Phosphorylation of Spo0A by the histidine kinase KinD requires the lipoprotein Med in Bacillus subtilis

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ABSTRACT

The response regulatory protein Spo0A of Bacillus subtilis is activated by
phosphorylation by multiple histidine kinases via a multicomponent phosphorelay. Here
we present evidence that the activity of one of the kinases KinD depends on the
lipoprotein Med, a mutant of which was known to cause a cannibalism phenotype. We
show that the absence of Med impaired, and that the over production of Med stimulated,
the transcription of two operons (sdp and skf) involved in cannibalism whose
transcription is known to depend on Spo0A–P. Further, these effects of Med were
dependent on KinD but not on kinases KinA, KinB and KinC. Additionally, we show
that deletion or over production of Med, respectively, impaired or enhanced biofilm
formation, and that these effects too depended specifically on KinD. Finally, we report that over production of Med bypassed the dominant-negative effect on transcription of sdp of a truncated KinD retaining the transmembrane segments but lacking the kinase domain. We propose that Med directly or indirectly interacts with KinD in the cytoplasmic membrane and that this interaction is required for KinD-dependent phosphorylation of Spo0A.

INTRODUCTION

The soil dwelling, gram-positive bacterium *Bacillus subtilis* is able to draw on a wide repertoire of adaptive responses to cope with adverse environmental circumstances. Several of these responses, such as spore formation, cannibalism, and biofilm formation require the same master regulatory protein, Spo0A (11, 13, 15, 18, 35-36). Spo0A is a member of the response regulator family of transcription factors and is active in its phosphorylated state (Spo0A-P) (14, 22). When phosphorylated, Spo0A can act as both an activator and repressor and is known to directly regulate about 120 genes (13, 25) and indirectly over 500 genes (12, 25)

Phosphorylation of Spo0A is governed by a multicomponent phosphorelay (5). At least four autophosphorylating sensor kinases, KinA, KinB, KinC and KinD (the contribution of a fifth, KinE is less certain) transfer phosphoryl groups to the relay protein Spo0F (21). Spo0F, in turn, transfers the phosphoryl group to Spo0B, which then phosphorylates Spo0A (5). It is thought that the activity of the kinases is regulated by specific signals whose nature is largely mysterious. KinA and KinB are particularly
important for entry into sporulation (29, 37) whereas KinC and KinD contribute to
triggering biofilm formation (23). Three of the kinases are integral membrane proteins
(KinB, KinC and KinD) whereas the fourth, KinA, is cytosolic. Flux through the relay is
also governed by phosphatases, which dephosphorylate Spo0F–P or Spo0A–P (30-31).
The activity of KinA is subject to inhibition by two other proteins: Sda and KipI
(6, 32, 39). Both proteins bind to KinA, thereby blocking autophosphorylation. The
synthesis of Sda is induced in response to DNA damage, preventing sporulation if
chromosome replication is impaired (6, 16, 19, 33). The production of KipI appears to be
linked to the utilization of nitrogen, but the specific signal that results in its inhibition of
KinA is not known (39).
The activity of KinB is also influenced by interaction with other proteins. In
addition to inhibiting KinA, Sda also inhibits the autophosphorylation reaction of KinB
(3, 6, 20). To be functional, KinB also requires the presence of a lipoprotein, KapB. The
kapB gene is in an operon with the kinB gene, and the two proteins are thought to form a
complex in the cytoplasmic membrane (10).
Here we report that the activity of KinD also depends on a partner, the lipoprotein
Med. Med was originally identified as a positive regulator of the competence gene
comK, but its function remained obscure (27-28). Our attention to Med was drawn
through our studies on the phenomenon of cannibalism in which cells that have activated
Spo0A in response to nutrient limitation produce a toxin and a killing factor that kill
sibling cells that have not activated Spo0A (11, 15). Colonies of cells that exhibit
cannibalism are delayed in sporulation. It is thought that nutrients released by the dead
cells delay sporulation by reversing or slowing the activation of Spo0A in the toxin- and
killing factor-producing cells. The toxin and the killing factor are produced by operons called sdpABC (hereafter sdp) and skfABCDEFGH (hereafter skf), respectively. Both operons are indirectly under the control of Spo0A via AbbA and AbrB (2, 13). Additionally, Spo0A–P directly binds to and activates the skf promoter at low levels, and acts as a direct repressor of skf transcription at high levels. Colonies of cells mutant for sdp or skf are mutant for cannibalism and exhibit accelerated sporulation. A previous survey of members of the Spo0A regulon for genes involved in cannibalism revealed that a mutation in the gene med caused accelerated sporulation (25). Here we show that Med acts by stimulating phosphorylation of Spo0A and that the target of Med is KinD.

