMC05-SD-*csbB* D97A and D44A, pHCMC05-SD-*csbB* plasmid DNA was used as template for inverse PCR using primers, csbB-D97A-F and csbB-D97A-R, and csbB-D44A-F and csbB-D44A-R, respectively. The resultant PCR product was self-ligated as described previously [16]. Resultant plasmids were used as templates to fuse a FLAG tag to the C-terminal end of CsbB by inverse PCR using primers csbB-FLAG-inverse-F and csbB-tag-inverse-R, and self-ligation, yielding pHCMC05-SD-*csbB*FLAG D97A and D44A, respectively.

A chimeric clone of GtrB–CsbB was constructed using the SLiCE system [17]. The N-terminal portion of GtrB and the plasmid backbone were amplified by PCR using *E. coli* JM109 genomic DNA and pHCMC05-SD-*csbB* as templates and the primers gtrB-N-SLiCE-F and gtrB-N-SLiCE-R, and csbB-inverse-F and csbB-SD-inverse-R, respectively. The two fragments were mixed with the standard SLiCE reaction mixture for in vitro recombination and used to transform *E. coli* C600 to connect with each other, yielding a plasmid designated pHCMC05-SD-*gtrB*^{N}-*csbB*^{C}. In order to adjust the SD sequence of *csbB* and *gtrB*, the SD sequence of *csbB* was substituted with the artificial SD sequence on the plasmid when comparing the expression of CsbB and GtrB, since the SD sequence of *E. coli gtrB* gene is ambiguous. Elimination of the native SD sequence of the *csbB* gene from plasmids pHCMC05-SD-*csbB* and pHCMC05-SD-*gtrB*^{N}-*csbB*^{C} was performed by inverse PCR using the primers csbB-F and pHCMC05-R, and self-ligation, yielding pHCMC05-*csbB* and pHCMC05-*gtrB*^{N}-*csbB*^{C}, respectively. In the resultant plasmids, both inserted genes are expressed only from the plasmid-born SD sequence.

2.7. Microscopic analysis

Cells were grown at 37 °C in LB medium with or without the addition of 1 mM IPTG. Five hundred microliters of the culture

was centrifuged, and 400 μl of the supernatant was aspirated off. The cells were then resuspended in the remaining 100 μl. Portions (2 μl) of each sample were spotted on glass slides. Microscopy was performed with a Nikon ECLIPSE phase-contrast microscope with a 100× UplanApo objective. Images were captured with an ORCA charge-coupled device camera and software (Hamamatsu).

3. Results and discussion

3.1. Isolation and analysis of mini-Tn10 mutants with altered SigM activity

Searching for as yet undiscovered mechanisms that regulate cell wall integrity, we isolated mutations giving rise to changes in SigM activity of the strain BSU41 harboring *amyE*::P*sigM′-lacZ* by mini-Tn*10* mutagenesis. Among approximately 20,000 colonies harboring mini-Tn*10*, about 400 colonies with obviously altered β-galactosidase activity were picked up and gridded to new LB plates containing Sp, 0.7 M NaCl and 0.005% X-gal, a condition that activates SigM in the wild type strain. In this step, about 200 colonies exhibiting different SigM expression levels than the parental strain (BSU41) were identified. Since these colonies might carry multi mini-Tn*10* insertions in their genome, genomic DNA was extracted from each colony and used to transform BSU41 to exclude unrelated mutations. These isolates were subjected to β-galactosidase assays yielding 11 mutants with increased β-galactosidase activity and 8 mutants with reduced activity in the presence of NaCl. Most of these were genes for cell wall and cell membrane related functions. The subset for which mini-Tn*10* insertion sites were determined is listed in Table 1. From among these, we chose the *yfhO* gene for further analysis, since it codes for a membrane protein of unknown function which is widely conserved among Gram-positive bacteria, and since it showed the strongest effect on SigM activity.

