The bacterial tyrosine kinase activator TkmA contributes to biofilm formation largely independent of the cognate kinase PtkA in *Bacillus subtilis*

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Summary

In *Bacillus subtilis*, biosynthesis of exopolysaccharide (EPS), a key biofilm matrix component, is regulated at the post-translational level by the bacterial tyrosine kinase (BY-kinase) EpsB. EpsB in turn relies on the cognate kinase activator EpsA for activation. A concerted role by a second pair of BY-kinase and its activator, PtkA and TkmA, respectively, in biofilm formation was also implicated in previous studies. However, the exact function of PtkA and TkmA in biofilm formation remains unclear. In this work, we show that the kinase activator TkmA contributes to biofilm formation largely independent of the cognate kinase PtkA. We further show that the biofilm defect by Δ*tkmA* can be rescued by complementation by *epsA*, suggesting a functional overlap between TkmA and EpsA and providing a possible explanation for the role of TkmA in biofilm formation. We also show that the importance of TkmA in biofilm formation depends highly on medium conditions; the biofilm defect of Δ*tkmA* is very severe in the biofilm medium, LBGM, but marginal in another commonly used biofilm medium MSgg. The molecular basis for the medium dependence is likely due to differential expression of *tkmA* and *epsA* in the two different media and complex regulation of these genes by both Spo0A and DegU. Our studies provide genetic evidence for a possible crosstalk between a BY-kinase activator (TkmA) and a non-cognate kinase (EpsB), and an example of how environmental conditions may influence such a crosstalk in regulating biofilm formation in *B. subtilis*. 
Importance

In bacteria, biosynthesis of secreted polysaccharides is often regulated by bacterial tyrosine kinases (BY-kinase). BY-kinases in turn rely on cognate kinase activators for activation. In this study, we investigated the role of a BY-kinase activator in biofilm formation in *Bacillus subtilis*. We present evidence that different BY-kinase activators may functionally overlap with each other, as well as an example of how activities of the BY-kinase activators may highly depend on environmental conditions. Our study broadens the understanding of the complexity of the regulations of the BY-kinases/kinase activators and the influence on bacterial cell physiology.
Introduction

Biofilms are multicellular communities of bacteria with highly complex structures and distinct morphological features (1-4). One of the most important characteristics of the biofilms is the presence of a self-produced extracellular matrix, which allows individual cells within the biofilm to stick to each other (2, 3, 5). In *Bacillus subtilis*, the biofilm matrix consists of an exopolysaccharide (EPS), amyloid fiber-like protein TasA, and a small hydrophobin BslA (6-11). The regulatory circuit that controls the expression of those matrix-encoding genes has been well studied in *B. subtilis* (5, 12-14). EPS and TasA fibers are produced by the protein products of two matrix operons, *epsA-O* and *tapA-sipW-tasA*, respectively (8, 15). These two operons are normally repressed by the biofilm repressor, SinR, and by the transition state regulator, AbrB (8, 16-18).

Repression by SinR is relieved when, under biofilm induction, a small anti-repressor SinI is expressed, which antagonizes SinR through direct protein-protein interactions (19-21). The gene *sinI* is activated by the master regulator Spo0A in response to environmental and cellular signals (22). The gene for the small hydrophobin BslA was shown to be under the control of the response regulator DegU and two transcription repressors, AbrB and SinR, either directly or indirectly (23).

The master regulator Spo0A is positioned at the heart of the genetic network for control of alternative cell fates (motile cells, matrix producers, spore formers, etc.) in *B. subtilis* (24). Spo0A governs endospore formation by regulating hundreds of genes involved in sporulation in *B. subtilis* (25, 26). Spo0A is also involved in biofilm formation through its control on several regulatory genes, including *sinI* and *abrB* (16, 27, 28). The
activity of Spo0A is under complex regulation at multiple levels. The Spo0A protein is activated by protein phosphorylation, which depends on the Kin family of sensor histidine kinases (from KinA to KinE) directly, or indirectly via a phosphor-relay (mediated by the phosphor-transfer proteins Spo0F and Spo0B) (29-31). Those histidine kinases sense a variety of environmental or physiological signals and subsequently promote signal transduction, leading to Spo0A activation (13, 32-37).

DegU is a global response regulator, whose phosphorylation is carried out by its cognate sensor histidine kinase DegS (38, 39). DegU is involved in motility, chemotaxis, biofilm formation, synthesis of secreted enzymes, and production of γ-poly-DL-glutamic acid (23, 38, 40, 41). The role of DegU in biofilm formation is quite complex because DegU controls multiple targets that were shown to be involved in biofilm formation, both positively and negatively (23, 40, 41). For example, the gene for BslA is under the positive control of DegU (23). In addition to functioning as an activator, high levels of DegU were shown to inhibit the matrix operons, epsA-O and tapA-sipW-tasA, although the biological significance for such negative regulation is less well understood (40).

Bacterial tyrosine kinases (BY-kinase) belong to a minor family of protein kinases in bacteria (42, 43). They are often associated with the biosynthesis of secreted polysaccharides (44). In Gram-positive bacteria, BY-kinases pair with cognate transmembrane kinase activators for activation. The activator protein contains an extracellular domain, which is speculated to sense environmental signals. The activity of BY-kinases also seems to be self-regulated by auto-phosphorylation at the tyrosine
residues. BY-kinases in turn regulate the target proteins by protein phosphorylation. In

*B. subtilis*, EPS biosynthesis is controlled, at the post-translational level, by the BY-

kinase EpsB and its activator EpsA in addition to the known regulations on the *epsA-O*

operon at the transcriptional level (45, 46). A recent study proposed a feedback

mechanism for the regulation of EPS biosynthesis in *B. subtilis* (47). According to that

study, the self-produced exopolysaccharide molecules act as a quorum-sensing-like

signal to alter the activity of EpsA, which then interacts with EpsB and stimulates the

activity of EpsB (45, 47). EpsB in turn regulates the activity of EpsE by protein

phosphorylation (47). EpsE is known for its dual function both as a molecular clutch for

the control of the flagellar motility and as a glycosyl-transferase involved in EPS

biosynthesis (48, 49).