MATERIALS AND METHODS

Strains and media

All strains used in this study are listed in Table 1. Bacillus subtilis PY79, 3610 and derivatives were grown in Luria-Bertani broth (LB) at 37°C for propagation. For measurements of gene activity with lacZ reporter genes, cells were induced to sporulate by nutrient exhaustion at 37°C in Difco sporulation medium (DSM) (26). For colony formation, cells were grown on solid MSgg medium (5 mM potassium phosphate, 100 mM morpholinepropanesulfonic acid, pH 7 [MOPS], 2 mM MgCl2, 50 µM MnCl2, 50 µM FeCl3, 700 µM CaCl2, 1 µM ZnCl2, 2 µM thiamine, 0.5% glycerol, 0.5% glutamate, and 50 µg/ml [each] threonine, tryptophan, and phenylalanine) containing 1.5% Bacto agar. When appropriate, isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) was added to a final concentration of 1mM, and xylose was added to a final concentration of 20mM. When needed, antibiotics were added at the following concentrations for growth.
of *B. subtilis*: 10 μg per ml of tetracycline, 100 μg per ml of spectinomycin, 10 μg per ml of kanamycin, 5 μg per ml of chloramphenicol, 0.4 μg per ml of phleomycin and 1 μg per ml of erythromycin.

**Strain construction**

General methods for molecular cloning and strain construction were performed according to published protocols (34). Long-flanking PCR mutagenesis was applied to make insertional knockout mutations in the *med* and *kinD* genes (38). Primers used in strain construction are listed in Table 2. Introduction of DNA into PY79 derivatives was conducted by transformation (17). SPP1 phage-mediated general transduction was used to introduce antibiotic resistance marker-linked mutations or over-expression constructs into derivatives of 3610 (40).

All oligonucleotide primers used in this study used in strain construction are listed in Table 2. Construction of the P<sub>sdp</sub>-lacZ and P<sub>skf-lacZ</sub> reporters was described previously (11, 15). To construct the IPTG-inducible P<sub>hyper-spank-med</sub> construct, a PCR product containing an optimized ribosome binding site and the *med* open reading frame was generated using primers AVB007/AVB008 and cloned into the NheI and SalI sites of either pDR111, an *amyE* locus integration plasmid, or pDP150, a *thrC* integration plasmid (modified pDR111; gift of D. Rudner). The resulting P<sub>hyper-spank-med</sub> plus *lacI* fragment was integrated into the chromosome at either *amyE* or *thrC* by double recombination to create strains AB392 and AB340, respectively. To construct the xylose-inducible P<sub>xylose-kinD</sub>* strain, a PCR product containing an optimized ribosome binding site and the first 882 base pairs (plus a stop codon) of the *kinD* open reading
frame was generated using primers AVB176/AVB177 and cloned into the HindIII and SphI sites of pEH211 (modified pDR150; gift of D. Rudner). The resulting $P_{\text{rcl}}$-kinD* plus xylR fragment was integrated into the chromosome at thrC by double recombination.

To construct the strain (AB434) that contains $P_{\text{sdp}}$-lacZ, $P_{\text{hyper-spank-med}}$, and $P_{\text{xylA-kinD*}}$, the $amyE$::$P_{\text{hyper-spank-med}}$ construct was first introduced by transformation into AHB282 (8), a derivative of PY79, which contains an additional $amyE$ site ($ywrk$::$Tn917$::$amyE$::cm) at position 317° on the circular chromosome marked by a chloramphenicol resistance gene. Transformants were selected for Spec resistance and Cm sensitivity (CmR SpecS) for integration of $P_{\text{hyper-spank-med}}$ (SpecR) into the $amyE$ sequence at the 317° position, but not into the native $amyE$ locus.

