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A protein complex supports the production of Spo0A-P and plays additional roles for biofilms and the K-state in *Bacillus subtilis*

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Abstract

Bacillus subtilis can enter three developmental pathways to form spores, biofilms or K-state cells. The K-state confers competence for transformation and antibiotic tolerance. Transition into each of these states requires a stable protein complex formed by YlbF, YmcA and YaaT. We have reported that this complex acts in sporulation by accelerating the phosphorylation of the response regulator Spo0A. Phosphorelay acceleration was also predicted to explain their involvement in biofilm formation and the K-state. This view has been challenged in the case of biofilms, by the suggestion that the three proteins act in association with the mRNA degradation protein RNaseY (Rny) to destabilize the *sinR* transcript. Here we reaffirm the roles of the three proteins in supporting the phosphorylation of Spo0A for all three developmental pathways and show that in their absence *sinR* mRNA is not stabilized. We demonstrate that the three proteins also play unknown Spo0A-P-independent roles in the expression of biofilm matrix and in the production of ComK, the master transcription factor for competence. Finally, we show that domesticated strains of *B. subtilis* carry a mutation in *sigH*, which influences the expression kinetics of the early spore gene *spoIIG*, thereby increasing the penetrance of the *ylbF, ymcA* and *yaaT* sporulation phenotypes.

Graphical Abstract

In *Bacillus subtilis*, the proteins YlbF, YmcA and YaaT form a complex needed for the formation of biofilms, for sporulation and for genetic transformation. This paper strongly supports a role for these proteins in stimulating the phosphorylation of the key transcription factor, Spo0A. The present data contradict an alternative published model that proposed a role for the complex in destabilizing the mRNA for SinR, a repressor of biofilm formation.

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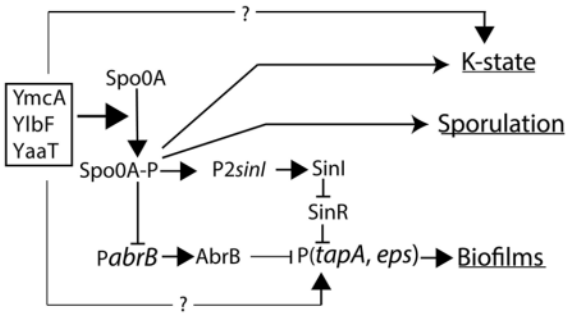

Keywords

biofilm; K-state; sporulation; Spo0A; YmcA-YlbF-YaaT

Introduction

Bacillus subtilis is an important model bacterium, largely because of its ability to choose among several developmental states; sporulation, biofilm formation, the K-state (which includes competence for transformation (Berka *et al.*, 2002)) and motile or sessile modes of life (Mirouze & Dubnau, 2013). The *B. subtilis* genome encodes a signal integration network that permits each cell to respond to the environment and its own metabolic state by adjusting the probability that it will enter each of the above-mentioned developmental pathways. The most important of the regulatory proteins in this signal transduction network is Spo0A, which is required for all forms of development and is active as a transcription factor when phosphorylated by a multicomponent phosphorelay (Burbulys *et al.*, 1991). In this phosphorelay, one or more of several kinases phosphorylates the response regulator protein Spo0F. The Spo0F-P phosphoryl group is then passed to the phosphotransferase protein Spo0B and then to the response regulator Spo0A. Several pathways feed pertinent information to the phosphorelay, including those that act on the kinases and those that dephosphorylate Spo0F-P or Spo0A-P. The phosphorelay thus serves as a central signal integration device that delivers the appropriate level of Spo0A-P at the right time and with an appropriate distribution within the population of cells (Ireton *et al.*, 1993, Chastanet & Losick, 2011, Chastanet *et al.*, 2010).

The present study explores the roles of three proteins, YlbF, YmcA and YaaT, which like Spo0A are required for the K-state, sporulation and the formation of normal biofilms. A screen for genes needed for K-state expression yielded *ylbF*, which was also shown to be required for spore formation (Tortosa *et al.*, 2000). A later screen for biofilm genes identified both *ylbF* and *ymcA* (Branda *et al.*, 2004, Kearns *et al.*, 2005), while *yaaT* was revealed in a screen for sporulation genes (Hosoya *et al.*, 2002). It was reported recently from our laboratory that individual knockouts of *ylbF, ymcA* and *yaaT* are deficient in sporulation and in establishing biofilms and the K-state. They also exhibit excessive chaining under certain conditions, suggesting that they are biased toward a sessile lifestyle (Carabetta *et al.*, 2013). YlbF, YmcA and YaaT are associated in *B. subtilis*, as shown by a complete set of reciprocal immunoprecipitations (IPs) followed by mass spectrometry, using each of the three proteins

as bait (Carabetta *et al.*, 2013). This study also demonstrated that when expressed in *Escherichia coli* the proteins could be purified as a stable 80 kDa ternary complex, which displayed a monodisperse peak by size exclusion chromatography.

The *in vitro* stability of the 80kDa complex suggested strongly that the three proteins were not merely transiently associated, but rather form a stable complex *in vivo*. The study by Carabetta et al (2013) focused on the roles of YlbF, YmcA and YaaT in sporulation and showed that the early spore gene *spoIIG,* which is dependent for its transcription only on Spo0A-P, was poorly transcribed in null mutants of each of the three genes, suggesting that they play a role in the formation of Spo0A-P. Hosoya *et al* (2002) had previously concluded that a *yaaT* knockout was blocked in the phosphorelay and had demonstrated that such a mutant could be suppressed for sporulation by the *sof-1* allele of *spo0A*. This asparagine to lysine mutation at position 12 of Spo0A, bypasses the need for Spo0F and Spo0B by facilitating the direct phosphorylation of Spo0A by one or more kinase (Spiegelman *et al.*, 1990). The *sof-1* mutation had also been reported to partially suppress *ylbF* inactivation for spore formation (Tortosa *et al.*, 2000). Further support for a role of YlbF, YmcA and YaaT in Spo0A-P formation was derived from the finding that a knockout of *spo0E* partially bypassed the *ylbF*, *ymcA* and *yaaT* knockouts for *spoIIG-luc* expression (Carabetta *et al.*, 2013, Hosoya *et al.*, 2002). Since Spo0E dephosphorylates Spo0A-P (Perego, 2001) and is a major drain on the phosphorylated form of this protein, we would expect that its elimination would at least partially compensate for a decreased rate of Spo0A phosphorylation. This expectation was confirmed (Carabetta *et al.*, 2013, Hosoya *et al.*, 2002). These observations, and the known dependencies on Spo0A-P for biofilm formation (Chu *et al.*, 2008), the K-state (Mirouze *et al.*, 2012) and sporulation (Hoch, 1993), suggested that the YlbF-YmcA-YaaT complex played a positive role at one or more steps in the phosphorylation cascade that leads to the production of Spo0A-P. Support for this hypothesis was generated using *sad-67*, a short deletion that removes residues 63–81 from the coding sequence of *spo0A* thereby rendering the Spo0A protein active without phosphorylation (Ireton *et al.*, 1993). This mutant form of Spo0A was shown to bypass the requirements for *ylbF, ymcA and yaaT* for expression of *spoIIG* (Carabetta *et al.*, 2013). Importantly, and most directly, the purified complex was shown to stimulate the phosphorelay *in vitro*. Although the experimental data of Carabetta et al (2013) were obtained for sporulation, the dependence of biofilm formation and the K-state on Spo0A-P and on YlbF, YmcA and YaaT prompted extrapolation of the findings to the latter forms of development and the proposal that a general role for the protein complex was to stimulate the production of Spo0A-P. Clearly these results did not exclude the possibility that additional roles existed.

Recently, the proposed role for YlbF, YmcA and YaaT in supporting the production of Spo0A-P has been challenged for biofilm formation (DeLoughery *et al.*, 2016). These authors confirmed some of the IP results of Carabetta et al (2013) using tagged YlbF to show association with YmcA and YaaT, and further documented the contacts among the three proteins using bacterial two-hybrid experiments. However, they did not detect an effect of the *ylbF*, *ymcA* and *yaaT* knockouts on the transcription of *sinI*, a Spo0A-P-dependent gene required for biofilm formation, nor did they detect an effect on the repression of *abrB-lacZ* transcription by Spo0A-P, the latter in direct contrast to the previous data for *ylbF* (Carabetta *et al.*, 2013). Instead, DeLoughery et al (2016) proposed that the YlbF-YmcA-

YaaT complex interacts with the ribonuclease Rny and that the absence of this interaction results in stabilization of the *sinR* transcript. This was proposed to result in the accumulation of SinR, a known repressor of biofilm matrix formation (Kearns *et al.*, 2005). Consistent with the Rny model, IP data suggested an *in vivo* interaction of Rny with YaaT (Carabetta *et al.*, 2013) and of Rny with YlbF (DeLoughery *et al.*, 2016). DeLoughery et al (2016) also used bacterial two-hybrid (B2H) experiments to show an interaction of Rny with YlbF and YmcA. The Rny hypothesis predicted that in knockouts of *ylbF*, *ymcA* and *yaaT*, the abundance of the *sinR* mRNA and its translated product would be increased. Enhanced levels of *sinR* mRNA and of the biofilm matrix gene repressor SinR were indeed reported in knockouts of *ylbF* and *ymcA*. The Rny hypothesis was further buttressed by the similar slow growth phenotypes of *ylbF*, *ymcA*, *yaaT* and *rny* knockouts and the reported synteny of *rny* and *ymcA*. Also consistent with this hypothesis are the observations that the deletion of *rny*, causes severe deficiencies in sporulation, competence and biofilm formation (DeLoughery *et al.*, 2016, Figaro *et al.*, 2013), while the overproduction of Rny induces biofilm formation (Lehnik-Habrink *et al.*, 2011b).

The general importance of YlbF, YmcA and YaaT for development in *B. subtilis* and the challenge posed by the alternative model prompted the further exploration of the roles of these three proteins in biofilm formation, the K-state and sporulation and a re-examination of the phosphorelay hypothesis. The new data, presented here, reaffirms an important role for these proteins in stimulating the production of Spo0A-P for all three forms of development. It is shown that the transcription of *abrB* and *sinI* are indeed affected by the knockouts under biofilm-forming conditions in the undomesticated strain NCIB3610 (hereafter 3610). We also show that all three knockouts are at least partially bypassed for *spoIIG*, *sinI* and *abrB* by the *sof-1* or *sad-67* alleles of *spo0A* and that pellicle formation, a sensitive indicator of matrix formation, is partially bypassed in *ylbF* and *ymcA* knockout strains by *sad-67*. Tellingly, the new data show that the knockouts do not phenocopy a deletion of *rny* for the stabilization of the *sinR* transcript. The present work also reveals additional but uncharacterized roles for YlbF, YmcA and YaaT in development beyond the acceleration of Spo0A phosphorylation.