*B. subtilis* also possesses a second pair of BY-kinase and the kinase activator,

PtkA and TkmA, respectively (46, 50). Genes for the two proteins are located in a

presumptive operon with two other genes, *ptpZ* and *ugd* (Fig. 1A). The *ptpZ* gene

encodes a phosphatase, which counteracts the activity of BY-kinase by

dephosphorylating the kinase as well as the targets of the kinase (46), and *ugd* encodes

a UDP-glucose dehydrogenase (*Ugd*) (Fig. 1A) (51). The biological function of TkmA,

PtkA, and PtpZ has been investigated in previous studies. It was shown that the protein

targets of the PtkA kinase include *Ugd*, TuaD (involved in teichuronic acid biosynthesis)

as well as single strand DNA-binding proteins SsbA and SsbB (46, 50, 52). Recent

studies also indicated the role of TkmA and PtkA in biofilm formation. However, the

exact function of TkmA and PtkA in biofilm formation remains unclear (45, 53).
In this work, we show that TkmA contributes significantly to biofilm formation in \textit{B. subtilis}, but largely independent of the cognate kinase PtkA. We provide genetic evidence suggesting that TkmA may functionally overlap with EpsA during biofilm formation. Second, we show that the importance of TkmA in biofilm formation is highly medium-dependent; the \textit{tkmA} deletion mutant had a very severe biofilm defect in the biofilm medium LBGM, but the defect was marginal in another commonly used biofilm medium MSgg. We investigated the molecular basis for the medium-dependence effect.

\textbf{Materials and Methods}

\textbf{Strains, media, and growth conditions.} For general purposes, \textit{B. subtilis} strains PY79, 3610, and their derivatives were grown at 37°C in lysogenic broth (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter broth) or on solid LB medium supplemented with 1.5% agar. For assays of biofilm formation, LBGM or MSgg media were used. LBGM is composed of LB with supplementation of 1% glycerol (v/v) and 100 \mu M MnSO₄ (37). The recipe for MSgg is as follows: 5 mM potassium phosphate (pH7.0), 100 mM MOPS (pH 7.0), 2 mM MgCl₂, 700 \mu M CaCl₂, 50 \mu M MnCl₂, 50 \mu M FeCl₃, 1 \mu M ZnCl₂, 2 \mu M thiamine, 0.5% glycerol, 0.5% glutamic acid, 50 \mu g/ml tryptophan, 50 \mu g/ml threonine, and 50 \mu g/ml phenylalanine (54). MSgg was supplemented with 1.5% Bacto-agar for solid plates. \textit{Escherichia coli} DH5α was used as a host for molecular cloning and was grown at 37°C in LB medium. When required, antibiotics were added at the following concentrations for growth of \textit{B. subtilis}: 1 \mu g/ml of erythromycin, 5 \mu g/ml of tetracycline, 5 \mu g/ml of chloramphenicol, 100 \mu g/ml of spectinomycin, and 10 \mu g/ml of kanamycin. For growth of \textit{E. coli}: 100 \mu g/ml of ampicillin, 50 \mu g/ml of kanamycin and 10
μg/ml of tetracycline. A list of strains and plasmids used in this work are summarized in Tables S1 and S2, respectively.

**DNA manipulation.** To extract genomic DNA from overnight cultures of *B. subtilis*, 1.5 ml cells were harvested in a microcentrifuge tube at 13,000 x g for 2 min and resuspended in 450 μl of distilled water. 50 μl of 0.5 M EDTA (pH 8.0) and 60 μl freshly prepared lysozyme (20 mg/ml) were added. The mixture was incubated for 30 min at 37°C, and followed by addition of 650 μl nucleus lysis solution and 250 μl protein precipitation solution (Promega). The mixture was rigorously vortexed for 1 min, followed by centrifugation at 15,000 x g for 5 min. 900 μl soluble fraction was transferred into a new microcentrifuge tube and 600 μl isopropyl alcohol was added. Centrifugation was repeated at 15,000 x g for 5 min. The pellet was washed with 70% ethanol and air-dried for 30 min before being dissolved in 100 μl of nuclease-free water.

General methods for molecular cloning followed the published protocols (55). Restriction enzymes (NEB) were used according to the manufacturer’s instructions. Transformation of plasmid DNA into *B. subtilis* strains were performed as described previously (56). SPP1 phage-mediated transduction was also used to transfer antibiotic-marked DNA fragments among different strains (8, 57). Long-flanking PCR mutagenesis was used to generate insertional deletion mutations (58). The generated PCR constructs were introduced into *B. subtilis* by genetic transformation and integrated to neutral integration sites (e.g. *amyE*) in the genome of *B. subtilis* by double-crossover recombination. Oligonucleotides used in this study are listed in Table S3.
**Strain construction.** To construct the insertional deletion mutant of the \(tkmA-ugd\) operon (FC72) and the insertional deletion mutants for each of the individual genes in the operon [TG34(\(ΔtkmA\)), TG35(\(ΔptkA\)), TG37(\(ΔptpZ\)) and TG38(\(Δugd\))], long-flanking PCR mutagenesis was applied to generate those mutations. Primers used to generate each of the mutations were as follows: delta-\(tkmA\)-P1, delta-\(tkmA\)-P2, delta-\(ugd\)-P3, and delta-\(ugd\)-P4 for \(ΔtkmA-ugd\), delta-\(tkmA\)-P1 to delta-\(tkmA\)-P4 for \(ΔtkmA\), delta-\(ptkA\)-P1 to delta-\(ptkA\)-P4 for \(ΔptkA\), delta-\(ptpZ\)-P1 to delta-\(ptpZ\)-P4 for \(ΔptpZ\), and delta-\(ugd\)-P1 to delta-\(ugd\)-P4 for \(Δugd\). The plasmid pDG1515(Tet\(^R\)) or pAH52(Mls\(^R\)) was used as the template during PCR.

To construct the reconstituted strains YC997, YC999 and TG43 with the point mutation (G>A) in the 5’ untranslated region of \(sinR\) in the native locus, the plasmid pYC192 (20) was used as the template for \(sinR\) mutagenesis. pYC192 contains a ~5-kb flanking sequence of \(sinR\), spanning from the upstream \(yqhG\), \(yqhH\), and \(sinI\) genes to the downstream \(tasA\) gene. A kanamycin resistance marker was also inserted to an intergenic region between \(sinR\) and \(tasA\). The insertion does not alter the biofilm robustness of the cells (20). Mutagenesis primers PsinR2-F and PsinR2-R were used in the PCR reaction to introduce the point mutation (G>A) into the 5’ untranslated region of \(sinR\) in pYC192 (the original sequence is “ggaaggtgatgacattg”, \(g\) for nucleotide substitution is highlighted), following the application of the site-directed mutagenesis kit (Qiagen). The resulting plasmid pYC288 was directly introduced into 3610, FC72 (\(ΔtkmA-ugd\)), and TG34 (\(ΔtkmA\)) by transformation, generating strains YC997, YC999, and TG43, respectively. Successful introduction of the point mutation in the 5’
untranslated region of $\text{sinR}$ at the native chromosomal locus in the above strains was confirmed by PCR amplification of the region and DNA sequencing.