**Kinetic β-galactosidase assay**

To measure β-galactosidase activity, we used a kinetic assay described previously (7). Briefly, cells were cultured in DSM medium at 37°C in a water bath with shaking. 1-ml culture was collected every 30 minutes. Cells were spun down and pellets were resuspended in 1 mL Z-buffer (60mM Na$_2$HPO$_4$, 40mM NaH$_2$PO$_4$, 10mM KCl, 1mM MgSO$_4$, 50 mM β-mercaptoethanol at pH 7.0). Ten microliters of these cells were added to individual wells of a clear 96-well plate containing 90 uL of 0.2 mg/mL lysozyme in Z-buffer. Cell lysis was allowed to proceed for 20-30 min at 37°C. Twenty microliters of 4 mg/mL 2-Nitrophenyl β-D-galactopyranoside (ONPG; Sigma-Aldrich) in Z-buffer were then added to each well and mixed thoroughly. Absorbance at 420 nm for each reaction...
was read once per minute for 1 h at 37°C in a Synergy 2 plate reader (BioTek). β-
Galactosidase activity (in arbitrary units [AU]) is reported as the rate of ONPG
conversion (i.e., Vmax, with units of ΔOD420 per minute) divided by the OD600 of the
sample at the time of collection. Standard curve analysis revealed that 1 AU ≈ 4 Miller
units. β-galactosidase activity for each strain was measured a minimum of three times in
separate experiments. A representative experiment is shown.

Colony formation

For colony formation, cells were first grown to exponential growth phase in LB
broth, and 1 μL of these cultures were spotted onto solid MSgg medium containing 1.5%
Bacto agar. The plates were incubated at 30°C for 72 hours. Images of the colonies were
taken using a SPOT camera (Diagnostic Instruments).

Spore and total cell quantification

Cells were grown as biofilms on MSgg plates and incubated at 30 °C for 72 hours prior to
harvesting for quantification. Samples were kept on ice during the following procedure.
Entire colonies were harvested, resuspended in 1 mL Phosphate Buffered Saline (PBS),
and subjected to mild sonication to disrupt the matrix and obtain single cells. For
quantification of spores, each preparation was incubated at 80 °C for 20 min to kill
vegetative cells. To determine viable cell counts, serial dilutions were plated from each
preparation before and after the 80°C incubation.

RESULTS AND DISCUSSION

The cannibalism operons sdp and skf are under the indirect control of Med
We asked if the accelerated sporulation phenotype of a med mutant was due to impaired expression of the sdp and skf operons. To do this, we examined the effect of a med deletion mutation (Δmed) on the expression of lacZ fused to the promoters for sdp (P_{sdp}-lacZ) and skf (P_{skf}-lacZ) during sporulation in DS medium. We observed that deletion of med led to decreased transcription from both promoters (Figure 1), providing an explanation for the cannibalism defect of a med mutant. To ensure that the effect of the deletion was not due to a polar effect on the expression of the downstream gene comZ, we deleted comZ, and observed no effect on expression of sdp and skf (data not shown). Also the med deletion mutation was complemented by a copy of med that had been inserted at the thrC locus (data not shown).

Next, we determined the effect of overproducing Med on the expression of sdp and skf. To do this, we constructed a fusion of the med gene to the IPTG-inducible promoter P_{hyper} and examined the effect of inducing this construct on the expression of P_{sdp}-lacZ and P_{skf}-lacZ during sporulation. We observed that expression of both genes was markedly elevated when Med was over expressed (Figure 1).

Med acts independently of KinC and in the same pathway as KinD to promote expression of sdp.

Because transcription of both sdp and skf is dependent on the activity of Spo0A, we wondered if Med might act by enhancing Spo0A-P levels. We asked if Med acted through either KinC or KinD as both of these kinases, like Med, are dispensable for sporulation but are thought to contribute to lowering AbrB levels (via Spo0A-P-
mediated repression) during the transition to stationary phase (21). We decided to focus on sdp expression, as its regulation by Spo0A is less complex than for skf.