Results

The present study investigates the roles of *ylbF*, *ymcA* and *yaaT* in biofilm formation by measuring the transcription of *abrB* and *sinI*, two genes that are required for the correct expression of the biofilm matrix (Kearns *et al.*, 2005, Chu *et al.*, 2008, Hamon *et al.*, 2004) and which are regulated by direct binding of Spo0A-P to their promoters (Strauch *et al.*, 1990, Shafikhani *et al.*, 2002, Fürbass *et al.*, 1991). Spo0A-P represses the transcription of *abrB* and activates that of *sinI.* Because AbrB represses downstream operons required for biofilm matrix formation and because SinI antagonizes the action of SinR, a repressor of these same operons (Bai *et al.*, 1993), Spo0A-P is a necessary initiator of biofilm formation (Fig. 1). No transcription factor other than Spo0A-P has been reported to repress the transcription of *abrB* except for AbrB itself (Strauch *et al.*, 1989) and only Spo0A-P appears to activate the transcription of *sinI*, although two repressors, AbrB and ScoC (Hpr) have been reported to bind to its promoter (Shafikhani *et al.*, 2002). Thus, *sinI* and *abrB* transcription provide reliable readouts for the availability of Spo0A-P.

yaaT, *ylbF* and *ymcA* are needed to repress *abrB* transcription

It was found previously that in a *ylbF* knockout, *abrB* is incompletely repressed during growth in DSM, a medium that induces spore formation, consistent with a role for YlbF in supporting the phosphorylation of Spo0A (Carabetta *et al.*, 2013). In order to extend these studies to biofilm formation, we have now investigated the roles of *ylbF*, *ymcA* and *yaaT* in the undomesticated strain 3610 growing in MSgg medium, which supports the robust expression of matrix genes (Kearns *et al.*, 2005). To measure the transcription of *abrB* in real time, a fusion of its promoter to the firefly luciferase (*luc*) gene was used. In order to interpret these data it is important to recognize that unlike fusions to *lacZ*, each data point in the luciferase assay has been shown to largely reflect a transcription rate (Mirouze *et al.*, 2011), rather than gene product accumulation.

ylbF, *ymcA* and *yaaT* knockouts mitigate the Spo0A-P-dependent decrease in the *abrB* transcription rate (Fig. 2A). It was reported previously that the rate of transcription of many genes fluctuates during growth, probably in response to minor changes in growth rate (Mirouze *et al.*, 2011). Despite the swings in the rate of *abrB* transcription that are evident in Fig. 2A, it is clear that the knockout strains with the 3610 background exhibit a sustained increase in the rate of *abrB* transcription during growth in MSgg medium compared to the wild-type strain. This is also true for the transcription of *abrB* in 3610 strains growing in DSM, a medium that supports sporulation (Fig. 2B). In Fig. 2B, the rate of *abrB* transcription in the three knockouts was intermediate between the wild-type and the *spo0A* knockout strains, consistent with the high affinity of the *abrB* promoter for Spo0A-P (Fujita *et al.*, 2005) and with the incomplete dependence of the phosphorelay on the Y-complex *in vitro* (Carabetta *et al.*, 2013). As the cultures approached the stationary phase of growth, the rates of *abrB* transcription decreased in all the strains (Fig. 2B). This unexplained decrease is independent of Spo0A-P because it also occurred in the *spo0A* knockout strain. It is important to note that the *ylbF*, *ymcA* and *yaaT* knockouts do not affect the transcription of *spo0A* or the amount of Spo0A protein early in stationary phase and that YlbF, YmcA and YaaT must therefore stimulate the phosphorylation of Spo0A or the activity of Spo0A-P (Carabetta *et al.*, 2013).

The transcription rate of P*abrB* would be expected to be sensitive to small fluctuations in the concentration of Spo0A-P during the course of growth because of the high binding affinity of Spo0A-P for this promoter (Fujita *et al.*, 2005) and because in the absence of YlbF, YmcA or YaaT a low level production of Spo0A-P probably takes place. For this reason there was more variation from experiment to experiment in the rate curves for P*abrB* than for other promoters. Nevertheless, the patterns shown in Fig. 2 are reproducible: the transcription rates in the *ylbF*, *ymcA* and *yaaT* mutants were consistently elevated compared to the wild-type in repeated experiments. Fig. S1 shows average data for the P*abrB-luc* reporter plotted with the standard deviations at each time point.

If *ylbF*, *ymcA* and *yaaT* affect *abrB* transcription by stimulating the phosphorylation of Spo0A rather than the activity of Spo0A-P, we would expect that the effects of the knockouts might be suppressed by the *sof-1* allele of *spo0A*. The *sof-1* mutation is believed to relax the specificity of Spo0A so that it can be directly phosphorylated by one or another kinase at a higher rate than the wild-type Spo0A protein, bypassing the intermediate phosphorelay

proteins Spo0F and Spo0B (Hoch *et al.*, 1985, Spiegelman *et al.*, 1990). Indeed a *ymcA* knockout is completely bypassed for repression of *abrB* transcription by the *sof-1* mutation in MSgg (Fig. 2C) and in DSM (Fig. 2D). In fact, the transcription rates in the presence of *sof-1* are even lower than in the wild-type strain (Fig. 2C). Because the *sof-1* strains used for this experiment are deleted for *spo0F* these lower rates suggest that the complete phosphorelay may normally restrain the rate of Spo0A phosphorylation.

yaaT, *ylbF* and *ymcA* are needed for the activation of *sinI* transcription

The *sinIR* locus is transcribed from three promoters (Fig. 3A), producing three overlapping transcripts (Gaur *et al.*, 1988, Shafikhani *et al.*, 2002, Lehnik-Habrink *et al.*, 2011b, DeLoughery *et al.*, 2016). An upstream promoter (P1) drives expression of both *sinI* and *sinR*, to produce a transcript of ~1.2–1.5 kb. A Spo0A-P-dependent promoter (P2) also drives expression of both genes, producing a ~0.7 kb transcript and a third, presumably constitutive promoter (P3), drives the expression of only *sinR* to produce a transcript of ~0.4 kb (Fig. 3A). Since transcription from P2, one of the two promoters that drive the expression of *sinI* is dependent on the direct binding of Spo0A-P, we would expect that transcription of *sinI* would be decreased in *ylbF*, *ymcA* and *yaaT* knockouts. Three tools have been used to test this crucial prediction of the phosphorelay model; a P2*sinI-luc* fusion, a P2*sinI-gfp* fusion examined by fluorescence microscopy and quantitative real-time PCR (qRT-PCR) measurements of native *sinI*- and *sinR*-containing transcripts. Note that both P2 fusions were inserted at the native locus.

The inactivation of *ymcA, ylbF* or *yaaT* caused decreased expression of a *luc* fusion to the Spo0A-P-dependent P2 promoter (Fig. 3B), as predicted by our hypothesis. This decrease is slightly less extreme than that imposed by inactivation of the phosphorelay using a *spo0F* knockout (compare panels B and D), suggesting that the deficit in Spo0A-P is not complete in *ylbF*, *ymcA* and *yaaT* knockouts, consistent with prior *in vitro* results (Carabetta *et al.*, 2013). Some *ylbF*, *ymcA* and *yaaT* independent transcription is detectable even in the *spo0F* knockout, presumably due to the presence of P1. Importantly, the Spo0A-P-dependent rise in *sinI* transcription that takes place as cells approach stationary phase is absent in the knockout strains (Fig. 3B). The *sad-67* allele of *spo0A* bypasses the phosphorelay by making Spo0A constitutively active and therefore independent of the need to be phosphorylated (Ireton *et al.*, 1993). If the effect of the three knockouts on *sinI* transcription were indeed due to the decreased production of Spo0A-P, we would expect the *sad-67* mutation to suppress the effects of *ylbF*, *ymcA* and *yaaT* inactivation on P2 transcription. The *ylbF* and *ymcA* knockouts were indeed suppressed when the *sad-67* gene was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG, Fig. 3C (+I lines)), strongly supporting a role for YlbF, YmcA and YaaT in regulating matrix and biofilm formation via their positive effects on the production of Spo0A-P. It is worth noting that the wild-type *spo0A* gene is present in the *sad-67* strains. Consequently, the lower than wild-type expression of *sinI-luc* in the *ylbF* and *ymcA* knockouts when IPTG is not added reinforces the conclusion that the these genes affect Spo0A-P phosphorylation (compare Figs. 3B and C).

In addition, the *sof-1* allele of *spo0A* also bypasses *ymcA* for the expression of *sinI-luc* (Fig. 3D), lending additional support for the conclusion that YlbF, YmcA and YaaT contribute to

biofilm formation by enhancing the production of Spo0A-P. Although suppression of a *spo0F* knockout by the *sof-1* allele yields higher than wild-type expression, the bypass of the *spo0F ymcA* double knockout is partial. A possible explanation is that YmcA may assist in the phosphorylation of a kinase or in the transfer of a phosphoryl group from a kinase to Spo0A. In Fig. 3D it is evident that the levels of *sinI-luc* expression in *spo0F* and s*po0F ymcA* strains are the same, consistent with these two genes acting on the same pathway to produce Spo0A-P.

The conclusion that YlbF, YmcA and YaaT support the production of Spo0A-P is further buttressed by the use of qRT-PCR. The total abundance of *sinI* transcript sequences, which derive from P1 and P2, was reduced about five-fold in *ylbF*, *ymcA* and *yaaT* knockouts as well as in a knockout of *spo0F* (Fig. 4A). Importantly, the abundance of *sinR* sequences was also reduced, although more modestly, reaching statistical significance for *yaaT* and *ylbF* (Fig. 4B). The lower level of the *sinR* transcript would be expected if the knockouts were to decrease transcription from the P2 promoter (Fig. 3A). Importantly therefore, we have not only shown a dependence of *sinI* transcription on YlbF, YmcA and YaaT, but we have failed to reproduce the 2.8-fold increased abundance of *sinR* mRNA sequences reported by DeLoughery et al (2016) in the absence of YlbF, based on their use of Northern blots. Lehnik-Habrink et al (2011b) have shown that depletion of the *rny* gene product leads to stabilization of the *sinR* transcript. We have confirmed this result using qRT-PCR (Fig. 4B), demonstrating clearly that the effects of *rny* and of *ylbF*, *ymcA* and *yaaT* inactivation on *sinR* mRNA are opposite. Thus the *ylbF*, *ymcA* and *yaaT* knockouts do not phenocopy an *rny* knockout and their biofilm defects cannot be ascribed to the stabilization of *sinR* mRNA. Note that in these qRT-PCR experiments we have normalized our data using sequences from *fusA*, the gene for elongation factor G (EFG). In independent experiments, Western blotting with anti-EFG has shown that the *ylbF*, *ymcA* and *yaaT* knockouts do not contain elevated levels of this protein (not shown).

As a third approach to test whether these knockouts affect transcription from the P2*sinIR* promoter, we employed fluorescence microscopy with a fusion of *gfp* to this promoter. This is a telling approach because it allows the study of P2 expression in individual cells. It has been shown that expression from this promoter is heterogeneous, presumably due to cell-to-cell variation in the levels of Spo0A-P (Chai *et al.*, 2008). We therefore expected to see at least some cells exhibiting enhanced fluorescence as Spo0A becomes phosphorylated and we further predicted that this enhancement would be dependent on *ylbF*, *ymcA* and *yaaT*. Fig. 5 confirms these predictions and shows that the heterogeneous high-level expression of GFP-fluorescence seen in wild-type 3610 is absent in the knockout strains. In fact the entire distribution of fluorescence intensity is shifted to lower values in the knockouts. The histograms shown in Fig. 5 suggest that as strain 3610 grows in MSgg, the population distribution of *sinI* expression does not bifurcate but rather that the entire distribution shifts, with a pronounced skew toward higher intensities, presumably leading some cells to pass a threshold concentration of SinI adequate to trigger matrix gene transcription. This shift is dependent on Spo0F (and hence on Spo0A-P) as well as on YlbF, YmcA and YaaT (Fig. 5).