Construction of the reconstituted strains YC998, YC1200, and TG44 with the point mutation (G>C) in the 5' untranslated region of $\text{sinR}$ in the native locus was similar to what was described above except that the mutagenesis primers PsinR3-F and PsinR3-R were used in the PCR reaction to introduce the point mutation (G>C) into the 5' untranslated region of $\text{sinR}$ in pYC192, following the application of the site-directed mutagenesis kit (Qiagen). The resulting plasmid pYC289 was then directly introduced into 3610, FC72 ($\Delta \text{tkmA-ugd}$), and TG34 ($\Delta \text{tkmA}$) by transformation, generating strains YC998, YC1200, and TG44.

To complement the $\Delta \text{tkmA}$ mutation with the wild-type copy of $\text{tkmA}$, the promoter sequence and the open reading frame of $\text{tkmA}$ were amplified by PCR using genomic DNA of $\text{B. subtilis}$ 3610 as the template and primers PtkmA-P1 and tkmA-P4. The PCR products cloned into the integration plasmid pDG1662 (59) by isothermal assembly following a published protocol (60), resulting in the recombinant plasmid pTG05. The recombinant plasmid was then introduced into the $\text{B. subtilis}$ laboratory strain PY79 by genetic transformation for the double-crossover recombination of the DNA sequences at the $\text{amyE}$ locus. The $\text{amyE}$ integration fragment was then introduced into 3610 by SPP1 phage-mediated transduction, generating TG47. To complement the $\Delta \text{tkmA}$ mutation with the wild type copy of $\text{epsA}$, the promoter sequence of $\text{tkmA}$ and the coding sequence of $\text{epsA}$ were amplified by PCR using the genomic DNA of $\text{B. subtilis}$ 3610 as the template and primers PtkmA-P1 and PtkmA-P2, and primers epsA-P3 and epsA-P4, respectively. The PCR products were cloned into the integration plasmid pDG1662 by
isothermal assembly (60), resulting in the recombinant plasmid pTG06. The resulting plasmid was similarly introduced into 3610 as described above, generating TG48.

To compare expression of the sinR gene in the wild type and the two reconstituted strains containing the nucleotide substitution of either “G>A” or “G>C” in the untranslated region of sinR, the regulatory sequence and the coding sequence for the first seven amino acid residues of sinR (followed by a stop codon, see sequence of the primer PsinR-R5) were amplified by PCR using primers PsinR-F5 and PsinR-R5, and genomic DNAs of B. subtilis strains 3610, YC997 and YC998, respectively, as the templates. The PCR products were digested with EcoRI and BamHI, and cloned into the plasmid pDG268 (61), which carries a chloramphenicol-resistance marker and a polylinker upstream of the lacZ gene between two arms of the amyE gene, to make a P_{sinR}-lacZ transcriptional fusion, resulting in the recombinant plasmids pTG01, pTG02 and pTG03, respectively. In this reporter plasmid, translation of lacZ is independent of the translation of the first seven amino acid residues of sinR.

To overexpress epsAB in B. subtilis, the epsAB genes were amplified by PCR using genomic DNA of B. subtilis 3610 as the template and primers epsAB-P1 and epsAB-P2. The PCR product was then cloned into the HindIII and BamHI sites of pDR150, which contains a xylose-inducible xylA promoter (a gift of D Rudner, HMS), generating the recombinant plasmid pTG04. The recombinant plasmid was then introduced into the B. subtilis laboratory strain PY79 by genetic transformation for a double-crossover recombination of the DNA sequences at the amyE locus. The amyE integration fragment was then introduced into strains TG34 and FC72 by SPP1 phage-mediated transduction.
To construct the reporter fusion for the poly-glutamate biosynthesis gene cluster (\textit{ywsCAB-ywtC}), the promoter sequence of the operon was amplified by PCR using primers PywsC-F1 and PywsC-R1. The PCR product was digested with \textit{Hind}III and \textit{Bam}HI, and cloned into the vector plasmid pDG268, which was also linearized by \textit{Hind}III and \textit{Bam}HI. The recombinant plasmid was first introduced into PY79 and then into 3610 in a similar fashion as described above.

\textbf{Characterization of the suppressor mutation.} To characterize the suppressor mutation in the strain YC820, the genomic DNA of YC820 was prepared according to the protocol described above. The genomic DNA was used as the template and primers PsinR-F1 and sinR-R1 were used to amplify both the regulatory and the coding sequences of the \textit{sinR} gene. The PCR product was then applied for DNA sequencing (Genewiz). The point mutation of G to A in the 5’ untranslated region of \textit{sinR} was confirmed by DNA sequencing.

\textbf{Assays of β-galactosidase activities.} For assays of the β-Galactosidase activities, cells were incubated in MSgg or LBGM at 37 °C in a water bath with shaking. One milliliter of culture was collected at each indicated time point after inoculation. Cells were spun down and pellets were resuspended in 1 ml Z buffer (40 mM NaH$_2$PO$_4$, 60 mM Na$_2$HPO$_4$, 1 mM MgSO$_4$, 10 mM KCl and 38 mM β-mercaptoethanol) supplemented with 200 μl/ml freshly made lysozyme. Resuspensions were incubated at 30 °C for 15 min. Reactions were started by adding 200 μl of 4 mg/ml ONPG (2-nitrophenyl β-D-galactopyranoside) and stopped by adding 500 μl of 1 M Na$_2$CO$_3$. Samples were briefly
spun down. OD\textsubscript{420} values of the samples were recorded using a Pharmacia ultraspectrometer 2000. The β-galactosidase-specific activity was calculated according to the equation (OD\textsubscript{420}/time × OD\textsubscript{600}) × dilution factor × 1000. Assays were conducted at least in duplicate.

Assays of pellicle and colony biofilms. \textit{B. subtilis} cells were grown in LB broth at 37 °C to mid-log phase. For colony formation, 2 μl of the cells was spotted onto LBGM medium (or MSgg medium) solidified with 1.5% agar. Plates were incubated at 30 °C for 72 hours prior to analysis. For pellicle formation, 6 μl of the cells mixed with 6 ml of LBGM broth (or MSgg broth) in 6-well plates (VWR). Plates were incubated at 30 °C for about 48 hours. Images were taken using either a SONY NEX-5 digital camera or a SPOT camera (Diagnostic Instruments).

Results

The deletion mutant for the BY-kinase activator TkmA shows a very severe biofilm defect in LBGM.

The exact role of the BY-kinase, PtkA, in biofilm formation in \textit{B. subtilis} remains elusive for the following reasons. First, the alteration in the biofilm phenotype caused by \textit{ΔptkA} is somewhat mild, which was shown in previous studies (45, 53) and in this study as well (Figs. 2 and S1). Second, none of the previously identified protein targets of PtkA seems to play a significant role in biofilm formation, including Ugd (UDP-glucose dehydrogenase), whose gene is located in the same operon with \textit{ptkA} (Figs. 1A and 2) (53). In most of the previous work trying to characterize the function of TkmA and PtkA
in biofilm formation, the commonly used biofilm-inducing medium MSgg was applied in the biofilm assays (54). MSgg is derived from a defined minimal medium originally applied for biofilm studies in Pseudomonas aeruginosa with supplementation of 0.5% glycerol and 0.5% glutamic acid (54). We recently formulated a new biofilm-inducing medium, LBGM (LB supplemented with 1% glycerol and 100 μM MnSO₄), and showed that it promotes strong biofilm formation in B. subtilis (37). We decided to perform biofilm assays by the mutants deleted either for the entire tkmA-ugd operon or for each of the individual genes in the operon in this new biofilm medium.