We first compared P_{sdp-lacZ} expression in a kinC mutant, a med, kinC double mutant and a kinC mutant that also contained P_{hyper-spank-med}. We observed that removing or overexpressing med, respectively, diminished or stimulated P_{sdp-lacZ} expression in the absence of KinC (Figure 2A). We conclude that because the contributions of Med and KinC were additive, these proteins evidently act in separate pathways to promote sdp expression. We next compared P_{sdp-lacZ} expression in a kinD mutant, a med, kinD double mutant, and a kinD mutant that also contained P_{hyper-spank-med}. We observed that removing or overexpressing med had little effect on P_{sdp-lacZ} expression in the absence of KinD (Figure 2B). In other words, the effect of the kinD mutation was epistatic to that of the med mutation. These results suggest that Med and KinD act in the same pathway to promote expression of sdp.

We also tested the dependence of KinA and KinB on Med. Mutations of kinA and kinB impaired sdp expression to only a small extent. Nonetheless, the effects of removing or overexpressing med on P_{sdp-lacZ} expression in the absence of KinA (Figure 3A) or KinB (Figure 3B) were similar (that is, additive) to what we had observed in the absence of KinC. We conclude that Med acts separately from KinA, KinB and KinC and is instead specifically required for the function of KinD.

Med acts independently of KinC and in the same pathway as KinD to influence biofilm formation.
A survey of genes required for efficient biofilm formation in *B. subtilis* revealed that a *med* mutation has a modest defect in biofilm colony morphology (9). Biofilms are composed of long chains of cells that are held together by an extracellular matrix consisting of protein and polysaccharide. Production of matrix is governed by a regulatory network that, like cannibalism, requires Spo0A activity. We wondered whether Med influenced biofilm formation through KinD. To test this we examined the colony morphology of derivatives of the wild strain (3610), which forms architecturally complex colonies on solid, biofilm-inducing (MSgg) medium. We first compared colony morphology of a *med* mutant, a *kinD* mutant, and a *kinC* mutant in the 3610 background. We observed that all three single mutations resulted in a similar subtle defect in colony morphology in which the colony was flatter and wider than that of the wild type (Figure 4, top). We then compared a *kinC kinD* double mutant (which is known to exhibit a severe block in biofilm formation (24)), a *kinC med* double mutant and a *kinD med* double mutant. We observed that the both the *kinC kinD* double mutant as well as the *kinC med* double mutant had a far more severe defect in colony morphology than either single mutant alone (Figure 4, middle). In contrast, a *med, kinD* double mutant was indistinguishable from the *med* or *kinD* single mutants. Lastly, we compared a strain that contained P<sub>hyper-spank-med</sub>, a strain that contained P<sub>hyper-spank-med</sub> as well as a *kinC* mutation and a strain that contained P<sub>hyper-spank-med</sub> as well as *kinD* mutation. We observed that overexpression of Med resulted in a hyper-wrinkly colony morphology. Deletion of *kinC* had little, if any, effect on the colony morphology of a Med overexpression strain. However, deletion of *kinD* resulted in a colony that was
indistinguishable from a *kinD* mutation alone (Figure 4, bottom). Thus it appears that Med acts through KinD, and independently of KinC to promote biofilm development.