The data presented above show that the rate of *sinI* transcription, the abundance of *sinI* mRNA and the translation of GFP protein produced from the *sinI* P2 promoter are all

reduced in *ylbF*, *ymcA* and *yaaT* knockouts. These three experimental approaches, together with the *sof-1* and *sad-67* suppression data, strongly indicate that YlbF, YmcA and YaaT augment the yield of Spo0A-P for the activation of *sinI* transcription. The results shown in Fig. 2 suggest the same for *abrB* repression under sporulation and biofilm forming conditions. The data in Fig. 4B clearly demonstrate that the *ylbF*, *ymcA* and *yaaT* knockouts do not phenocopy *rny* inactivation for the increase in *sinR* mRNA abundance shown for depletion strains by Laalami et al (2013) and by the stability study of Lehnik-Habrink et al (2011b). We conclude that YlbF, YmcA and YaaT do not exert their effects on biofilm formation by destabilizing the *sinR* transcript and we further conclude that there is no evidence that Rny mediates the developmental defects of knockouts of *ylbF*, *ymcA* or *yaaT*.

YlbF, YmcA and YaaT affect P*tapA-luc* expression via a SinR and Spo0A-P-independent mechanism

The *tapA-sipW-tasA* operon is required for the formation of biofilm matrix and is directly repressed by SinR and AbrB (Chu *et al.*, 2006, Hamon *et al.*, 2004, Chu *et al.*, 2008) (Fig. 1). TapA and TasA are protein components of the matrix (Romero *et al.*, 2010, Romero *et al.*, 2011). As expected, transcription of the *tapA* operon is severely inhibited by *ylbF*, *ymcA* and *yaaT* knockouts (Fig. 6A). However, a *sinR* knockout does not completely bypass the inactivation of the three genes for *tapA* expression (Fig. 6B). Although appreciable bypass is evident, the levels of expression in the *sinR* strains carrying the knockouts are considerably less than in the *sinR* strain that is wild-type for *ylbF*, *ymcA* and *yaaT*. In fact the bypassed levels do not even reach the expression rate of the strain that is wild-type for both *sinR* and the three genes, particularly in the case of *yaaT* (compare Fig. 6A and B). This incomplete bypass can also be seen from examination of biofilm colonies on MSgg agar (Fig. 6E). The colonies of the *sinR* strains which are also deficient for *ylbF*, *ymcA* or *yaaT* are less wrinkled than the wild-type parent and much less so than the hyper-wrinkled colonies of the *sinR* strain that is wild-type for these three genes. If the stimulation of the phosphorelay provided the entire explanation for the roles of YlbF, YmcA and YaaT, we would expect this bypass to be complete. It is likely that the partial nature of the *sinR* bypass effect on colony morphology and on the expression of *tapA* is due to both the higher concentration of AbrB present in the *ylbF, ymcA* and *yaaT* knockouts and to a Spo0A-independent role of YlbF, YmcA and YaaT in supporting the expression of *tapA*. Such a role is strongly suggested by the failure of *sof-1* to bypass *ymcA* (Fig. 6C) and by the very slight *sad-67* bypass of *ymcA* for *tapA* operon expression (Fig. 6D). Although slight, this bypass is real and is evident for pellicle formation by *ylbF sad-67* and *ymcA sad-67* strains when IPTG is present during growth (Fig. S2). Interestingly, there is little or no bypass of pellicle formation by *sad-67* in the *yaaT* knockout, suggesting that the YaaT protein may have a role independent of its two binding partners or may be able to function to some extent in the presence of either YlbF or YmcA. As expected, no bypass of pellicle formation by *sof-1* was detected (not shown). Since both *sof-1* and *sad-67* efficiently bypass the loss of *spo0F* for *tapA* expression, albeit with altered kinetics, it is clear that the corresponding mutant forms of Spo0A are active (Fig. 6C and D). We conclude that while YlbF, YmcA and YaaT are required for the expression of the *tapA* operon and in biofilm formation by supporting the production of Spo0A-P, they have an additional important role in matrix gene expression.

ylbF, *ymcA* and *yaaT* and the K-state

ComK is the master regulator of the K-state. The regulation of *comK* transcription involves Spo0A-P-dependent and ComK-dependent components (Mirouze *et al.*, 2012). As cells approach stationary phase the level of Spo0A-P rises and activates expression of *comK* by direct binding to three operator sites upstream from the start site for transcription. This initiates a low level of ComK synthesis. As the level of Spo0A-P continues to rise, it binds to two repression sites in the *comK* promoter region, shutting down transcription. Thus, within a temporal window of opportunity set by the kinetics of Spo0A-P accumulation and by the binding constants for Spo0A-P, some cells in the population (~15% in the domesticated strains of *B. subtilis*) achieve a level of ComK sufficient to activate a positive feedback loop, mediated by the association of ComK with its own promoter. These cells enter the K-state, stop growing (Hahn *et al.*, 2015) and become competent for transformation. The transient Spo0A-P-dependent "uptick" in *comK* expression can be readily measured by using a *PcomK-luc* reporter in a background in which *comK* has been inactivated.

The *ylbF*, *ymcA* and *yaaT* knockouts express *comK* poorly (Carabetta *et al.*, 2013). If the three proteins affect *comK* expression by supporting the production of Spo0A-P, we would expect that in the knockouts the initial rise in this basal level expression of *comK* would be eliminated or perhaps delayed. If the rise were merely delayed, the subsequent decrease in transcription rate might be mitigated. The uptick, measured in a *comK* background, is in fact delayed in a *ymcA* knockout and the decline in the uptick is indeed slowed (Fig. 7A), consistent with a decreased rate of Spo0A-P formation. Both of these effects, which have been seen in five independent experiments, are consistent with a positive role for YmcA, and presumably for YlbF and YaaT as well, in the production of Spo0A-P. However, this result does not explain the K-state deficiency of the *ylbF*, *ymcA* and *yaaT* mutants because the area under the rate curve shown for *ymcA* in Fig. 7A is obviously greater than in the wild-type strain. Consequently, in a $comK^{+}$ strain, a *ymcA* knockout should have resulted in an increase in the number of K-state cells and thus in an increase in the expression of *comK*. Such an increase does not take place (Fig. 7B). This contradiction demonstrates that although *ymcA* is needed for normal K-state regulation due to the part it plays in regulating the production of Spo0A-P, it has an additional role in the K-state. Although *sof-1* can appreciably suppress the loss of *spo0F* for *comK* expression in a background that is wild-type for *comK,* it does not suppress *ymcA* for *comK* expression (Fig. 7B). Thus, it appears that in addition to playing a role in the proper timing of *comK* expression by controlling the synthesis of Spo0A-P, YmcA does something else for the K-state.

ylbF, ymcA and yaaT and sporulation

Strains carrying knockouts of these three genes are deficient in sporulation and exhibit a severe block at the level of Spo0A phosphorylation, manifested as a defect in transcription from the *spoIIG* and *spoIIE* promoters. This defect was largely bypassed by the *sad*-67 allele of *spo0A* (Carabetta *et al.*, 2013, Tortosa *et al.*, 2000). To investigate this further, it was determined whether the *sof-1* allele could also bypass *ymcA* for the transcription of a *spoIIG-luc* reporter. The *sof-1* strains used for these studies carried a knockout of *spo0F.* The *spoIIG-luc* transcription rate increases sharply in the wild-type strain as the culture enters stationary phase, while the rates of the *ymcA* and *spo0F* strains are nearly zero (Fig. 8). Fig.

8 also shows that transcription in the *sof-1 spo0F* and *ymcA sof-1 spo0F* strains increases only after delays compared to the wild-type. Once these delays are completed, the slopes of all the curves are similar. These results suggest that the rates of Spo0A phosphorylation are not restored to the wild-type level by *sof-1*, so that the amount of Spo0A-P reaches the high threshold required for *spoIIG* transcription more slowly in the *spo0F* and *ymcA* strains. Although the bypass of *ymcA* is therefore less complete than that of the *spo0F* knockout, substantial suppression by *sof-1* is evident, consistent with a role for YmcA in the phosphorylation of Spo0A. The delayed expression of *spoIIG-luc* expression in the *spo0F* knockout strain in the presence of *sof-1* may be due to the inability of kinases to directly phosphorylate the mutant Spo0A protein as rapidly as they do Spo0F. The even longer delay in the suppressed *ymcA* mutant suggests that YmcA may stimulate the autophosphorylation of KinA or the transfer of the phosphoryl group from KinA-P, as suggested above to explain the partial suppression of *sinI* expression in the *ymcA* knockout by *sof-1*. In addition, *sof-1* exhibits a substantial suppression of the sporulation deficiencies of the *ylbF*, *ymcA* and *yaaT* knockouts (Table 1). In the presence of the *sof-1* allele, the sporulation frequency for *ymcA* is suppressed to 36% of the wild-type level, whereas a *spo0F* knockout mutation is completely bypassed. The absolute numbers of spores/ml for the *ymcA spo0F sof-1* strain are about 6% of the numbers reached by the *spo0F sof-1* strain but 118-fold greater than that of the *ymcA* strain, showing substantial but incomplete suppression of the *ymcA* knockout. Suppression of a *yaaT* knockout for sporulation by *sof*-1 has been reported previously (Hosoya *et al.*, 2002). Taken together, the *sof-1* and *sad-67* suppression results for *spoIIG* transcription and spore formation provide strong evidence that YlbF, YmcA and YaaT are needed for efficient Spo0A phosphorylation..

The measurements of spore formation presented to this point were carried out in domesticated strains of *B. subtilis*. During the course of this work, it was observed that the effects of *ymcA* and *ylbF* knockouts in 3610 on spore formation determined after 24 hours, were much milder than in the domesticated background (Fig S3). Although significantly greater, the effect of a *yaaT* knockout was also slightly less extreme than in the domesticated background. We have shown that the stronger penetrance of the *ylbF*, *ymcA* and *yaaT* knockouts in the domesticated strain is due to a T to C transition mutation in *sigH*, resulting in a V117A substitution present in all the sequenced domesticated strains and absent in undomesticated *B. subtilis* strains and their close relatives (Fig. S4). In wild-type 3610 the *spoIIG* transcription rate increases more sharply and reaches a higher level than in the domesticated background (Fig. S5A). When the V117A substitution was introduced into the *sigH* gene of strain 3610, the response of *spoIIG* was blunted (Fig. S5A). We interpret these kinetic differences as evidence that under sporulation conditions, the amount of Spo0A-P reaches its maximal level more rapidly in 3610 than in the domesticated strain, because it expresses a wild-type SigH. What is more, the SigH V117A change explains the difference in penetrance of the *ylbF*, *ymcA* and *yaaT* mutations, as demonstrated for both sporulation and *spoIIG* transcription (Figs. S3 and S5B–D). It is noteworthy that in the 3610 background with and without the *sigH* mutation, the initial rise in *spoIIG* transcription is followed by a pronounced decline (Fig. S5A). This decline may be due to the presence of RapP in 3610, which is known to dephosphorylate Spo0F-P (Parashar *et al.*, 2013, Omer Bendori *et al.*,

2015). RapP may also cause the lower maximal expression of *spoIIG* in 3610 carrying the *sigH* mutation, compared to the expression in the domesticated strain.

Most likely the commonly used domesticated strains express a crippled SigH. This may slow the production of Spo0A-P in several ways. The RNA polymerase holoenzyme with SigH transcribes *kinA, spo0F* and *spo0A* (Predich *et al.*, 1992) and as a result the increase in these phosphorelay proteins during sporulation of the domesticated strains is probably slowed. Also, *phrE,* which encodes an antagonist of the RapE phosphatase, is transcribed by SigH-RNA polymerase (McQuade *et al.*, 2001) and as a result RapE may remove phosphoryl groups from Spo0F-P more rapidly in the domesticated strain. We suggest that because the rate of Spo0A-P synthesis is challenged by the *sigH* mutation, the domesticated system is more dependent on the phosphorelay-accelerating roles of YlbF, YmcA and YaaT. This indirect argument provides further confirmation that these proteins accelerate the production of Spo0A-P. Despite the relatively small effects of *ymcA* and *ylbF* on the final frequency of spore formation in 3610, their kinetic effects on *spoIIG* transcription are substantial (Fig. S5), illustrating the importance of these genes for sporulation even in the undomesticated strain.