Our results show that the mutant deleted for the entire operon (ΔtkmA-ugd) has a very severe defect in the formation of both colony and pellicle biofilms in LBGM (Fig. 2). Interestingly, the deletion mutant for the single tkmA gene (ΔtkmA) also has a severe biofilm defect, nearly identical to that of the deletion mutant of the operon (Fig. 2). On the contrary, the other three individual deletion mutants (ΔptkA, ΔptpZ, and Δugd) formed biofilms that may be best described as mildly altered for the surface architecture (Fig. 2). We next complemented ΔtkmA using the wild type tkmA gene integrated at the ectopic amyE locus. The complementation strain (ΔtkmA, amyE::tkmA) formed robust biofilms that were identical to those of the wild type cells (Fig. 2). Our results suggest that in the newly formulated biofilm medium LBGM, the kinase activator TkMA plays a major role in biofilm formation, and its role is largely independent of the PtkA kinase or any other protein encoded by genes in the operon.
We hope to point out that in previous studies, it was also shown that even in MSgg, the biofilm phenotype of the ΔtkmA mutant was somewhat more severe than that of the ΔptkA mutant (45, 53). The authors speculated that TkmA might have a PtkA-independent activity in contributing to biofilm formation (45, 53). Our current results not only supported this idea, but suggested further that this PtkA-independent activity of TkmA may be the chief reason for its importance in biofilm formation. In addition, the importance of TkmA in biofilm formation is medium condition-dependent since the biofilm defect caused by ΔtkmA was very severe in LBGM (Fig. 2), but marginal in MSgg (Fig. S1).

A suppressor mutant of ΔtkmA-ugd forms robust biofilms.

Next, we decided to further characterize the putative PtkA-independent activity of TkmA in biofilm formation. We took an unbiased genetic approach by searching for suppressor mutants of ΔtkmA-ugd that are capable of forming robust biofilms again in LBGM. When the ΔtkmA-ugd mutant was streaked out on LB agar plate for routine cell proliferation, the colonies demonstrated little surface morphology (upper panel in Fig. S2A). We obtained a spontaneous suppressor mutant, which showed quite distinct and robust colony morphology on the same plate (upper and lower panels in Fig. S2A). Upon further characterization, we found that the mutant formed robust colony and pellicle biofilms in LBGM, very different from the parent strain (Fig. 3B). This indicates that the putative suppressor mutation fully rescued the biofilm defect caused by ΔtkmA-ugd. This suppressor mutant also showed extensive cell chains in shaking culture, again, very different from the parent strain (Fig. S2B).
Based on observations in our previous studies (62, 63), in which spontaneous mutants bearing point mutations in either the sinR or abrB gene were shown to form both robust biofilms and extensive cell chains, we decided to map the suppressor mutation by trying the targeted approach first and sequencing those biofilm-related regulatory genes in the suppressor mutant. Interestingly, our sequencing results indeed revealed a single nucleotide substitution in a region immediately upstream of sinR. The mutation (G>A, Fig. 3A) seems to be located in the 5’ untranslated region of sinR between the transcription start (+1, indicated by the horizontal arrow) and the translation start (TTG), next to the Shine-Dalgarno-like sequence (5’-GGAAGG-3’) for ribosome binding.

To confirm that the single nucleotide change in the 5’ untranslated region of sinR is solely responsible for the suppressor phenotype, we reconstituted the point mutation on the chromosome of the ΔtkmA-udg mutant. To do so, we introduced two different single nucleotide changes (G to A as was in the original suppressor mutant, as well as G to C) to the 5’ untranslated region in the native sinR locus, following a previously published protocol (63) (see Materials and Methods). We then tested the biofilm phenotype of the two reconstituted strains. Both strains formed robust colony and pellicle biofilms in LBGM, identical to that of the original suppressor mutant (Fig. 3B). This result confirms that the single nucleotide change in the 5’ untranslated region of sinR is solely responsible for the suppression of the biofilm defect caused by ΔtkmA-udg. The reconstituted mutations also completely rescued the biofilm defect by the single ΔtkmA deletion mutation (data not shown).
The suppressor mutation lowers sinR expression.

Since the suppressor mutation is located in the 5'-untranslated region of sinR, we suspected that the mutation might alter sinR expression at the post-transcriptional level by affecting either mRNA stability or translation initiation of sinR. To test the first possibility, we created a transcriptional reporter by fusing the regulatory region and the coding sequence for the first seven amino acids of sinR (followed by addition of a stop codon) to the lacZ gene. Since this fusion contains a separate ribosome binding site and the translation start for the lacZ gene, it is purposed to report the mRNA abundance of the lacZ transcript starting from the untranslated region of sinR. In parallel, we also introduced the same point mutations (either G to A, or G to C) into the 5' untranslated region of sinR in the reporter fusion (see Materials and Methods). B. subtilis derivatives bearing either the wild type reporter or the reporter fusion with the point mutation were assayed for β-galactosidase activities. The results (Fig. 3C) showed that the two reporter strains harboring the point mutation (TG41 and TG42, unfilled squares and triangles, respectively) had lower activities than the wild type reporter strain (TG40, unfilled diamonds), an indication that the suppressor mutation negatively impacted sinR expression, possibly by affecting mRNA stability of sinR. In theory, it is also possible that the mutation impacts both mRNA stability and translation initiation of sinR (e.g. by also influencing ribosome binding). We chose not to test the latter possibility since the putative change in mRNA stability is sufficient to explain what we have observed. How the point mutations in the 5' untranslated region of sinR may affect sinR mRNA stability is still unclear to us. A previous study showed that mRNA stability of sinR was regulated by specific RNA nucleases, whose activities significantly affect biofilm formation in B.
subtilis (64). Thus, we speculated that sinR could be one of the prime targets for mRNA stability control in B. subtilis.

epsA complementation rescued the biofilm defect caused by ΔtkmA.