**Med inhibits sporulation during biofilm formation in a KinD-dependent manner**

It has been previously reported that deletion of *kinD* results in a small increase in the number of spores formed under biofilm-inducing conditions. More importantly, deletion of *kinD* partially restores sporulation to an *eps* *taxA* double mutant, which is blocked in matrix production. An *eps* *taxA* double mutant is normally conspicuously delayed in sporulation under biofilm-inducing conditions (1). These observations have led to the idea that KinD is a checkpoint protein that links spore formation to matrix production, with KinD acting as a phosphatase in the absence of matrix and a kinase as matrix accumulates (1). We wondered if Med had a similar effect on sporulation in a biofilm. To investigate this, we harvested biofilms after 72 hours of growth at 30˚C and calculated the number of heat resistant spores as a fraction of total living cells. We then expressed this number as a ratio relative to the wild type strain (3610). We first compared a *med* mutant, a *kinD* mutant, a *med, kinD* double mutant, an *eps* *taxA* double mutant, an *eps* *taxA* *kinD* triple mutant and an *eps* *taxA* *med* triple mutant. We found that both single mutations resulted in a slight increase in the number of heat-resistant spores when compared to wild type, and that the *med kinD* double mutant was indistinguishable from either of the single mutants. We also observed that deletion of either *kinD* or *med* resulted in a partial restoration of sporulation in an *eps* *taxA* mutant background (Table 3). We next tested the effect of overexpressing *med* on sporulation in a biofilm. We compared a strain that contained P<sub>hyper-spank-med</sub>, a strain that contained P<sub>hyper-spank-med</sub> as
well as a kinD mutation and a strain that contained $P_{\text{hyper-spank-med}}$ as well as an eps and a 
tasA mutation. We observed a 10-fold reduction in the number of heat resistant spores 
relative to wild type when Med was overproduced. However, when we introduced a kinD 
mutation into a $P_{\text{hyper-spank-med}}$ background, sporulation was restored to levels comparable 
to that of a kinD mutation alone. Overexpression of med in an eps tasA double mutant 
background did not have a noticeable additional inhibitory effect on sporulation (Table 
3). In sum, these results indicated that the effect of Med on sporulation during biofilm 
formation is similar to that of KinD and that the effect of Med is dependent on KinD.

Overexpression of truncated KinD has a dominant-negative effect on sdp expression 
that is rescued by overproduction of Med

As a further test of the idea that med and kinD interact genetically, we constructed 
a fusion of the first 882 base pairs of the kinD open-reading frame to the xylose-inducible 
promoter $P_{\text{xyLA}}$. This construct (hereafter $\text{kinD}^*$) contained both transmembrane segments 
and the extracellular double PAS-like sensor domain of KinD, but did not contain the 
histidine kinase or ATPase domains. We noticed that when we induced expression of 
this construct, $P_{\text{sdp-lacZ}}$ expression was impaired. We wondered if the impaired level of 
$P_{\text{sdp-lacZ}}$ expression was dependent on Med. We tested the effect of inducing $P_{\text{xyLA}}$-
kinD*, in a med mutant as well as in a strain that contained $P_{\text{hyper-spank-med}}$. We observed 
that inducing kinD* had no effect in the absence of med, and, strikingly, that 
overproduction of Med restored $P_{\text{sdp-lacZ}}$ expression in a KinD* producing strain (Figure 
5). This result suggests that expression of kinD* has a dominant-negative effect on 
expression of $P_{\text{sdp-lacZ}}$, but that this effect is dependent on Med. These results are
consistent with the following ideas: (1) Med and KinD interact, (2) the non-functional, truncated KinD, KinD* and wild type KinD compete for a limiting number of Med molecules, thereby titrating Med, and (3) when Med is overproduced, titration is overcome, restoring KinD activity.

Conclusions

The principal contribution of this work is the elucidation of a function for the previously mysterious lipoprotein Med. Med was originally identified as a positive regulator of the competence gene \textit{comK} by Ogura \textit{et al} in 1997. At the time, the authors rejected the idea that Med might be acting through Spo0A to induce \textit{comK} expression because a \textit{med} mutation did not affect sporulation. Our results show that Med does indeed act through Spo0A, and this is most likely the basis for the modest effect of a \textit{med} mutation on \textit{comK} expression that the authors observed. The authors also later characterized Med as a cell-surface localized lipoprotein (27-28).

How does Med act to promote the activity of KinD? The simplest interpretation of our results is that Med interacts directly with KinD. However, efforts to demonstrate such an interaction by chemical crosslinking and by means of “pull down” experiments with His-tagged Med have so far been unsuccessful. Therefore, we cannot exclude the possibility that the interaction is indirect. Whether the interaction is direct or indirect, the use of a truncated KinD shows that the transmembrane and extracellular sensor domains of KinD are all that is required to interact with Med, whose N-terminal region is anchored in the cytoplasmic membrane and whose C-terminal region projects from the outside surface of the membrane. Assuming that Med and KinD are in contact, we propose that
the functional form of KinD is as a complex with Med. If so, KinD is not alone in its
requirement for a lipoprotein partner. KinB, another kinase capable of activating Spo0A,
requires a lipoprotein, KapB, for its activity. Although there is no evidence of a direct
interaction between KinB and KapB, the fact their genes are in the same operon has led
to the idea that the two proteins physically interact (10).