YlbF, YmcA and YaaT do not appear to be membrane-localized

Based on fluorescence microscopy, it has been reported that YaaT-GFP is associated with the cell membrane (Hosoya *et al.*, 2002) and Rny is known to be an integral membrane protein (Lehnik-Habrink *et al.*, 2011a). Deloughery et al (2016) have offered the reported membrane localization of YaaT as support for their hypothesis. We have re-examined the localization issue, using fusions of YFP to the C-termini of YlbF, YmcA and to the N-terminus of YaaT and an Rny-YFP construct (a kind gift from Jörg Stülke) as a control for membrane localization. All three of our fusion proteins are functional (Carabetta *et al.*, 2013). Fig. S6 shows deconvolved images of cells from the four strains. Although the Rny-YFP signal is faint, it appears to be restricted to the cell periphery as expected, while the YlbF, YmcA and YaaT fusions are located in the cytoplasm, presenting a lumpy or punctate distribution. These images do not support the contention that these proteins are associated with the membrane. We cannot exclude that they do so transiently or to a limited extent. It is worth pointing out that our YaaT fusion is to the N-terminus of YaaT and that of Hosoya et al (2002) is to the C-terminus.

Bacterial two-hybrid (B2H) analysis suggests an association of YlbF, YmcA and YaaT with Spo0F, Spo0B and possibly with KinA

Our previously published B2H data (Carabetta *et al.*, 2013), obtained in *E. coli* using the adenyl cyclase system (Karimova *et al.*, 1998), detected an interaction of YmcA with itself, with Spo0F and Spo0B, but not with Spo0A and KinA. In contrast, no interactions of YlbF and YaaT with the phosphorelay proteins were detected. However, we have recently determined that a number of the restriction sites contained within the multiple cloning site of the vector p*T25N-kan* are not in-frame with the *cya* fragment, contrary to the published description (Karimova *et al.*, 1998). This caused the T25 fragment to be out of frame with the cloned genes in our p*T25* constructs. Inspection of the p*T25N-ymcA* plasmid revealed an overlooked deletion of a cytosine nucleotide near the 3′ end of the *ymcA* gene. This

mutation restored the reading frame for the T25 fragment, at the same time changing the last 4 amino acid residues at the C-terminus of YmcA. The remaining intact YmcA sequence apparently still interacted with partner proteins and with itself. The self-interaction was not unexpected because YmcA has been crystallized as a dimer (Seetharaman, 2009). To determine whether interactions involving YlbF and YaaT had been missed because of our error, new T25 fusions to *ymcA*, *ylbF* and *yaaT* were constructed and the B2H experiments were repeated in 4 independent trials. A statistically significant increase relative to the empty vector control was interpreted as a positive interaction. No significant interactions were detected with Spo0A (Table S3), consistent with our recent *in vitro* observation that the purified Y-complex stimulates phosphorylation of the phosphorelay intermediates in the absence of Spo0A (not shown). However, with these corrected constructs, significant interactions were detected for all three proteins with both Spo0F and Spo0B, as well as among YlbF, YmcA and YaaT (p-values < 0.0001). The association signals for the interaction of YlbF, YmcA and YaaT with Spo0B and Spo0F were similar in strength to that observed for YmcA with YaaT. No significant interactions were detected with KinA when the *cya* fragment was fused to the N-terminus (T18-KinA). Switching the fragment to the C-terminus (KinA-T25) resulted in an apparently significant interaction with YmcA and YlbF (p-value = 0.048 and 0.005, respectively). However, because the KinA-T25 and T18 pair exhibited an unusually high background signal, we regard these KinA interactions as tentative. The strongest association was seen for the YmcA-YlbF heterodimer (Table S3).

Discussion

A central finding of the present study is that YlbF, YmcA and YaaT do support the production of Spo0A-P and that this role is important for biofilm formation and the K-state as well as for sporulation. A second result is that the three proteins play additional uncharacterized roles in *comK* expression for the K-state, in expression of the *tapA* operon and for biofilm formation. A third finding is the suggestive evidence from B2H experiments that YlbF, YmcA and YaaT all interact with Spo0F, Spo0B and possibly with KinA. Finally it has been shown that domesticated strains carry a mutant form of *sigH*, which affects the penetrance of the *ylbF*, *ymcA* and *yaaT* phenotypes for sporulation.

YlbF, YmcA and YaaT support the production of Spo0A-P

Any interference with the production of Spo0A-P would result in an increase in the rate of transcription from the *abrB* promoter and a decrease in the transcription from P2*sinI* (Fig. 1), thus abrogating the formation of biofilms. DeLoughery et al (2016) have reported that in the absence of YlbF or YmcA, the transcription of a *sinI-lacZ* fusion was unaffected and that in the absence of YlbF or YaaT, the expression of an *abrB-lacZ* fusion was likewise not altered. In contrast, we report here that in the absence of any one of the three proteins, *sinI* transcription is decreased and that of *abrB* is enhanced (Fig. 2, 3, 4 and 5). In the case of *sinI,* these effects have been documented using P2*sinI-gfp* and P2*sinI-luc* reporters and by qRT-PCR. The failure to detect these effects may lie in both the methods used by DeLoughery et al (2016) and in the kinetics of *sinI* and *abrB* transcription. Although the use of *lacZ* fusions is powerful, its determination is inherently less accurate than the use of fusions to *luc* because light output in the luciferase assay directly reports the rate of

transcription whereas β-galactosidase accumulates, making the determination of rate differences between two strains less accurate when these differences are transient. With the plate reader assay, readings are taken every two minutes in real-time, rather than at widely-spaced intervals, increasing the allover accuracy of data collection. The transcription rate of *sinI* is quite low in MSgg and consequently inherently difficult to measure accurately. In the case of *abrB*, differences in the rates of transcription using *lacZ* are also difficult to detect because the total β-galactosidase in growing cultures is initially high and then decreases as Spo0A becomes phosphorylated. Thus, in the wild-type strain, even if transcription of *abrB* were to cease entirely, decreases could only result from dilution as cells divide or if the β-galactosidase were unstable. Rate differences would be particularly challenging to observe when the effect is partial, as with the *ylbF*, *ymcA* and *yaaT* knockouts. Whatever the cause of the discrepancies, we believe that the present results, obtained in the 3610 background with cultures growing in MSgg, are particularly robust; they have been documented for all three knockouts and in the case of *sinI*, using three methods. Thus, a total of five developmental promoters that are directly regulated by Spo0A-P have been shown to respond predictably to knockouts of *ylbF*, *ymcA* and *yaaT*: P*spoIIG*, P*spoIIE*, P*abrB*, P*sinI* and P*comK* (this work, Carabetta et al (2013), Hosoya et al (2002) and Tortosa et al (2000)).

In addition to the *in vivo* data presented in this study that support a role for the YlbF, YmcA and YaaT in the accumulation of Spo0A-P, it has been shown that the complex of these three proteins accelerates the phosphorelay *in vitro* and that inactivation of *spo0E*, which encodes a Spo0A-P phosphatase, partially bypasses *ylbF*, *ymcA* and *yaaT* knockouts (Carabetta *et al.*, 2013). An independent study concluded that *yaaT* is an early spore gene that supports the formation of Spo0A-P (Hosoya *et al.*, 2002). An interesting further indication is the finding that the three knockouts increase cell chaining as cells approach stationary phase (Carabetta *et al.*, 2013). This observation prompted the investigation of a *spo0A* knockout, which was also found to exhibit increased chaining, a phenotype that to our knowledge had not been reported previously. Thus, our model led to a verified prediction. We conclude that the preponderance of evidence supports a role for these proteins in accelerating the phosphorelay.

Is the Rny model correct?

An important point of difference in the two models lies in the effect of *ylbF*, *ymcA* and *yaaT* knockouts on the amount of *sinR* mRNA. In the report by DeLoughery et al (2016) it was claimed that the level of this transcript, measured in Northern blots, was increased about 2.8-fold in a *ylbF* mutant and by an unspecified amount in a *ymcA* knockout. Although a loading control (stained rRNA) is included in the relevant figure (Fig. 3 in DeLoughery et al (2016)), the signals were apparently not normalized to the load, which appears from the figure to be slightly less for the wild-type strain. The present qRT-PCR data fail to show an increase in mRNA abundance and instead show that the level of the *sinR* mRNA is somewhat decreased in all three knockouts compared to the wild-type. This decrease is consistent with our model, because the P2 promoter drives the transcription of both *sinI* and *sinR*. Here again, we propose that the discrepancies in our findings can be explained by the methods used. Northern blotting is inherently less sensitive than qRT-PCR and is difficult to quantitate accurately (Reue, 1998), particularly in the case of low abundance transcripts like

those from the *sin* locus. Nevertheless, in Fig. 3C of the DeLoughery et al paper, the 700 bp *sinIR* transcript disappears in the absence of YlbF and YmcA, as would be expected from the phosphorelay model and consistent with the present data derived from the use of luciferase fusions, qRT-PCR and microscopy with a *gfp* fusion to the *sinIR* promoter. Lehnik-Habrink et al (2011b) have shown that when Rny is depleted, the 400 bp *sinR* transcript is stabilized. Both we (Fig. 4) and DeLoughery et al (2016) have confirmed this result for an *rny* knockout. Because the qRT-PCR data (Fig. 4) show that the *sinR* transcript actually decreases in abundance in the *ylbF*, *ymcA* and *yaaT* knockouts, as do transcripts carrying the *sinI* sequence, we conclude that the absence of YlbF, YmcA and YaaT does not phenocopy the *rny* null mutation.

It is worth pointing out that the Rny model, even if the evidence supported it, could not be generalized to the K-state. *sinR* is an essential gene for competence expression while a *sinI* knockout, which phenocopies SinR overproduction, has no obvious K-state phenotype (Bai *et al.*, 1993). Thus, the stabilization of the *sinR* transcript could not explain the K-state phenotype of *ylbF*, *ymcA* and *yaaT* knockouts.

DeLoughery et al (2016) offer the slow growth phenotypes of the *rny* and *ylbF*, *ymcA* and *yaaT* mutants as further evidence that the knockouts phenocopy *rny*. Although slow growth is a rather non-specific phenotype, it is worth noting that the *ylbF*, *ymcA* and *yaaT* knockouts grow at room temperature on MSgg or LB agar, albeit more slowly than the wild-type. In contrast, an *rny* knockout strain does not form colonies at room temperature (not shown), thus exhibiting a phenotype shared with knockouts of other degradosome components (Awano *et al.*, 2007, Luttinger *et al.*, 1996, Purusharth *et al.*, 2007, Wang & Bechhofer, 1996).

DeLoughery et al (2016) offer an additional argument in favor of the Rny hypothesis that relies on synteny between *ymcA* and *rny*. Although synteny is at best only a suggestive indication of common function, we believe that their analysis has overstated the synteny for these two genes, because they have limited their study to only 3 *Firmicutes* other than *B. subtilis*. We have reexamined this issue with a more complete list of bacterial species, representing all of the major families of *Firmicutes* (Figs. S7 and S8). *ymcA* and *rny* are quite distant from one another in the genetic maps of some of the representative *Firmicutes* shown in Fig. S7. In fact *ymcA* appears to be more consistently syntenic with *mutS* and *mutL* than with *rny* although we are aware of no evidence that YmcA is involved in mismatch repair. Other genes in the neighborhood are also present as often as *rny,* notably *cotE* and *spoVS* among the spore formers and again there is no evidence that YmcA plays a role in spore coat assembly.