Our results suggest that lowered expression of sinR may be the cause for the suppressor phenotype. This may in turn be due to overexpression of some of the SinR-repressed genes since SinR acts as a biofilm master repressor. The SinR regulon has been well characterized, including the tapA-sipW-tasA operon for production and deployment of TasA protein fibers, the epsA-O operon for biosynthesis of EPS, and the lutABC operon for lactate utilization (8, 15, 65). We decided to focus on the epsA-O operon partly because epsAB, the first two genes in the operon, are homologous to tkmA-ptkA. To test whether epsA-O is overexpressed in the suppressor mutant, we introduced a transcriptional reporter for the epsA-O operon (P_{epsA}-lacZ) into the strains with either the wild type sinR or the sinR mutant alleles at the native locus that we constructed previously, resulting in strains YC952 (WT), YC1201 (G>A), and YC1202 (G>C) respectively. We compared β-galactosidase activities of the above three strains.

To avoid cell clumping during shaking growth due to hyper activities of SinR in some of the above strains (8), an ΔepsH mutation was introduced into the reporter strains. Our results showed that the two strains with the point mutation in the 5' untranslated region of sinR (YC1201 and YC1202, unfilled squares and triangles) had much higher activities than that of the wild type strain (YC952, unfilled diamonds) (Fig. 3D). The reason why a mild decrease in the expression of sinR may lead to such a drastic increase in the

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expression of epsA-O is due to hypersensitivity of SinR-mediated repression, a unique feature of SinR activity characterized in our previous studies (20, 63).

We speculated that overexpression of epsA-O (or more specifically epsAB) in the suppressor mutant may compensate for the loss of tkmA. To test our hypothesis, we created an inducible epsAB construct by fusing epsAB to a xylose inducible promoter (P_xylA-epsAB, see Materials and Methods) and integrated the fusion to the chromosomal amyE locus in the ΔtkmA-ugd and the ΔtkmA mutants. We then tested biofilm formation by those engineered strains in LBGM in the absence or presence of xylose. Interestingly, in the presence of xylose, the biofilm defect in both the ΔtkmA-ugd and the ΔtkmA mutants was largely rescued (Fig. 4), whereas in the absence of xylose, no suppression of the biofilm defect was seen. We conclude that loss of tkmA (or the entire tkmA-ugd operon) can be fully compensated by overexpression of epsAB.

To further narrow down to either epsA or epsB in the role of compensation, we repeated the complementation experiment for the ΔtkmA mutant. This time, we placed the epsA gene under the control of the tkmA promoter (P_tkmA-epsA) and integrated the engineered construct to the amyE locus of the ΔtkmA mutant. We similarly tested biofilm formation by this strain in LBGM. As shown in Fig. 2, this complementation strain formed fairly robust colony and pellicle biofilms, indicating that epsA itself (at the comparable expression level) is able to compensate for the loss of tkmA. It also implies that the PtkA-independent activity of TkmA in biofilm formation may have to do with the
ability of TkmA to complement EpsA activities. If so, it is conceivable that TkmA may
crosstalk with EpsB, the cognate BY-kinase of EpsA, to regulate EPS biosynthesis.

Expression of *tkmA* is higher in LBGM than in MSgg while the opposite is true for
*epsA*.

Based on our genetic evidence, we propose that the PtkA-independent activity of
TkmA when contributing to biofilm formation is likely due to its ability to complement
EpsA activities. But it is still unclear to us why the importance of *tkmA* in biofilm
formation differs significantly in the two different biofilm media (LBGM vs MSgg). One
possible scenario we can think of is that the *tkmA* gene is differentially expressed in the
two different media, being higher in LBGM but lower in MSgg. Higher expression of
*tkmA* presumably leads to proportionally more significant contributions of TkmA to EPS
biosynthesis and biofilm formation in LBGM than in MSgg. To test our hypothesis, we
constructed a transcriptional reporter fusion for *tkmA* by amplifying and fusing the
promoter sequence of *tkmA* (from about 250-bp upstream to the start of *tkmA*, Fig. 1) to
*lacZ*. We then integrated this reporter (*P_{tkmA}-lacZ*) to the chromosomal *amyE*
locus of the *B. subtilis* 3610 and conducted a bioassay comparing the expression of *tkmA* in
LBGM and MSgg by using the engineered *B. subtilis* reporter strain (TG49). Our results
show that the expression of *tkmA* increased over time in both media (Fig. 5A).
Interestingly, the increase was clearly stronger in LBGM (Fig. 5A, unfilled squares) than
in MSgg (Fig. 5A, filled diamonds). This result indicates that *tkmA* is expressed higher in
LBGM than in MSgg.
If TkmA were to compete with EpsA for cross-talking with EpsB, then the expression of epsA in LBGM and MSgg may also matter. We applied a similar approach to test whether there is a differential expression of epsA in the two different media. The *B. subtilis* strain bearing the $P_{\text{epsA-lacZ}}$ transcriptional fusion [YC110, (66)] was cultured in LBGM and MSgg, and assayed for β-galactosidase activities. Interestingly, and opposite to what was seen for the $P_{\text{tkmA-lacZ}}$ reporter, cells harboring the $P_{\text{epsA-lacZ}}$ reporter showed much higher activities in MSgg (Fig. 5B, filled diamonds) than in LBGM (Fig. 5B, unfilled squares). Taken together, we conclude that tkmA is expressed at a higher level in LBGM than in MSgg, whereas for epsA, it is expressed at a lower level in LBGM than in MSgg. An inference from above is that in LBGM, TkmA may compete more effectively with EpsA for interaction with EpsB and thereby contributes more to the regulation of EPS biosynthesis and biofilm formation than in MSgg. If true, this may explain why the importance of TkmA in biofilm formation differs significantly between LBGM and MSgg.

**tkmA and epsA are regulated by Spo0A and DegU in a complex fashion.**

To further understand what caused the differential expression of tkmA and epsA in LBGM and MSgg, we decided to revisit the transcriptional regulation of tkmA and epsA in the two different biofilm media. Transcriptional regulation of the tkmA-ugd and the epsA-O operons has been investigated in previous studies (25, 41, 53). Results from microarray analysis suggest that the tkmA-ugd operon belongs to the Spo0A regulon (25). It was also shown that Spo0A~P bound to the regulatory sequence of the tkmA-ugd operon in gel mobility shift assays, indicating direct regulation of the operon...
by Spo0A (25). However, that result may need further examination as we elaborate
next. Our analysis of the regulatory region of *tkmA* (the intergenic sequences between
*tkmA* and *ywqB*) revealed two putative Spo0A–P binding sites that perfectly match the
consensus sequence of the so-called OA box (“TGTCGAA”, Fig. 1B). The first binding
site is located upstream of a σ^A^-dependent promoter that drives expression of a putative
small open reading frame with unknown function (highlighted in yellow in Fig. 1A-B)
(67). The second putative binding site of Spo0A–P is located upstream of a σ^A-
dependent promoter that possibly drives the transcription of the *tkmA-ugd* operon (Fig.
1A-B). To test whether Spo0A activates transcription of the *tkmA-ugd* operon from the
second putative OA box, we introduced the previously constructed P_{tkmA-lacZ} reporter
fusion (note that this reporter fusion only contains the intergenic region between *tkmA*
and the small ORF) into the wild type strain and the *spo0A* mutant, and compared β-
galactosidase activities of the two strains. As shown in Fig. 6A, in the *spo0A* mutant, the
P_{tkmA-lacZ} fusion was expressed at a much lower level, confirming that Spo0A positively
regulates the *tkmA-ugd* operon likely by binding to the second OA box. DegU was also
shown to positively regulate the *tkmA-ugd* operon in previous studies (41, 53). We also
confirmed that the activity of the same P_{tkmA-lacZ} reporter fusion decreased to very low
levels in the *degSU* mutant compared to that in the wild type cells (Fig. 6A). Thus, the
*tkmA* gene is under the strong positive regulation by both DegU and Spo0A.