Med acts by stimulating phosphorylation (and under some condition,
dephosphorylation) of Spo0A indirectly through KinD. Previous work has shown that
Spo0A–P directly represses transcription of med (13, 25). The negative regulation of
med by Spo0A thus constitutes a negative feedback loop wherein the activity of KinD is
curtailed as Spo0A–P levels rise. This may allow other kinases, such as KinA and KinB,
to take over phosphorylation of Spo0F and drive Spo0A–P levels high enough for
sporulation to occur.

Acknowledgements. This work was support by NIH grants GM18568 to RL and
MH090948 to J. Liu and RL.

TABLES

Table 1. Strains used in this study

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<tr>
<td>AB433</td>
<td>Δmed::tet, thrC::PxylA-D* erm, amyE::PsdpABC-lacZ cm</td>
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<td>AB434</td>
<td>thrC::PxylA-D* erm, yvrK::amyE::P&lt;sub&gt;hyper&lt;/sub&gt;-spanA::med spe, amyE::PsdpABC-lacZ cm</td>
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<tr>
<td>AB481</td>
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<tr>
<td>AB482</td>
<td>ΔkinA::tet, Δmed::erm, amyE::PsdpABC-lacZ cm</td>
<td>AB142 x AB484</td>
</tr>
<tr>
<td>AB483</td>
<td>ΔkinA::tet, thrC::P&lt;sub&gt;hyper&lt;/sub&gt;-spanA::med spe, amyE::PsdpABC-lacZ cm</td>
<td>AB340 x AB481</td>
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<tr>
<td>AB484</td>
<td>ΔkinB::cm, amyE::PsdpABC-lacZ cm</td>
<td>RL4261 x RL4264</td>
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<tr>
<td>AB485</td>
<td>ΔkinB::cm, Δmed::erm, amyE::PsdpABC-lacZ cm</td>
<td>AB142 x AB484</td>
</tr>
<tr>
<td>AB486</td>
<td>ΔkinB::cm, thrC::P&lt;sub&gt;hyper&lt;/sub&gt;-spanA::med spe, amyE::PsdpABC-lacZ cm</td>
<td>AB340 x AB484</td>
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<tr>
<td>AB498&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Δeps::tet, ΔttsA::kan</td>
<td>Lab stock</td>
</tr>
<tr>
<td>AB535&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ΔkinD::erm</td>
<td>This study</td>
</tr>
<tr>
<td>EH 456</td>
<td>ΔkinD::tet</td>
<td>This study</td>
</tr>
<tr>
<td>RL2886</td>
<td>amyE::PsdpABC-lacZ cm</td>
<td>(15)</td>
</tr>
<tr>
<td>RL3554</td>
<td>amyE::PsdpABC-lacZ cm</td>
<td>(11)</td>
</tr>
<tr>
<td>RL4261</td>
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<td>Lab stock</td>
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<tr>
<td>RL4263</td>
<td>ΔkinC::cm</td>
<td>Lab stock</td>
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<td>amyE::PsdpABC-lacZ cm</td>
<td>(15)</td>
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<td>RL4552&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ΔkinD::tet</td>
<td>EH 456 x 3610</td>
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<tr>
<td>RL4566</td>
<td>ΔkinD::tet</td>
<td>Lab stock</td>
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<sup>a</sup> All strains are isogenic with PY79 unless otherwise marked. <sup>b</sup> Strains isogenic with 3610. <sup>c</sup> The x indicates that DNA from the first strain was introduced into the second strain.

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Table 2. Primers used in this study

<table>
<thead>
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<th>Primer</th>
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<td>med p3</td>
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<tr>
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<td>Δmed</td>
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<tr>
<td>Δeps, ΔtasA, Δmed, Med overexpression</td>
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**Table 3.** Med inhibits sporulation during biofilm formation in a KinD-dependent manner.