Although the weight of evidence is against the Rny model as stated, the IPs of Rny by tagged YlbF and YaaT (DeLoughery *et al.*, 2016, Carabetta *et al.*, 2013) and the bacterial two hybrid (B2H) experiments showing association of Rny with YlbF and YmcA (DeLoughery *et al.*, 2016) are indeed suggestive of association between these two proteins and Rny. The demonstration by DeLoughery et al (2016) that the inactivation of *ylbF* and *ymcA*, like the inactivation of *rny*, affect processing of the *cggR-gapA* transcript suggests that these interactions with Rny may have biological consequences. We emphasize that we

have no evidence excluding some sort of role for YlbF, YmcA and YaaT interaction with Rny in development. For example, this interaction may be involved in the Spo0A-P-independent activities of YlbF, YmcA and YaaT that are documented in this study. One problem is that the extreme pleiotropy of the *rny* mutant makes it difficult to falsify a role for its interactions other than by examining each mechanistic proposal in turn. In fact it has been reported that Rny-depletion alters the abundances of 51% of the annotated protein-encoding transcripts of *B. subtilis* (Laalami *et al.*, 2013).

Based on their Rny hypothesis, DeLoughery et al (2016) have suggested that the *ylbF*, *ymcA* and *yaaT* be renamed *rcsA, rcsB and rcsC* for "RNase Y-containing complex subunits A, B and C". We do not agree with this nomenclature, in part because we are not convinced that YlbF, YmcA and YaaT activate Rny. We therefore prefer not to include "Rny" in the acronyms in the absence of evidence showing that this interaction is central to the roles of YlbF, YmcA and YaaT. Importantly, a quaternary complex of these three proteins with Rny has not been shown to exist. A stable association, suggested by the word "subunits", has not been confirmed biochemically and there is no evidence that a quaternary complex actually exists *in vivo*, as implied by the Rcs nomenclature. For example it is possible that Rny associates independently with each of the three proteins or only with YlbF and YaaT. We believe that it is premature to rename these genes until more mechanistic insights are achieved.

Interactions of YlbF, YmcA and YaaT with phosphorelay components

If YlbF, YmcA and YaaT were involved in supporting the phosphorylation of Spo0A, we would expect them to interact with one or more components of the phosphorelay. In their B2H experiments, DeLoughery et al (2016) did not confirm the interactions of YmcA with Spo0F and Spo0B that we had reported (Carabetta *et al.*, 2013). However, our two laboratories used different B2H systems; theirs was based on an interaction between the λ C1 protein and the α subunit of RNA polymerase (Deighan *et al.*, 2008), while ours was based on the reassembly of adenyl cyclase fragments (Karimova *et al.*, 1998). These B2H experiments have now been repeated with new constructs, correcting a sequence error in our previously used plasmid clones that led to an apparent absence of YlbF and YaaT interactions with phosphorelay components. The results shown in Table S3 extend the list of putative interactions with Spo0B and Spo0F to YlbF, YmcA and YaaT and hint that YlbF and YmcA may also contact KinA. We believe that these suggestive data must be interpreted with caution. Although the positive controls (interactions among YlbF, YmcA and YaaT) and the negative controls (with empty vectors) all behaved as expected, these interactions must be verified by independent means, most convincingly with purified proteins. We have so far not observed interactions of YlbF, YmcA and YaaT with phosphorelay proteins in our IPs. However, these interactions may occur at growth stages that were not analyzed or may be transient. Nevertheless, the consistent *in vitro* stimulation of the phosphorelay (Carabetta *et al.*, 2013) argues strongly that some sort of interaction does take place.

YlbF, YmcA and YaaT have multiple roles

Several experiments show that YlbF, YmcA and YaaT play Spo0A-P-independent roles in biofilm formation, in the K-state and possibly for sporulation. Clear evidence for this in the

case of biofilm formation derives from the study of suppressor mutations of *spo0A* and their effects on *sinI*, *abrB* and *tapA* transcription. *ymcA* and *spo0F* knockouts are bypassed by *sad-67* and *sof-1* for *sinI* transcription and by *sof-1* for *abrB* transcription, consistent with a role for YmcA in the synthesis of Spo0A-P (Figs. 2D, 3C and 3D). We expected that these suppression effects would extend to *tapA*, which is repressed by AbrB and SinR (Fig. 1). However, *sad-67* and *sof-1* give little or no bypass of *ymcA* for *tapA* expression, while a *spo0F* knockout is largely suppressed by *sof-1* (Fig. 6). These observations indicate that *ylbF*, *ymcA* and *yaaT* support *tapA* expression not only by activating the synthesis of Spo0A-P but also by an additional mechanism. The regulation of *tapA* is not completely understood and at least two genes, *remA* and *remB* (Winkelman *et al.*, 2009, Winkelman *et al.*, 2013), have been shown to contribute to matrix gene expression independently of *spo0A* and *sinR*. Also, SlrR and YmdB are needed to make matrix (Kobayashi, 2008, Chai *et al.*, 2010, Diethmaier *et al.*, 2011). YlbF, YmcA and YaaT may act on one or more of these proteins or on their cognate genes, in addition to stimulating the production of Spo0A-P.

Similar data are available for the K-state, suggesting the existence of Spo0A-P-dependent and -independent roles for YlbF, YmcA and YaaT in establishing the K-state. The uptick in *comK* basal expression, which is governed by the concentration of Spo0A-P, exhibits a sustained elevation in a *ymcA* knockout, as expected if the Spo0A-P concentration were to increase slowly (Fig. 7A). Thus in wild-type cells, YlbF, YmcA and YaaT help determine the window of opportunity for transitions to the K-state and thus the fraction of cells that enter this state. However, in contrast to its suppression of *spo0F*, *sof-1* does not suppress the effect of a *ymcA* knockout for P*comK* expression suggesting an important Spo0A-P-independent role for this protein. YlbF, YmcA and YaaT may affect ComK production in a variety of obvious ways. One possibility is that the Y- proteins increase the stability of the *comK* or *comS* mRNAs either by limiting the activity of Rny or by some other mechanism.

The evidence for a Spo0A-P-independent role for YlbF, YmcA and YaaT in sporulation is more ambiguous. The data obtained with *sad-67* and *sof-1* show clearly that bypass of the knockouts for s*poIIG* transcription takes place in the presence of these alleles of *spo0A* (Fig. 8 and Carabetta *et al* (2013)). Although suppression is nearly complete in the case of *sad-67*, it is incomplete for *sof-1.* The partial nature of the *sof-1* bypass is probably due in part to a slow rate of Sof-1 protein phosphorylation, as shown by the delay in *spoIIG* transcription in the *sof-1 spo0F* strain compared with the wild-type strain (Fig. 8). But the fact that the delay is even longer in the *ymcA spo0F sof-1* strain hints that something else is taking place. As suggested above in connection with the incomplete bypass of *ymcA* inactivation for *sinI* expression, YmcA may affect the autophosphorylation of one or more of the kinases that donate a phosphoryl group directly to the Sof-1 protein or the transfer of a kinase phosphoryl group, consistent with the possible interactions of YlbF, YmcA and YaaT with KinA (Table S3). Thus, the data do not point clearly to a Spo0A-P-independent role for the three proteins in sporulation, but also do not exclude such a role.

More generally, a possible role for YlbF, YmcA and YaaT beyond the acceleration of Spo0A-P production is consistent with the fact that they are encoded by the genomes of bacteria that lack *spo0A* (not shown). It is also clear that the lack of YaaT has a more severe effect on pellicle formation than YlbF and YmcA in the presence of *sad-67* (Fig. S2),

implying additional complexity. It is noteworthy that *yaaT* is more widely conserved among the *Firmicutes* than are *ylbF* and *ymcA* (not shown), suggesting that it may have an independent role even in *B. subtilis*. Further work is clearly required to more fully reveal the biochemistry and physiology of these important proteins. Because the pathways for biofilm formation and for the K-state are quite distinct, it is tempting to suggest that YlbF, YmcA and YaaT are involved with some underlying aspect of metabolism, in addition to their roles in Spo0A-P formation.

Domesticated *B. subtilis* strains carry a mutant form of *sigH*

The point mutation present in all sequenced domesticated strains has a clear consequence for sporulation. As a result of the V117A replacement, the induction of *spoIIG* expression is slowed and its maximal transcription rate is decreased. This kinetic difference may be relevant to the fitness of *B. subtilis* in nature, where the rapid onset of sporulation in the face of stress may be advantageous. Remarkably, an A117V mutation was selected in the PY79 background, as a suppressor of the sporulation defect of a particular allele of *spo0A* (Bramucci *et al.*, 1995). Thus, the suppressor mutation restored the domesticated *sigH* to the "wild" state. Bramucci et al (1995) studied the effect of this *sigH* mutation on the transcription of *spoIIE* and *spoIIA*. In both cases they observed a elevated expression in the presence of this "wild" allele, consistent with the present data. In domesticated and undomesticated strains in which the two *sigH* alleles were swapped we found no difference in K-state induction and the mutation has no obvious effect on the appearance of biofilm colonies on agar (not shown) probably because less Spo0A-P is needed for these pathways than for sporulation. Because it is so widely distributed among laboratory strains, the V117A mutation may have been induced by the X-irradiation to which the parent of strain 168 was subjected (Burkholder & Giles, 1947).

Experimental procedures

Microbiological methods

Bacterial strains are listed in Table S1. The backgrounds used for all experiments were either IS75, a domesticated derivative of strain 168, or the undomesticated NCIB3610. Standard methods were used for plasmid constructions and the details are described in Supporting Information. Constructs were introduced into IS75 by transformation (Albano *et al.*, 1987) and into 3610 by transduction using bacteriophage SPP1 (Cozy & Kearns, 2010). One exception was for the swapping of the *sigH* alleles, which was carried out by transformation in both backgrounds, using the pMiniMad2 plasmid as described (Cozy & Kearns, 2010, Mukherjee *et al.*, 2013). A second exception was for the construction of certain strains with alleles of *spo0A* and the *tapA-luc* fusion. Because these two loci are linked it was convenient to combine them using transformation. In this case to avoid introducing unwanted mutations from IS75 by congression, the needed marker was first transduced into wild-type 3610 and then transferred from this intermediate strain by transformation. Pellicle and biofilm formation were assessed using MSgg medium (Kearns *et al.*, 2005) as described (Branda *et al.*, 2006), except that incubations were at room temperature (approximately 23°C). Spore frequency was determined as described (Carabetta *et al.*, 2013) using Difco Spore Medium (Schaeffer *et al.*, 1965) and K-state expression was measured in competence medium

(Albano *et al.*, 1987). All bacterial growth was at 37°C unless otherwise indicated. Antibiotic selections were carried out on Lysogeny Broth agar plates (Cozy & Kearns, 2010) containing ampicillin (100 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), erythromycin (10 μg ml^{-1}), tetracycline (25 μg ml^{-1}), kanamycin (5 μg ml^{-1}) or chloramphenicol (5 μg ml^{-1}). In some cases selection was for erythromycin (1 μg ml^{-1}) plus lincomycin (20 μg ml^{-1}). Solid media were solidified by the addition of 1.5% agar. When required, IPTG was added to a concentration of 1 mM. For movement of the *sof-1* allele, selection was applied for the erythromycin resistance marker associated with the accompanying Δ*spo0F* marker and the colonies were subjected to heat treatment as described for the original suppressor screen to select for those that had inherited *sof-1* (Kawamura & Saito, 1983). In each case the presence of the *sof-1* mutation was confirmed by sequencing.