Further sequence analysis of the operon revealed that between the *ptkA* and
*ptpZ* genes there lies a 52-base pair intergenic region (Figs. 1A and 1C). A stem loop-
like structure can be predicted based on DNA sequences in this intergenic region, which
could function as a putative transcriptional attenuator (Figs. 1C). If true, we wondered that there might be a second promoter in the intergenic region driving the expression of just the downstream ptpZ and ugd genes. To test that, we constructed a transcriptional reporter by fusing this 52-bp intergenic region with lacZ, creating P_{ptpZ-lacZ}, and introduced this reporter to the wild type strain, the Δspo0A, and the ΔdegSU mutants for integration at the amyE locus. Wild type cells harboring this reporter were assayed for β-galactosidase activities and showed very little activities in shaking culture, indicating that the this putative internal promoter (if exists) is not being actively transcribed under normal conditions (Fig. 6B). The same was seen in the spo0A mutant. Surprisingly, the reporter showed drastically increased activities when assayed in the degSU mutant (Fig. 6B), suggesting that DegU strongly represses the phosphatase gene (ptpZ) from the putative internal promoter while simultaneously positively regulating the genes for the kinase and the kinase activator (ptkA and tkmA) from the upstream promoter. So why do cells have such complex regulations on this operon by DegU? We presumed that in the DegU−P^{HIGH} cells, not only the kinase PtkA is highly expressed, but simultaneously, cells also make sure that the production of the counteracting phosphatase PtpZ is low due to repression by DegU−P as well as possible transcriptional attenuation immediately after ptkA. Together, this would allow strong activation of the kinase by auto-phosphorylation due to limited counteracting phosphatase activities as well as strong activation of the target proteins.

For the epsA-O operon, previous studies showed that it is regulated by both Spo0A and DegU (8, 40). Spo0A positively and indirectly regulates the operon through
its regulation on the sinI and abrB genes (17, 18, 22). A recent study showed that DegU negatively regulates the epsA-O operon (40). This was somewhat unexpected given that DegU is a positive regulator for biofilm formation in B. subtilis. By application of the P_{epsA-lacZ} reporter, we confirmed the positive regulation of epsA-O by Spo0A and strong negative regulation of the operon by DegU (Fig. 6C) (40). In summary, the epsA-O and the tkmA-ugd operons are under the complex regulations by both Spo0A and DegU. We provided a simple scheme to summarize the concerted regulations of the two operons (Fig. 6D). We predict that rising Spo0A activities will increase the production of both TkmA and EpsA. In contrast, rising DegU activities may oppositely affect levels of the two activator proteins, strongly increasing TkmA expression while holding back the production of EpsA. If so, it is conceivable that environmental conditions favoring high DegU activities will render the role of TkmA more important in biofilm formation.

Varied activities of Spo0A and DegU in LBGM and MSgg are responsible for the differential expression of tkmA and epsA.

Spo0A and DegU are two well-studied examples of the regulatory proteins whose activities are highly dependent on environmental conditions, and both of them regulate the epsA-O and the tkmA-ugd operons. Thus, it is possible that differential expression of tkmA and epsA is a consequence of varied activities of Spo0A and DegU under the two different medium conditions. In this section, we focus on the possible role of DegU in the differential expression of epsA and tkmA, whereas the corresponding role of Spo0A will be discussed in the next section.
For DegU, we hypothesize that the activities of DegU may be higher in LBGM than in MSgg, which in turn causes higher expression of tkmA but lower expression of epsA in LBGM than MSgg (Figs. 5A-B). To test our hypothesis, we constructed two transcriptional reporter fusions, $P_{ywsc}$-lacZ and $P_{flaB}$-lacZ. The first reporter, $P_{ywsc}$-lacZ, allows us to measure the expression of the $ywscAB-ywtC$ operon, whose gene products are involved in production of γ-poly-DL-glutamic acid (68). The $P_{flaB}$-lacZ transcriptional fusion reports the activity of the $fla/che$ operon, whose gene products are involved in motility and chemotaxis (41). Both operons are known to be positively regulated by DegU (41, 69). β-Galactosidase activities of the cells harboring the reporter grown in LBGM and MSgg were assayed. Our results showed that the expression of both reporters was higher in LBGM (unfilled squares) than in MSgg (filled diamonds), indicating that the activity of DegU varies in the two different media and is likely higher in LBGM than in MSgg (Figs. 5C-D). As a reminder, higher DegU activities may oppositely affect the levels of the two activator proteins, strongly increasing TkmA expression while repressing the production of EpsA.

For the role of Spo0A in the differential expression of tkmA and epsA in the two different media, we similarly hypothesized that Spo0A activities might be higher in LBGM than in MSgg. We took a similar genetic approach to compare the activities of Spo0A in LBGM and MSgg by applying a transcriptional reporter, $P_{abrB}$-lacZ. The $abrB$ gene is known to be directly and negatively regulated by Spo0A. Thus, expression of $abrB$ inversely correlates with Spo0A activities (28). Cells bearing the $P_{abrB}$-lacZ reporter were grown in LBGM and MSgg, and assayed for β-galactosidase activities. Our results...
show that the reporter strain had consistently lower activities when grown in LBGM (squares) than in MSgg (diamonds), indicating that Spo0A activities also differ in LBGM and MSgg, and were higher in LBGM than in MSgg (Fig. 5E).