**FIGURE LEGENDS**

**Figure 1.** Med is required for the maximal expression of the *sdpABC* and *skfABCDE* operons. Strains harbored either *amyE::Psdp-lacZ* (top) or *amyE::Psdp-lacZ* (bottom) and...
were either wild-type (RL4264), mutant for *med* (AB332), or harbored an overexpression construct P_{hyper-spank-med} (AB333). Cells were grown in liquid DS medium; hour 0 of stationary phase was the end of the exponential phase of growth. Expression of P_{hyper-spank-med} was induced by addition of 1mM (final concentration) IPTG to the medium. The experiment was repeated a minimum of three times. Shown is a representative experiment.

**Figure 2.** *Med* acts independently of KinC and in the same pathway as KinD to promote expression of *sdp*. (A) Strains harbored amyE::P_{sdp-lacZ} and were either wild-type (RL4264), mutant for *med* (AB332), harbored P_{hyper-spank-med} (AB333), mutant for *kinC* (AB341), mutant for *med* and *kinC* (AB342), or mutant for *kinC* and harbored P_{hyper-spank-med} (AB343). (B) Strains harbored amyE::P_{sdp-lacZ} and were either wild-type (RL4264), mutant for *med* (AB332), harbored P_{hyper-spank-med} (AB333), mutant for *kinD* (AB337), mutant for *med* and *kinD* (AB338), or mutant for *kinD* and harbored P_{hyper-spank-med} (AB339). Cells were grown in liquid DS medium; hour 0 of stationary phase was the end of the exponential phase of growth. Expression of P_{hyper-spank-med} was induced by addition of 1mM (final concentration) IPTG to the medium. The experiment was repeated a minimum of three times. Shown is a representative experiment.

**Figure 3.** *Med* acts independently of KinA and KinB to promote expression of *sdp*. (A) Strains harbored amyE::P_{sdp-lacZ} and were either wild-type (RL4264), mutant for *med* (AB332), harbored P_{hyper-spank-med} (AB333), mutant for *kinA* (AB481), mutant for *med* and *kinA* (AB482), or mutant for *kinA* and harbored P_{hyper-spank-med} (AB483). (B) Strains harbored amyE::P_{sdp-lacZ} and were either wild-type
mutant for med (RL4264), harbored P_{hyper-spank-med} (AB333), mutant for kinB (AB484), mutant for med and kinB (AB485), or mutant for kinB and harbored P_{hyper-spank-med} (AB486). Cells were grown in liquid DS medium; hour 0 of stationary phase was the end of the exponential phase of growth. Expression of P_{hyper-spank-med} was induced by addition of 1mM (final concentration) IPTG to the medium. The experiment was repeated a minimum of three times. Shown is a representative experiment.

Figure 4. Med acts independently of KinC and in the same pathway as KinD to influence biofilm formation. Shown are biofilms formed by strains grown on solid MSgg medium that were either wild-type (3610), mutant for med (AB349), mutant for kinC (AB399), mutant for kinD (RL4552), mutant for kinC and kinD (AB401), mutant for med and kinC (AB400), mutant for med and kinD (AB351), harbored P_{hyper-spank-med} (AB396), mutant for kinC and harbored P_{hyper-spank-med} (AB402), or mutant for kinC and harbored P_{hyper-spank-med} (AB398). Expression of P_{hyper-spank-med} was induced by addition of 1mM (final concentration) IPTG to the medium.

Figure 5. Overexpression of truncated KinD has a dominant-negative effect on sdp expression that is rescued by overproduction of Med. Strains harbored amyE::P_{sdp-lacZ} and were either wild-type (RL2886), mutant for med (AB307), harbored P_{hyper-spank-med} (AB430), harbored a construct that overexpressed truncated KinD (amino acids 1-294) P_{xylA-kinD*} (AB432), mutant for med and harbored P_{xylA-kinD*} (AB433), or harbored P_{hyper-spank-med} and P_{xylA-kinD*} (AB434). Cells were grown in liquid DS medium; hour 0 of sporulation was the end of the exponential phase of growth. Expression of P_{hyper-spank-med} was induced by addition of 1mM (final
concentration) IPTG to the medium. Expression of \( P_{\text{xylA-kinD}} \) was induced by addition of 20mM (final concentration) xylose to the medium. The experiment was repeated a minimum of three times. Shown is a representative experiment.

REFERENCES


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