Luciferase assays

Experiments were carried out as described (Mirouze *et al.*, 2011). Briefly, growth was at 37°C with shaking in the appropriate media, with the addition of luciferin (4.7 μM final concentration). Readings of optical density at 600 nm and of light output were taken at 2-minute intervals. A Perkin Elmer Envision 2104 plate reader, equipped for enhanced luminometry was used. The lids of the microtiter plates were maintained at 38°C to prevent condensation. All of the luciferase promoter fusions were inserted at their native loci by single reciprocal recombination using transformation with plasmid constructs. For each experiment growth and light output measurements were carried out in duplicate. Each plate reader experiment was repeated at least three times and representative results are shown in the figures.

qRT-PCR

Bacillus cells were grown in 10 ml of MSgg liquid media, with aeration for 6 or 8 hours at 37°C at which times they were in late log and early stationary phase respectively. Cells were harvested, and RNA was isolated using the FastRNA Pro Blue Kit (MP Biomedicals) as per manufacturer's instructions with one modification; samples were processed in the Fastprep instrument for 3 cycles (45, 45, and 30 seconds) at a speed of 6.0 to ensure efficient cell lysis. To ensure complete removal of genomic DNA contamination, samples were treated with the TURBO DNA-free Kit (Applied Biosystems) as per manufacturer's instructions. This step was essential because of the low abundance of the *sin* locus transcripts. The concentration and purity of RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). 2 μg of RNA was copied into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per manufacturer's instructions. For each RNA sample, a control reaction in the absence of reverse transcriptase was carried out to confirm the absence of genomic DNA contamination. Any remaining RNA was hydrolyzed by incubation with 0.1 M NaOH (final concentration) for 20 minutes at 70°C, and then neutralized by the addition of an equal volume of HCl. The reactions were cleaned using MinElute PCR purification spin columns (Qiagen) according to the manufacturer's instructions.

For qRT-PCR, all of the cDNA samples were diluted 1:5 (considered as 1x concentration) and then further diluted 1:10 twice (0.1x and 0.01x) to use as standards. The wild-type

cDNA was used for the standards and all other cDNAs were analyzed at the 0.1x concentration. Five μl of the cDNA dilutions or water (no-template control to ensure lack of contamination) were added to MicroAmp Optical tubes (Applied Biosystems) with 20 μl of a mixture of Power SYBR Green PCR master mix (Applied Biosystems) with 0.1 μM of each primer. Primers used were 5sinR-RT, 3sinR-RT, 5sinI-RT, 3sinI-RT, 5EFG-RT and 3EFG-RT (Table S2). Samples were analyzed on an ABI Prism 7700 sequence detector (Applied Biosystems) and data was analyzed (standard curve method) by using Sequence Detection Systems (SDS) software (v.1.9.1, Applied Biosystems). The *fusA* (EFG) transcript was used as an internal control to adjust for differing amounts of input cDNA. Experiments were performed from 3 biological replicates, and data points from individual runs were averages of triplicates.

Microscopy and image analysis

For the P2*sinI-GFP* fusion experiment, cells were grown in liquid MSgg media for 7 hours, at which point they were in the early stationary phase. An aliquot of cells was harvested, and diluted into PBS (81 mM Na_2HPO_4 + 24.6 mM NaH_2PO_4 + 100 mM NaCl). 1 μl of each culture was placed on an agarose pad made up in minimal medium and mounted on the microscope. Cells were imaged on a Nikon Eclipse Ti inverted microscope outfitted with an Orca Flash 4.0 Digital Camera (Hamamatsu) with a Nikon TIRF 1.45 NA Plan Neoflur 100 oil immersion objective. NIS-Elements AR (v 4.40, Nikon) was used for image acquisition and data analysis. The acquisition time for GFP fluorescence was identical for each analyzed strain. The images from all 5 strains were compared simultaneously, using the synchronizer tool. For each image, a background region of interest (“ROI”) was defined, and the average background was subtracted. The look-up tables (LUTs) were used for visualization, and all intensities were adjusted to an identical scale. Fluorescence intensity measurements were performed using the software’s automated General Analysis feature. Thresholds were established to define the objects of interest (cells), and were applied to each image. The program automatically measures the pixel intensity from the thresholded objects. At least 1200 cells per strain were counted. Similar observations were made from two independent analyses.

For YFP localization studies, cells were grown and prepared for microscopy as above. All acquisition times and parameters for the various samples were identical. Z-stacks were acquired in 27 steps using 0.2 μM slices, and images were deconvolved in NIS-Elements. Deconvolution was performed using the 3D Blind method with 10 iterations, allowing for background subtraction. Each image was enhanced in an identical fashion and for each a slice in the Z-stack from the center of the cell was selected for presentation in Fig. S6. Observations of localization were made 3 independent times.

Bacterial 2-hybrid analysis

B2H analysis was carried out exactly as described previously (Carabetta *et al.*, 2013). The construction of plasmids for B2H analysis is described in Supporting information.

Statistical methods

All statistical analyses were performed as described in Carabetta *et al* (2013). In short the logarithms of RT-PCR, spore counts and B2H data values were obtained and used for subsequent analysis. The software package Stata (version 13.1) was used to perform a one-way analysis of variance (ANOVA) followed by post hoc t-tests applying the Bonferroni correction to accommodate multiple comparisons. For the B2H data, a statistically significant fold increase relative to the empty vector control was interpreted as a positive interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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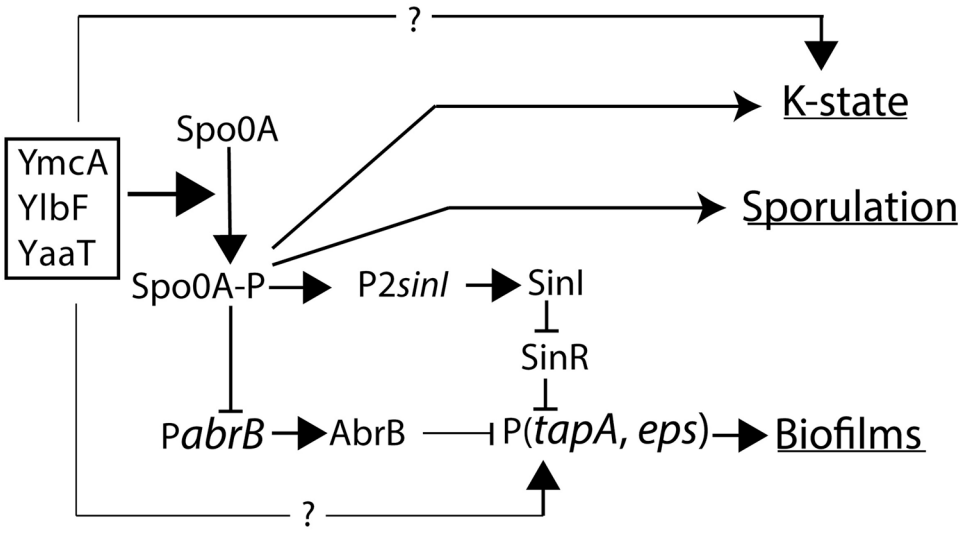

Fig. 1.

Regulation by YmcA, YlbF and YaaT. A complex of these three proteins stimulates the production of Spo0A-P by acting on the phosphorelay. Spo0A-P acts in turn to activate the transcription of *sinI.* SinI then sequesters SinR, preventing it from repressing the expression of the biofilm matrix operons. Spo0A-P also represses the transcription of *abrB*, which encodes another matrix gene repressor. Spo0A-P regulates the K-state and spore formation by acting both positively and negatively on the *comK* promoter and positively on early spore gene promoters. Finally, the three proteins act on matrix gene and K-state expression in at least one unknown, Spo0A-P-independent manner.

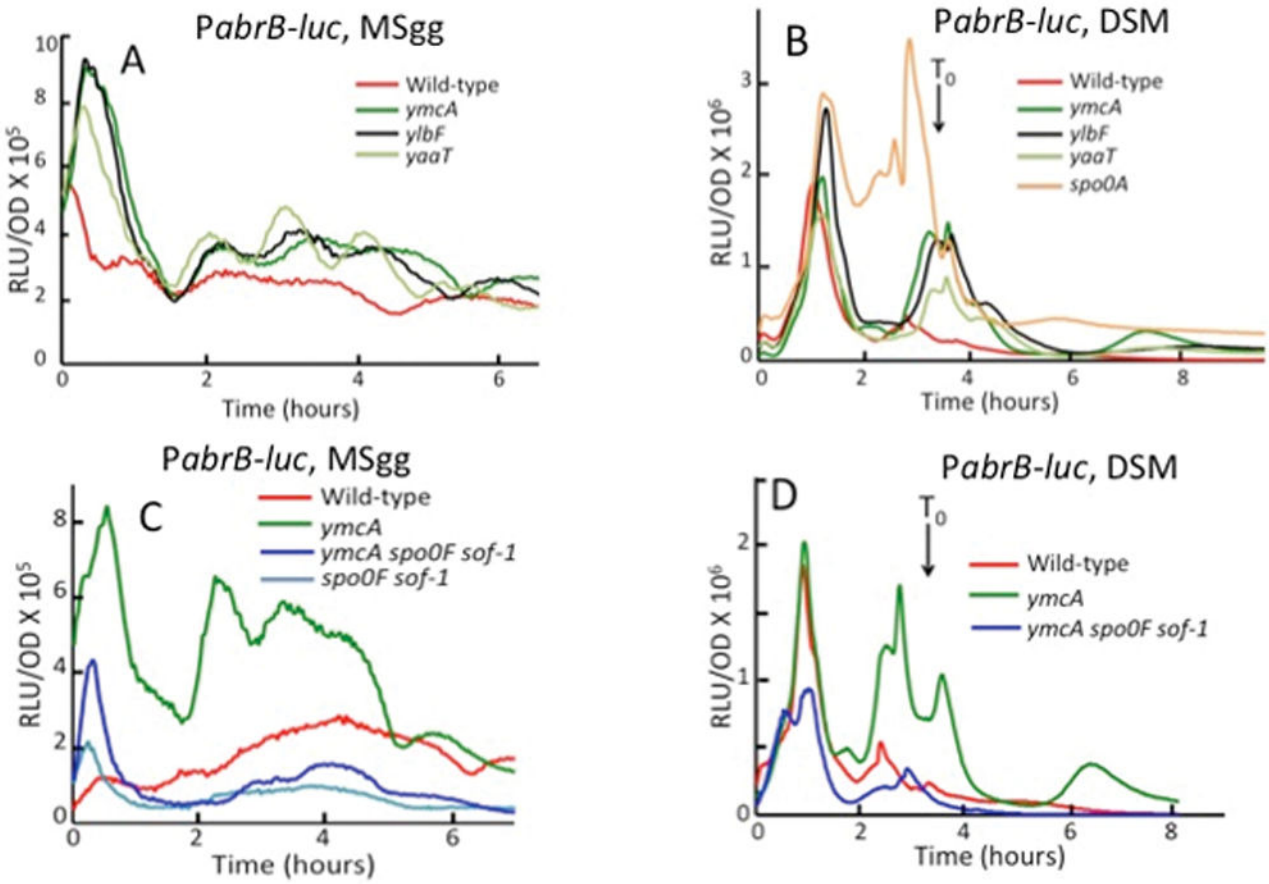

Fig. 2.