Similar to what DegU does, higher Spo0A activities likely also contribute to higher tkmA expression in LBGM than in MSgg. Since Spo0A is also a positive regulator for the epsA-O operon (Fig. 6C), one may intuitively predict that higher activities of Spo0A would lead to higher expression of epsA-O in LBGM than in MSgg. Spo0A indirectly regulates epsA-O in part through its regulation on sinl (20). SinI is a small anti-repressor, which antagonizes the master biofilm repressor SinR, leading to derepression of SinR-controlled genes (8, 19). Surprisingly, when we compared the β-galactosidase activities from the cells harboring the P_{sinI-lacZ} reporter in LBGM and MSgg, the activities of the reporter strain were mildly lower in LBGM (unfilled squares) than in MSgg (filled diamonds, Fig. 5F). This indicates that, for sinl (and likely SinR-repressed epsA-O), higher activities of Spo0A alone do not result in higher expression of those genes in LBGM than in MSgg. A possible answer to the above result may lie in the regulatory region of sinl, which contains a unique arrangement of both the activator and multiple operator sequences of Spo0A (20). Our previous study showed that optimal expression of sinl depends on intermediate levels of Spo0A, which preferentially binds to the high-affinity activator site, but not the low-affinity operator sites. Higher levels of Spo0A instead curtail sinl activation by binding to both the activator and operator sites (20).
In toto, we propose that differential expression of *tkmA* and *epsA* in the two different biofilm media may be the reason why the importance of TkmA in biofilm formation is highly medium-dependent, and this is contributed by both Spo0A and DegU, both of which showed higher activities in LBGM than in MSgg.

**Discussion**

BY-kinases play important roles in biosynthesis of secreted polysaccharides in bacteria. In *B. subtilis*, the BY-kinase and the kinase activator, EpsB and EpsA, are involved in the regulation of EPS biosynthesis and biofilm formation (45, 47, 50). Previous studies also suggested that the second BY-kinase PtkA augments the role of EpsB in biofilm formation (45, 53). However, the exact function of PtkA and TkmA in biofilm formation remains unclear. In this study, thanks to the application of the new biofilm-inducing medium LBGM, we show that the mutant for the kinase activator, TkmA, has a severe biofilm defect in LBGM, and that the importance of TkmA in biofilm formation is largely independent of the cognate BY-kinase, PtkA. Based on our genetic evidence, we postulated that the role of TkmA in biofilm formation might have more to do with shared regulation with EpsA on EpsB. Interestingly, while we were working on this project, a recent study investigating cross-regulation among different types of kinases in *B. subtilis* provided molecular evidence to support our hypothesis (70). By using yeast and bacterial two hybrid assays, strong interactions between TkmA and EpsB, between TkmA and PtkA, but not between EpsA and PtkA, were observed in that study (70). In terms of the biological significance for such interactions (crosstalk between the non-cognate TkmA and EpsB), one simple projection is that EPS
biosynthesis and biofilm formation may be regulated at the post-translational level in response to a broader range of environmental stimuli. In addition, recent studies also showed broad cross-phosphorylation among BY-kinases and other types of kinases (e.g. Ser/Thr kinases) as well as overlapping activities among different pairs of BY-kinases and the kinase activators. All these studies imply that complex regulations and biological significance of those BY-kinases may have been underestimated (70). Here we presented a schematic model to summarize our hypothesis (Fig. S3). In that model, EpsA is indicated to sense self-produced exopolysaccharide molecules in a quorum-sensing-like mechanism (47) while TkmA is speculated to sense unknown environmental signals. It will be interesting to know what signals TkmA senses and how these signals may be physiologically relevant to biofilm formation in future studies.

Another interesting observation in this study is that the importance of TkmA in biofilm formation is highly medium-dependent. Our evidence suggests that the medium-dependence effect may be due to differential expression of tkmA and epsA in LBGM and MSgg. We further propose that differential expression of tkmA and epsA in the two different media is a consequence of complex regulations of those genes by Spo0A and DegU, both of which showed higher activities in LBGM than in MSgg. A model to explain the molecular basis for the medium condition dependence for the importance of TkmA in biofilm formation is described in Fig. 7. Our study illustrated an interesting example of how medium (or environmental) conditions can significantly alter the activity of the BY-kinase activators in the regulation of the bacterial multicellular development process. What exactly differs between the two different biofilm media, which subsequently results
in varied activities of both Spo0A and DegU, is not known. It is also somewhat difficult to
directly compare these two media [one being a rich medium (LBGM) without defined
chemical ingredients and the other being a defined minimal medium (MSgg)]. However,
it is worth noting that manganese was shown to be critical for Spo0A activities (71).
Therefore, higher amounts of manganese in LBGM (100 μM) than in MSgg (50 μM) may
be partially responsible for the difference in Spo0A activities in the two different media.
In fact, when manganese was provided at 50 μM, the biofilm-inducing activity of the
modified LBGM was notably weaker (data not shown). Lastly, we cannot rule out the
possibility that LBGM contains an unknown signal at an abundant level for optimal
activation of TkmA whereas the putative signal is largely absent or much less abundant
in MSgg. The idea of an unknown signal for activation of TkmA is based on the
observation that the kinase activator contains an extracellular domain for potential
signal sensing and the presence of such a putative signal is thought to be important for
the activity of the kinase activator in interacting with, and activating the kinase.

Finally, our study also indicates that DegU is not just simply a positive regulator
for the tkmA-ugd operon, rather, DegU acts as a switch for the kinase/phosphatase
(PtkA/PtpZ) activities. We showed that in parallel to the positive regulation on ptkA from
the upstream promoter, DegU also seems to negatively regulate the expression of ptpZ
and ugd from a putative internal promoter located between the ptkA and ptpZ genes
(Figs. 1A and 1C). In addition, a putative transcriptional attenuator immediately
downstream of ptkA may further reduce the expression of the phosphatase gene (Fig.
1C). Therefore, when DegU activities are high, DegU preferentially promotes expression
of ptkA for the kinase activity while simultaneously represses ptpZ for the phosphatase activity. When DegU activities are low, it derepresses expression of the phosphatase gene from the putative internal promoter. We hope to point out that such an intergenic region between the gene for the kinase and the gene for the phosphatase, and the transcription attenuator-like stem-loop structure within the intergenic region seem to be fairly conserved in homologous gene clusters in other Bacillus species (Fig. S4).

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Figure legends

Fig. 1. Analyses of the regulatory sequences in the *tkmA-ugd* operon. (A). Genetic arrangement of the *tkmA-ugd* operon. Promoters and predicted transcription terminators in the intergenic regions between *ywqB* and *tkmA*, and between *ptkA* and *ptpZ* are indicated by arrows and stem loops respectively. A previously characterized small open reading frame (ORF) with unknown function (67) in the intergenic region of *ywqB* and *tkmA* is shown and highlighted in yellow. The protein products encoded by individual genes in the *tkmA-ugd* operon are noted underneath. BY, bacterial tyrosine; UDP, uridine diphosphate. (B). Sequence analysis of the intergenic region between *ywqB* and *tkmA*. The -35 and -10 motifs (underlined and highlighted in green) and the transcription start (+1) of the two σ^A^-dependent promoters are annotated, one for the expression of the small ORF highlighted in yellow and the other for the *tkmA-ugd* operon. The predicted transcription terminator for the small ORF is underlined and highlighted in purple. Two putative Spo0A~P binding sites that perfectly match the consensus sequence (OA box, ‘TGTCGAA’) are highlighted in orange. The 250-bp DNA sequence used for generating the P_{tkmA}-lacZ reporter fusion was outlined. (C). Sequence analysis of the intergenic region between *ptkA* and *ptpZ*. The DNA sequence immediately downstream of *ptkA* is highlighted in purple and folded into a stem-loop structure with indicated free energy (ΔG=-8.87 kcal/mol). Stop codon of *ptkA* is highlighted in blue while start codon of *ptpZ* is highlighted in red.