To make sure that the phenotype was not due to some additional mutation other than mini-Tn*10* insertion into the *yfhO* gene, we constructed an in-frame deletion mutant of the *yfhO* gene. In the experiments shown below, we used the strain HRI001, in which the selection marker for P*sigM–lacZ* was changed to erythromycin resistance, as a control strain in order to avoid a conflict with the selection marker for pHCMC05. The strain HRI006 (Δ*yfhO*) exhibited significantly higher SigM activity than the strain HRI001 (*yfhO*$^+$) even in the absence of NaCl (Fig. 1), similar to strain 57F. Additionally, activity of no other ECF sigma factor in *B. subtilis* was affected by deletion of the *yfhO* gene (data not shown). This suggests that SigM activity is negatively affected by YfhO in a specific manner.

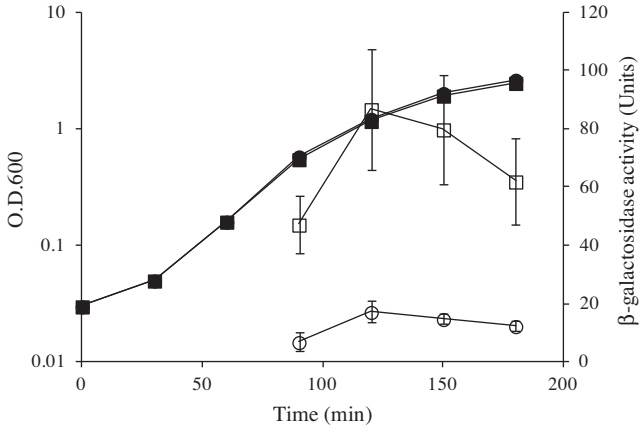

Fig. 1. Effect of *yfhO* mutation on SigM activity. The strains HRI001 (*yfhO*$^+$) (circles) and HRI006 (Δ*yfhO*) (squares) were grown in liquid LB medium and the growth curve (closed symbols) and β-galactosidase activity (open symbols) was measured at indicated time points. Values represent mean of three identical experiments with error bars.

3.2. Effect of disruption and overexpression of the csbB gene on SigM activity in yfhO$^+$ and ΔyfhO strains

Next, we turned our attention to the *csbB* gene located upstream of *yfhO*. Transcriptional analyses available at BSORF (http://bacillus.genome.ad.jp/) show that *csbB* and *yfhO* are co-transcribed as an operon. Moreover, comparative analysis of the *yfhO* locus in *B. subtilis* and its close relatives revealed that all species possessing *yfhO* also carry the *csbB* gene upstream of *yfhO* (Fig. 2). Consequently, we speculated that the function of the *csbB* gene is related to the function of *yfhO*.

To confirm this hypothesis, we constructed an in-frame and marker-free deletion mutant of the *csbB* gene to avoid a possible polar effect on the downstream *yfhO* expression (HRI005) and investigated whether disruption of the *csbB* gene affects SigM activity (Fig. 3A). A *csbB* single mutant exhibited no significant effect on SigM activity. However, in the double mutant in which both *csbB* and *yfhO* was deleted (HRI007), the high level of SigM activity shown by the *yfhO* single mutant was reduced to that of the strain HRI001. This suggests genetic interaction between YfhO and CsbB. In addition to this, our preliminary data using bacterial two-hybrid analysis indicated protein–protein interaction between CsbB and YfhO (data not shown). These results support the idea that CsbB and YfhO interactively affect SigM activity; they suggest that SigM is activated by the function of CsbB in the absence of *yfhO*.

To test for this, we introduced a multicopy plasmid that harbored an extra copy of the *csbB* gene under the IPTG inducible pro-

Table 1
Tn-*10* transposon mutants with altered SigM activity isolated in this study.