YlbF, YmcA and YaaT act negatively on the rate of *abrB* transcription by stimulating the production of Spo0A-P. Luciferase assays for P*abrB-luc* expression were performed as described in Experimental procedures. Cells were grown in MSgg (A and C) and DSM (B and D). The time at which cells enter stationary phase (T_0) is indicated in B and D. The graphs are color coded as indicated. The strains used in these experiments were as follows. (A and B) wild-type (BD7623), *ΔymcA* (BD7823), *ΔylbF* (BD7824), *ΔyaaT* (BD7825) *Δspo0A* (BD7788). (C and D) wild-type (BD7623), *ΔymcA* (BD7647), *ΔymcA Δspo0F sof-1* (BD7649), *Δspo0F sof-1* (BD7646). All the strains were constructed in the 3610 background.

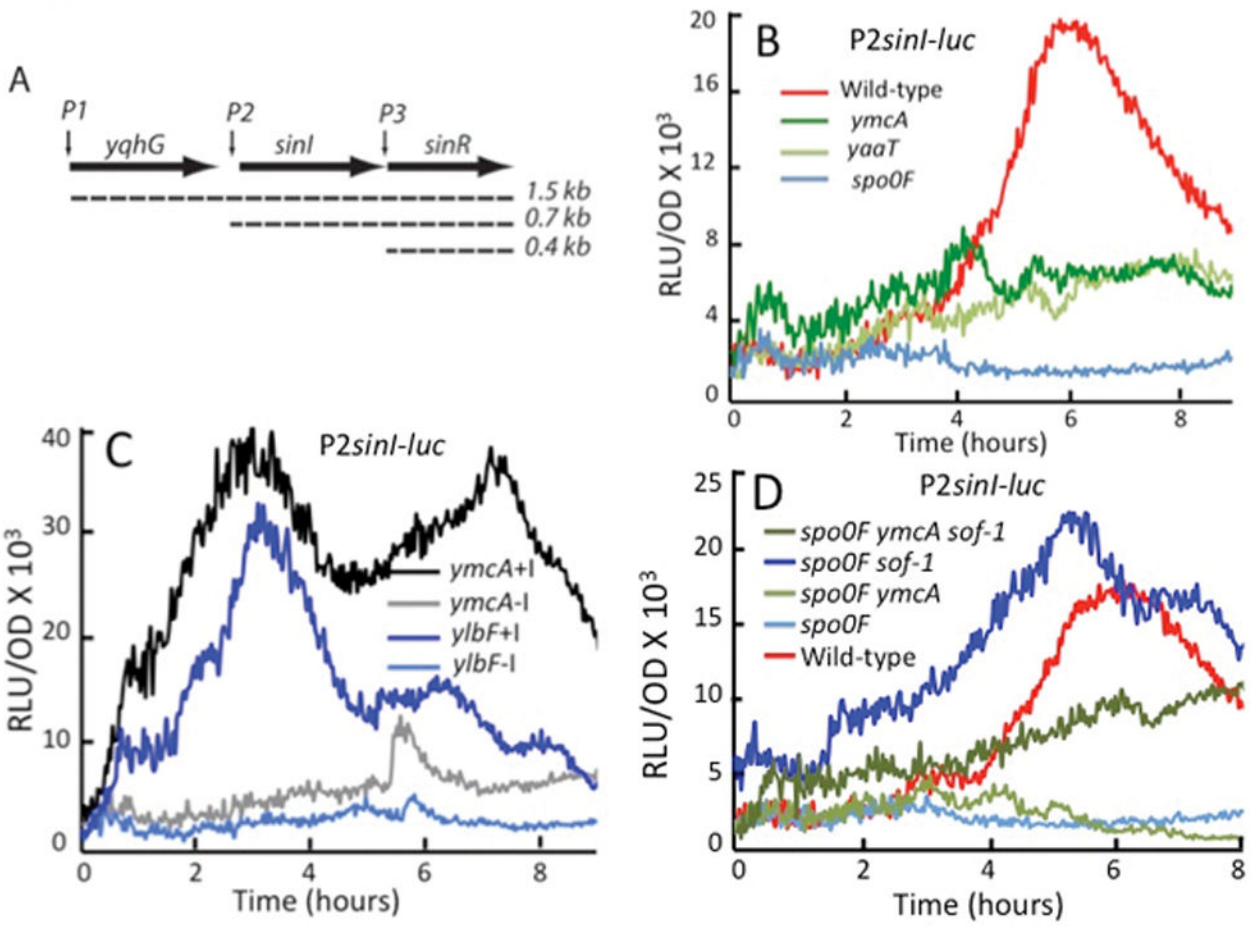

Fig. 3.

YlbF, YmcA and YaaT act positively on the rate of *sinI* transcription by stimulating the production of Spo0A-P. (A) A transcription schematic of the *sin* locus. (B) The effect of *ylbF*, *ymcA* and *yaaT* knockouts on transcription from a P2*sinI-luc* reporter construct. (C) Effects of IPTG induction of the *sad-67* mutant form of *spo0A* on expression from the P2*sinI* promoter in the presence of *ymcA* and *ylbF* knockouts. The lines labeled +I and −I indicate the presence and absence of IPTG. (D) Effects of the *sof-1* allele of *spo0A* on expression from P2*sinI* in the presence of *spo0F* and *ymcA* knockouts. Strains used were as follows. (B) wild-type P*sinI-luc* (BD7817), *ΔymcA* P*sinI-luc* (BD7821), *ΔyaaT* P*sinI-luc* (BD7820). (C) *ΔymcA sad-67* P*sinI-luc* (BD7842), *ΔylbF sad-67* P*sinI-luc* (BD7843). (D) wild-type P*sinI-luc* (BD7817), *Δspo0F* P*sinI-luc* (BD7853), *Δspo0F sof-1* P*sinI-luc* (BD7854), *Δspo0F sof-1 ΔymcA* P*sinI-luc* (BD7855), *Δspo0F ΔymcA* P*sinI-luc* (BD7856). The bacteria were grown in MSgg and all the strains were constructed in the 3610 background.

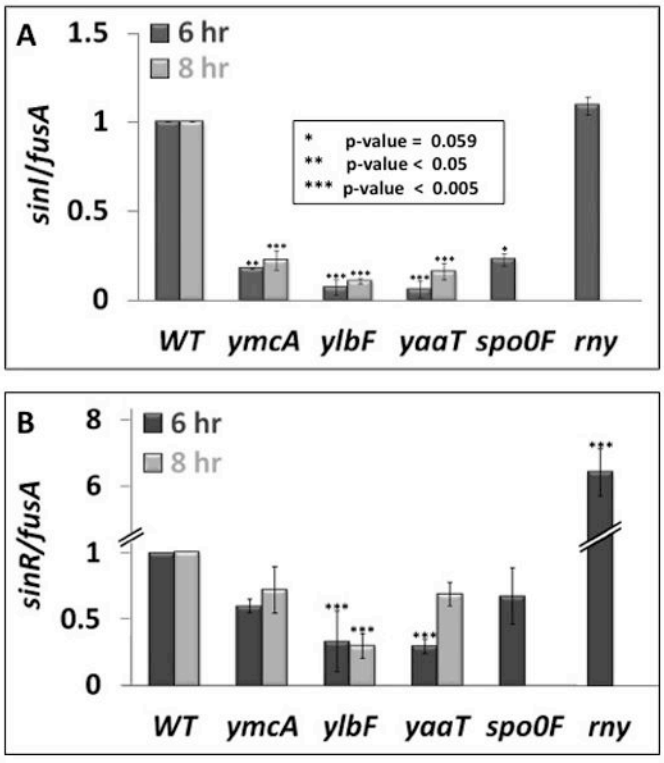

Fig. 4.

Effects of *ylbF*, *ymcA*, *yaaT*, *spo0F* and *rny* knockouts on the abundance of transcripts containing *sinI* (panel A) and *sinR* (panel B) sequences. The data were obtained by qRT-PCR and were normalized to the level of *fusA* mRNA as described in Experimental procedures. Samples for the preparation of RNA were taken from cultures growing in MSgg at the indicated times, which corresponded to late log and stationary phase. The strains used were: wild-type (BD7477), *ΔymcA* (BD7736), *ΔylbF* (BD7737), *ΔyaaT* (BD7731), *Δspo0F* (BD7593) and *Δrny* (BD7839). All experiments were repeated 3 independent times, and error bars represent standard deviation. Statistically significant interactions are displayed by asterisks as noted in the figure. Al the strains were constructed in the 3610 background.

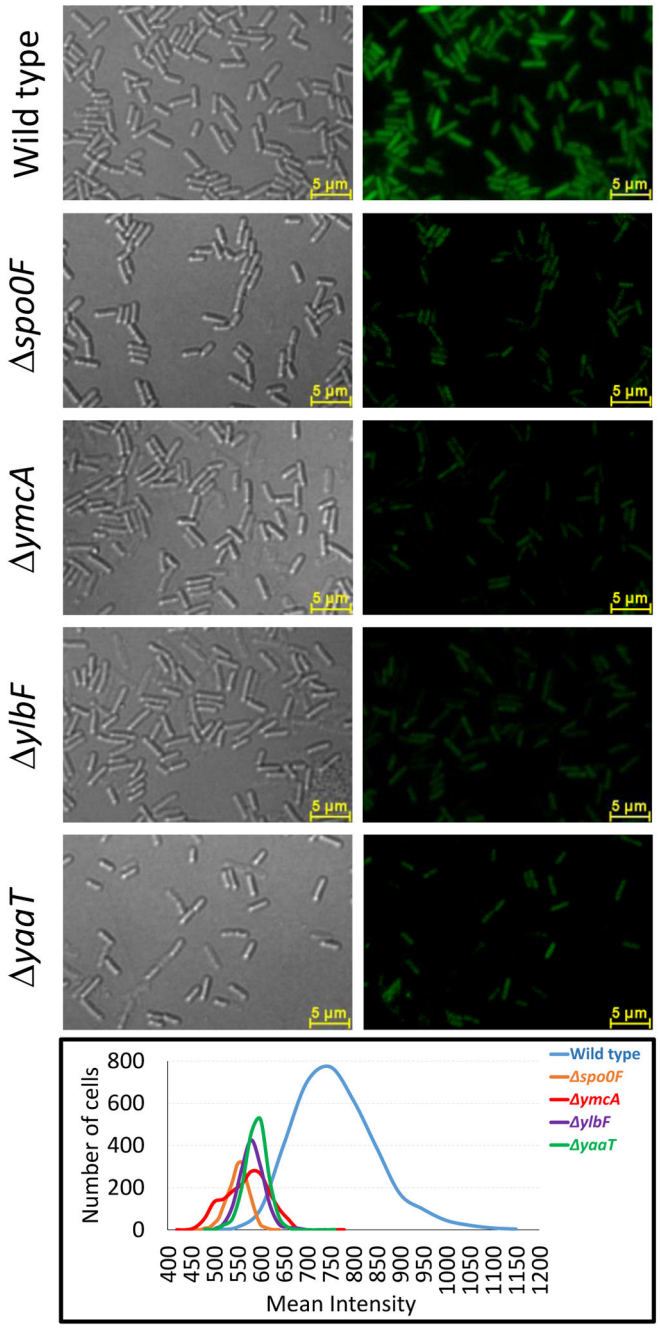

Fig. 5.