Fig. 2. Colony and pellicle biofilms formed by mutants with various deletions in the *tkmA-ugd* operon and complementation strains in LBGM. The strains tested in
this assay include WT (3610), various mutants deleted either for the entire tkmA-ugd operon (ΔtkmA-ugd, FC72) or each of the individual genes in the operon (ΔtkmA/TG34, ΔptkA/TG35, ΔptpZ/TG37, and Δugd/TG38), and two complementation strains of ΔtkmA, one by tkmA (TG47) and the other by epsA (TG48). Cells were inoculated in LBGM in 6-well polyvinyl plates (VWR) or on solidified LBGM agar plates and incubated at 30°C for about 48 hours (for pellicles) or 72 hours (for colony biofilms). Scale bars in all panels for colony biofilms is estimated to be 0.3 cm in length; scale bars in all panels for pellicle biofilms represent 1 cm in length.

Fig. 3. Characterization and reconstitution of the suppressor mutation. (A). The suppressor mutation in YC820 was characterized as a single nucleotide substitution (from G to A) in the 5’ untranslated region of sinR. The -35 and -10 motifs and the transcription start (+1) of the promoter of sinR, and the single nucleotide change (from G to A) are highlighted. The Shine-Delgarno sequence is shown in blue and the start codon (TTG) of sinR is shown in red. (B). Colony and pellicle biofilm phenotypes in LBGM of the wild type (3610), the tkmA-ugd deletion mutant (FC72), the suppressor mutant of ΔtkmA-ugd (YC820), and two reconstituted strains that contain the single nucleotide substitution of either G to A (YC997) or G to C (YC998) in the regulatory region of sinR. White bars in all panels for colony biofilms is estimated to be 0.3 cm in length; white bars in all panels for pellicle biofilms represent 1 cm in length. (C). Assays for the β-galactosidase activity were performed to compare the activities of the strains harboring the transcriptional fusion of either the wild type sinR gene (TG40, diamond) or the sinR alleles with the substitution from either G to A (TG41, square) or G to C (TG42,
triangle) in the regulatory region of sinR to the lacZ gene. The reporter fusion was integrated at the chromosomal amyE locus of a ΔepsH mutant (RL4548). (D). Assays of the β-galactosidase activity were performed to compare the activities of the strains that harbor the reporter fusion for the epsA-O operon (P_{epsA-lacZ}) integrated at the amyE locus and contain either the wild type sinR gene at the native locus (YC952, diamond) or the sinR mutant alleles with either the change of G to A (YC1201, square) or G to C (YC1202, triangle) in the 5’ untranslated region of sinR. Results are representative from three independent assays.

**Fig. 4.** Overexpression of epsAB rescues the biofilm defect caused by ΔtkmA. The epsAB genes were placed under the control of the xylose inducible promoter (P_{xylA-epsAB}) and the fusion was introduced into the ΔtkmA-ugd mutant and the ΔtkmA mutant, resulting in TG45 and TG48, respectively. In the presence of xylose (0.1%, v/v), both strains were able to form robust colony (A) and pellicle (B) biofilms in LBGM, comparable to that of the wild type cells, whereas in the absence of xylose, no strong biofilm was observed in the engineered strains. White bars in all panels for colony biofilms is estimated to be 0.3 cm in length; white bars in all panels for pellicle biofilms represent 1 cm in length.

**Fig. 5.** Genetic evidence for differential regulations of tkmA and epsA in LBGM and MSgg. Assays of the β-galactosidase activities of the wild type strain harboring one of the following fusions: P_{tkmA-lacZ} (A, TG49), P_{epsA-lacZ} (B, YC110), P_{ywsC-lacZ} (C, YC1275), or P_{flaB-lacZ} (D, YC605), P_{abrB-lacZ} (E, FC287), and P_{sinI-lacZ} (F, YC127).
Cells were grown in either LBGM (unfilled squares) or MSgg (filled diamonds) in shaking culture. Cell samples were periodically collected and assayed for β-galactosidase activity. Error bars present standard deviations from triplicate assays.

**Fig. 6. Genetic regulation of the tkmA-ugd operon.** (A-C) Assays of the β-galactosidase activities of the wild type strain, the spo0A mutant, and the degSU mutant that contain the transcriptional lacZ reporter fusion with the regulatory region of either tkmA (A, P_{tkmA}-lacZ), or ptpZ (B, P_{ptpZ}-lacZ), or epsA (C, P_{epsA}-lacZ). All fusions were integrated at the chromosomal amyE locus of the cells. Cells were grown in LBGM with shaking to O.D_{600}=1.0 before harvest for the assays. Error bars represent standard deviations from triplicate assays. (D). A schematic summary of the regulations on the tkmA-ugd and the epsA-O operons by Spo0A and DegU. Spo0A positively regulates both the tkmA-ugd and the epsA-O operons whereas DegU has opposite regulations on the two operons, acting positively on tkmA-ugd but negatively on epsA-O. DegU also negatively regulates the expression of ptpZ (and possibly ugd) from a putative internal promoter just upstream of ptpZ by an unknown mechanism. (+) represents positive regulation and (-) for negative regulation.

**Fig. 7. A proposed model for the molecular basis of the medium-dependence effect in biofilm formation.** Based on genetic evidence presented in this study, two different scenarios (A and B) corresponding to the two different medium conditions (LBGM and MSgg) are described here. For Spo0A, we propose that the levels of Spo0A are higher in LBGM (++++) than in MSgg (++). Consequently, Spo0A directly activates
the *tkmA-ugd* operon at higher levels in LBGM (++++) than in MSgg (+). In contrast, higher levels of Spo0A do not necessarily lead to higher expression of *sinI* due to the presence of both activator and operator sites of Spo0A in the regulatory region of *sinI* (20). Thus, Spo0A alone is not expected to cause differential expression of *epsA-O* in LBGM and MSgg. For DegU, we also predict higher activities of DegU in LBGM (++++) than in MSgg (+), which lead to higher expression of *tkmA-ugd* and lower expression of *epsA-O* due to strong repression of *epsA-O* by DegU in LBGM. This model predicts that in LBGM, the relative ratio of TkmA/EspA is much higher than that in MSgg. Therefore TkmA makes significantly more contributions to the regulation of EPS biosynthesis and biofilm formation by cross-talking with EpsB.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7