Strain	Tn-*10*-inserted gene	Annotation of gene product[a]	SigM activity[b]
Positive effect on SigM activity			
23D	*glpD*	Glycerol-3-phosphte dehydrogenase	1.3
43A	*ecsB*	ABC-2 type transport system permease protein	2.0
46A	*secDF*	Protein translocase subunit	2.5
57F	*yfhO*	Conserved membrane protein	4.0
57L	*pyrB*	Aspartate carbamoyltransferase	2.5
Negative effect on SigM activity			
9B	*ygxA*	Uncharacterized protein	0.7
56E	*yddH*	D, L-endopeptidase, cell wall hydrolase	0.6
57G	*minC*	Septum site-determining protein	0.6
60I	*miaA*	tRNA dimethylallyltransferase	0.7
62C	*iolR*	Repressor of myo-inositol catabolism operon	0.5
65B	*liaS*	Cell wall antibiotics resistance related sensor kinase	0.5

[a] Product annotation of each gene is with reference to the BSORF *Bacillus subtilis* genome database.
[b] SigM activity is given as a multiple of parent strain (BSU41) activity.

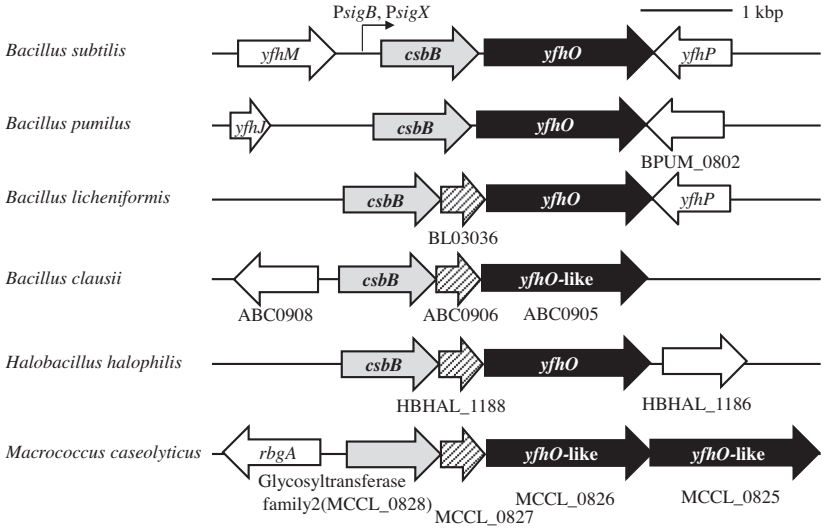

Fig. 2. Comparison of Gene organization around the *csbB* and *yfhO* genes in *B. subtilis* and other Gram-positive bacteria. Genes encoding proteins annotated as CsbB or glycosyltransferase family 2 are indicated by gray arrows. Genes encoding the proteins annotated as YfhO or possessing a YfhO motif (indicated as *yfhO*-like) according to the Pfam database (http://pfam.sanger.ac.uk/) are indicated by black arrows. Genes encoding proteins annotated as GtrA-like proteins are indicated by hatched arrows. White arrows indicate other genes located in the neighborhood of *csbB* and *yfhO*. Genome maps were created according to the KEGG genome database (http://www.genome.jp/kegg/genes.html).

moter into the strain HRI001 and the *csbB–yfhO* double deleted derivative (HRI007). In the *csbB*$^+$ and *yfhO*$^+$ background (HRI017), SigM activity was not affected by the overproduction of CsbB upon addition of IPTG (Fig. 3B). In contrast, in the Δ*csbB–yfhO* background (HRI019), the overproduction of CsbB elevated the SigM activity to about 20-fold of that of the control strain HRI010 harboring the empty vector pHCMC05 (Fig. 3B). These results strongly suggest that activation of SigM in the *yfhO* mutant is closely related to the function of CsbB.

3.3. Effect of glycosyltransferase activity on SigM activity

Homology search with web databases indicated that *csbB* encodes a family 2 glycosyltransferase belonging to the GtrB subfamily (Supplemental Fig. 1). GtrB is a protein that acts as a bactoprenol glucosyltransferase, which catalyzes the reaction C55-P + UDP-glucose → C55-P-glucose + UDP, and is involved in serotype conversion of Gram-negative bacteria [18]. This led us to hypothesize that CsbB is a bactoprenol glycosyltransferase and that this activity causes activation of SigM. We investigated whether GtrB of *E. coli* also as acts like CsbB. A significant effect of overexpression of GtrB on SigM activity was observed when bacteria were cultivated on solid LB plate (data not shown), but in liquid LB culture the effect was barely noticeable (Fig. 3B). Although both GtrB and CsbB are putative membrane proteins, we surmised that GtrB of *E. coli* might not localize properly into the *B. subtilis* cytoplasmic membrane or might be labile when the cells grow fast. Therefore, we fused the N-terminal portion of GtrB (catalytic domain) and C-terminal portion of CsbB (membrane-spanning domain). This chimeric protein induced SigM activity effectively when it was overexpressed in the *yfhO* mutant but this activation of SigM was not suppressed by the presence of *yfhO* (Fig. 3B). This suggested that YfhO did not interact with GtrB effectively.