The distribution of fluorescence in cells expressing P2*sinI-gfp* is shifted to lower values in *ylbF*, *ymcA*, *yaaT* and *spo0F* strains. All of the representative images shown were obtained with identical microscope settings and were processed in the same way. Fluorescence intensity was measured for a population of cells and all the distributions were plotted together in the bottom panel for comparison. The strains used were wild-type P2*sinI-gfp* (BD7799), *Δspo0F* P2*sinI-gfp* (BD7800), *ΔymcA* P2*sinI-gfp* (BD7806), *ΔylbF* P2*sinI-gfp*

(BD7807), *ΔyaaT* P2*sinI-gfp* (BD7805). Growth was in MSgg and all the strains were constructed in the 3610 background.

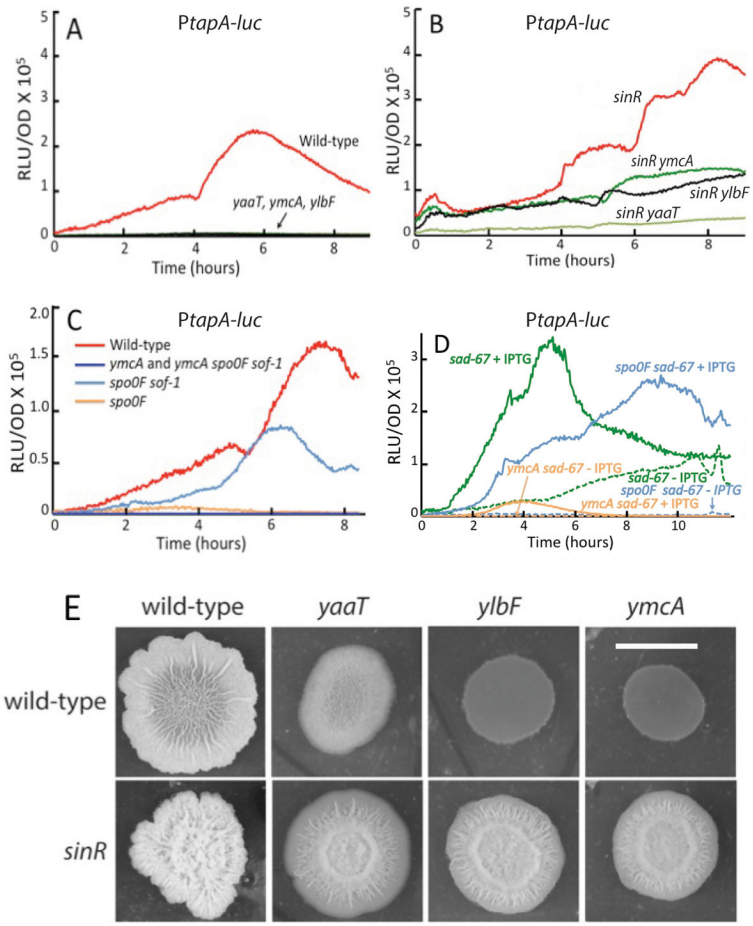

Fig. 6.

Effects of *ylbF*, *ymcA* and *yaaT* knockouts on expression of the *tapA* operon. (A–D) Luciferase assays for P*tapA*-luc expression were performed as described in Experimental procedures. The blue line at the bottom of panel C contains data for the *ymcA* and *ymcA spo0F* strains. (A) The effects of *yaaT, ylbF* and *ymcA* knockouts on a P*tapA*-luc expression reporter. (B) The effect of *sinR* inactivation on *tapA* expression in the *ylbF*, *ymcA* and *yaaT* knockouts. (C) and (D) show the extent of bypass of a *ymcA* null mutant by *sof-1* and *sad-67* respectively. The solid and dashed lines in Panel D show results with and without the addition of IPTG, respectively. The strains used were as follows. (A) wild-type *ΔepsH* P*tapA-luc* (BD7870), *ΔymcA ΔepsH* P*tapA-luc* (BD7872), *ΔyaaT ΔepsH* P*tapA-luc* (BD7871), *ΔylbF ΔepsH* P*tapA-luc* (BD7873). (B) wild-type *ΔsinR ΔepsH* P*tapA-luc* (BD7900), *ΔymcA ΔsinRΔ epsH* P*tapA-luc* (BD7974), *ΔylbFΔ sinR ΔepsH* P*tapA-luc* (BD7975), *ΔyaaT ΔsinRΔ epsH* P*tapA-luc* (BD7973). The strains used in panels A and B were deleted for *epsH* to avoid the clumping that occurs in *sinR* strains. We have shown that the elimination of *epsH* has no discernible effect on the expression of P*tapA-luc* (not shown). (C) wild-type P*tapA-luc* (BD7644), *ΔymcA* P*tapA-luc* (BD7652), *ΔymcA Δspo0F sof-1* P*tapA-luc* (BD7654), *Δspo0F sof-1* P*tapA-luc* (BD7651), *Δspo0F* P*tap -luc* (BD7650). Panel (D) wild-type *sad-67* P*tapA-luc* (BD7844), *Δspo0F sad-67* P*tapA-luc* (BD7845), *ΔymcA sad-67* P*tapA-luc* (BD7846). (E) Cells were grown in LB media until an OD_{600} of

1.0, and 5 μl were spotted on MSgg agar plates. Pictured is the colony morphology after four days of growth at room temperature. The scale bar corresponds to 1 cm and all the colonies were photographed at the same magnification. The *sinR+* strains used were BD7736 (*ΔymcA*), BD7737 (*ΔylbF*), BD7731 (*ΔyaaT*) and BD7477 (wild-type). The *sinR* strains were BD7837 (*ΔymcA*), BD7838 (*ΔylbF*), BD7836 (*ΔyaaT*) and BD7835 (Δ*sinR*). Growth was in MSgg and all the strains were constructed in the 3610 background.

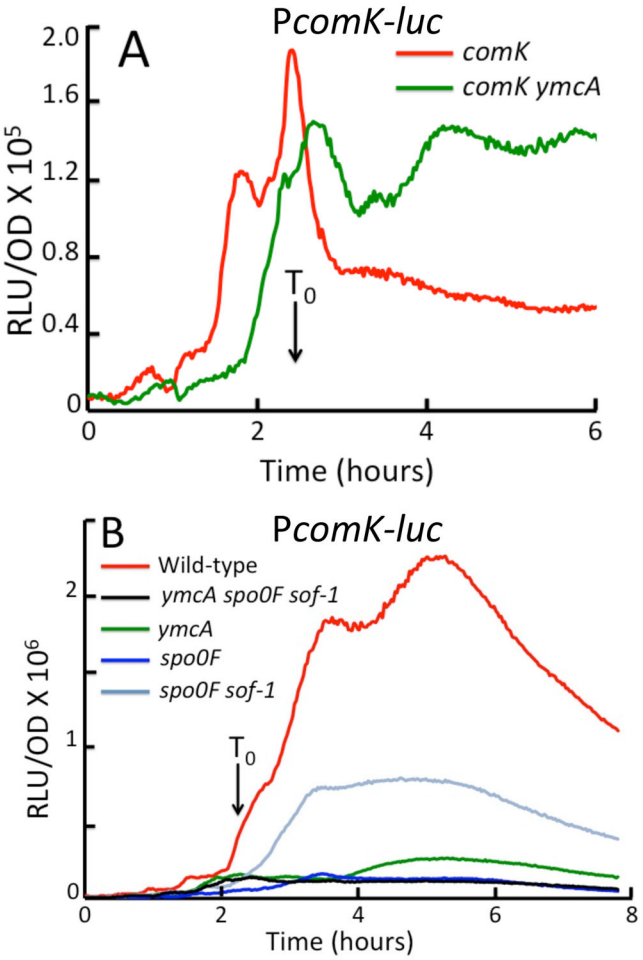

Fig. 7.

ymcA knockout effects on the expression from the *comK* promoter are not bypassed by *sof-1*. (A and B) Luciferase assays for P*comK-luc* expression were performed as described in Experimental procedures. (A) shows the effect of a *ymcA* knockout on P*comK-luc* expression in a *comK* knockout. (B) shows the effect of *ymcA* and *spo0F* inactivation in a *comK*$^{+}$ background, as well as the effects of *sof-1*. The strains used were as follows. (A) wild-type *ΔcomK* P*comK-luc* (BD4893), *ΔymcA ΔcomK* P*comK-luc* (BD7642). (B) wild-type P*comK-luc* (BD4773), *ΔymcA Δspo0F sof- 1* P*comK-luc* (BD7608), *ΔymcA* P*comK-luc* (BD7605), *Δspo0F* P*comK-luc* (BD7606), *Δspo0F sof-1* P*comK-luc* (BD7607). The strains were all derivatives of IS75 and were grown in competence medium.

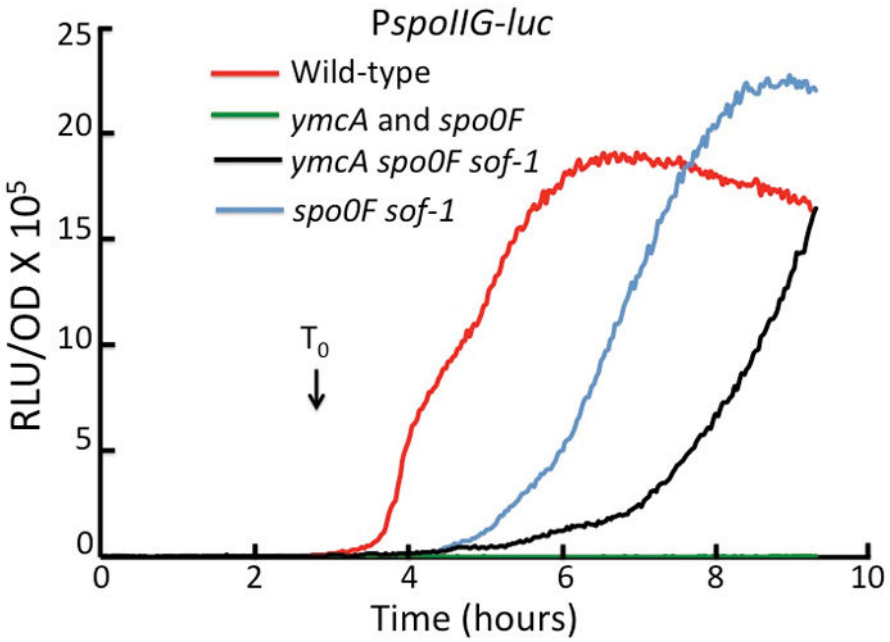

Fig. 8.

Partial bypass of *ymcA* and *spo0F* inactivation by *sof*-1 for *spoIIG-luc* expression in cultures growing in DSM. Luciferase assays for P*spoIIG-luc* expression were performed as described in Experimental procedures. The strains used were: wild-type (BD5813), *ΔymcA* (BD7624), *Δspo0F* (BD7710), *Δspo0F sof-1* (BD7711), *Δspo0F sof-1 ΔymcA* (BD7712).

Table 1

Suppression of sporulation by *sof-1*

Strain	Genotype	Sporulation (%) [a]
IS75	Wild-type (IS75)	79.6±6 (n=7)
BD3032	*ΔymcA*	0.023±0.003 (n=6)
BD7186	*Δspo0F*	0 (n=6)
BD7307	*Δspo0F sof-1*	79.5±10.8 (n=8)
BD7315	*ΔymcA spo0F sof-1*	28.7±18.3 (n=8)

[a] Sporulation is expressed as

$$\frac{(\text{heat resistant colony forming units})}{(\text{total colony forming units})} \times 100$$

All determinations were repeated at least 6 independent times, and are shown ± standard deviations. The strains were all grown for 24 hours in DSM at 37°C.