Furthermore, for the proteins belonging to the GtrB family, several aspartate residues have been shown to be essential for glycosyltransferase activity. In particular, aspartate residues located in the N-terminal region and the conserved D × D motif have been considered residues necessary for glycosyltranseferases which use NDP-sugar as substrate (Supplemental Fig. 1) [19,20]. Substitution of these aspartate residues with alanine in CsbB (D97A, D44A) abolished its ability to activate SigM (Fig. 3C). The FLAG epitope-tag was fused to each CsbB protein in order to detect the expression level, and it was revealed that CsbB proteins were not labile due to amino acid substitution (Fig. 3C). This fact strongly suggests that it is the glycosyltransferase activity of CsbB that is responsible for the activation of SigM in the absence of YfhO.

3.4. Effect of overexpression of putative glycosyltransferase on cell wall synthesis

According to the model proposed by Korres et al., GtrB and two more membrane proteins, GtrA and GtrX, where X varies with serotype, are involved in the O-antigen modification step of *Shigella flexneri*[18]. C55-P-glucose is produced at the inner surface of the cytoplasmic membrane, catalyzed by GtrB and is flipped or transported across the membrane to make it available to the GtrX. GtrX, which is a serotype specific enzyme, specifically attaches the glucosyl residue to the appropriate sugar residue of the O-antigen unit [18]. After serial reaction, released C55-P is thought to be recovered into the cytoplasmic side. On the other hand, Guan et al. have shown that expression of *gtrB* in the absence of *gtrX* in *S. flexneri* slowed growth and they proposed that this phenotype was due to disturbance of the intracellular bactoprenol pool [21].

Several observations led us to the conclusion that these phenomena were very likely analogous to the case of *csbB* and *yfhO*. First, CsbB has high similarity with GtrB. Second, YfhO is predicted to be a membrane protein which contains 13 membrane-spanning domains, similar to GtrX. Third, CsbB and YfhO interact with each other genetically. Although YfhO does not exhibit similarity with GtrA or GtrX, YfhO may act like GtrX (and GtrA) specific to the product of the reaction catalyzed by CsbB. In short, disruption of YfhO may interfere with C55-P recycling and thereby cause activation of SigM.

Bactoprenol is an isoprenoid composed of isoprene units. In *B. subtilis*, isoprene is synthesized by the action of the *ispE* gene and the *ispDF* operon, which code for the core enzymes in the biosynthetic pathway [22]. The strains YABHp and YACMp, where these genes were under the control of the IPTG-dependent spac promoter, showed asymmetrically globular daughter cells in phase-contrast micrographs when IPTG was removed from the culture medium (Fig. 4E and F). This reflects that inactivation of these

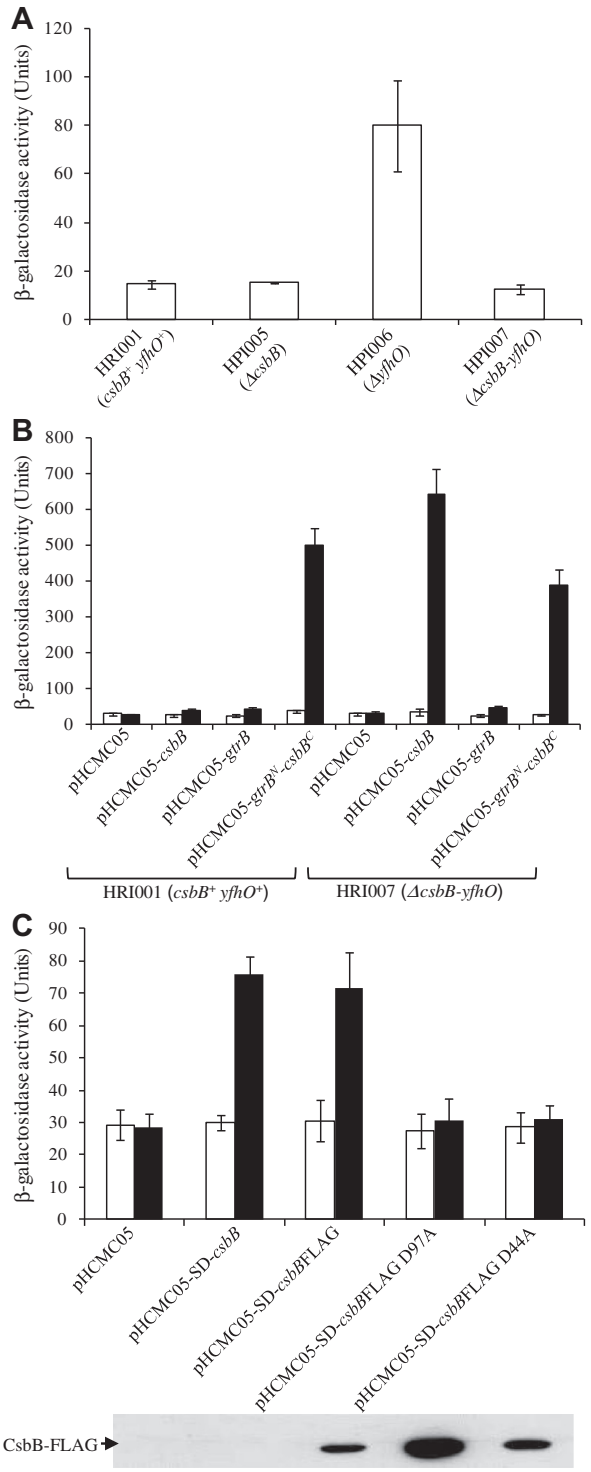

Fig. 3. Effect of bactoprenol glycosyltransferase activity on SigM activity. (A) Effect of *csbB* mutation on SigM activity. The activity of β-galactosidase in the strains HRI001 (*csbB*$^+$ *yfhO*$^+$), HRI005 (Δ*csbB*), HRI006 (Δ*yfhO*) and HRI007 (Δ*csbB–yfhO*) was measured at O.D.600 = 0.8. (B, C) Effect of overexpression of CsbB, GtrB and their mutant form in HRI001 (*csbB*$^+$ *yfhO*$^+$) and HRI007 (Δ*csbB-yfhO*) cells. In each strain, β-galactosidase activity was measured 90 min after addition of IPTG. White bars, IPTG minus; black bars, IPTG plus. Values represent mean of three identical experiments with error bars. (B) Strains are: HRI008 (pHCMC05), HRI017 (pHCMC05-*csbB*), HRI020 (pHCMC05-*gtrB*), and HRI023 (pHCMC05-*gtrB*^{N}-*csbB*^{C}) in the *csbB*$^+$ and *yfhO*$^+$ strain (HRI001); HRI010 (pHCMC05), HRI019 (pHCMC05-*csbB*), HRI022 (pHCMC05-*gtrB*), and HRI025 (pHCMC05-*gtrB*^{N}-*csbB*^{C}) in the Δ*csbB-yfhO* strain (HRI007). (C) Strains are: HRI010 (pHCMC05), HRI013 (pHCMC05-SD-*csbB*), HRI014 (pHCMC05-SD-*csbB*FLAG), HRI015 (pHCMC05-SD-*csbB*FLAG D97A) and HRI016 (pHCMC05-SD-*csbB*FLAG D44A) in the Δ*csbB–yfhO* strain (HRI007). Western blots using anti-FLAG antibody are shown on the bottom.

enzymes causes a shortage of bactoprenol leading to inhibition of PG biosynthesis [23]. Overexpression of CsbB in the *csbB*$^+$*yfhO*$^+$cells (HRI017) did not affect morphology (Fig. 4B), whereas in the *yfhO*$^-$ cells (HRI019), overexpression of CsbB caused morphological changes (Fig. 4D) similar to those observed in isoprene-depleted cells (Fig. 4E and F). This suggests that overexpression of CsbB in the absence of *yfhO* severely reduced the availability of bactoprenol for cell wall synthesis. In our preliminary experiments, SigM activity was elevated in the YABHp and YACMp strains when IPTG was depleted (data not shown). In a recent study, SigM was activated by friulimicin B treatment, which inhibits recycling of C55-P [9]. These observations suggest that SigM-regulating mechanisms sense availability of C55-P, which is influenced by external and cell wall stress.

The expression of *csbB* is controlled by two alternative sigma factors, SigB and SigX (Fig. 2) [24,25]. SigB is a general stress response sigma factor induced by various environmental stresses or nutrient starvation [26]. SigX has been reported to contribute to modification of the cell envelope and resistance to cationic antimicrobial peptide activity [27]. It has been shown that the *csbB* mutant strain exhibits sensitivity to NaCl and ethanol stress [24]. These observations indicate that CsbB is involved in the cell envelope stress response. The present data on CsbB–YfhO are insufficient to pin down exact roles and explain why absence of YfhO will cause severe stress. Nevertheless, we hope that this study is a meaningful step towards a comprehensive understanding of the biological functions of the bactoprenol glycosyltransferases.

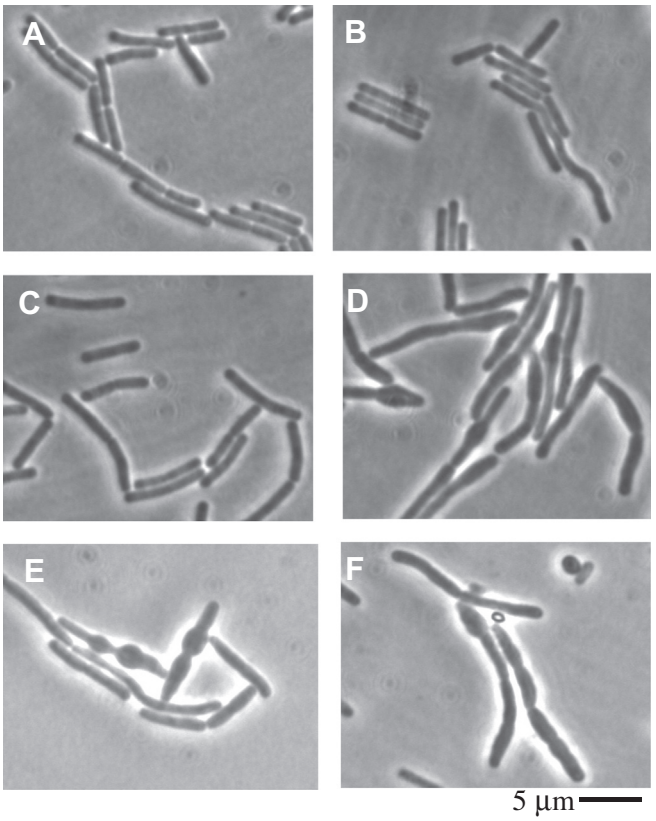

Fig. 4. Effect of overexpression of CsbB and depletion of bactoprenol on cell morphology observed by phase-contrast microscopy. (A–D) Effect of overexpression of *csbB* in *csbB*$^+$ *yfhO*$^+$ (A, B) and Δ*csbB–yfhO* (C, D) background. Pictures were taken after 90 min incubation with (B, D) or without (A, C) the addition of IPTG. (E, F) Effect of depletion of bactoprenol by suppression of *ispE* (E) and *ispDF* (F) expression, using YABHp (Pspac-*ispE*) and YACMp (Pspac-*ispDF*) strains, respectively. Pictures were taken 120 min after removal of IPTG.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.064.

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