Published in final edited form as:
Mol Microbiol. 2011 May ; 80(4): 1014–1030. doi:10.1111/j.1365-2958.2011.07627.x.

MecA dampens transitions to spore, biofilm exopolysaccharide and competence expression by two different mechanisms

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Summary

The adapter protein MecA targets the transcription factor ComK for degradation by the ClpC/ClpP proteolytic complex, thereby negatively regulating competence in *Bacillus subtilis*. Here we show that MecA also decreases the frequency of transitions to the sporulation pathway as well as the expression of *eps*, which encodes synthesis of the biofilm matrix exopolysaccharide. We present genetic and biophysical evidence that MecA down-regulates *eps* expression and spore formation by directly interacting with Spo0A. MecA does not target Spo0A for degradation, and apparently does not prevent the phosphorylation of Spo0A. We propose that it inhibits the transcriptional activity of Spo0A~P by direct binding. Thus, in its interaction with Spo0A, MecA differs from its role in the regulation of competence where it targets ComK for degradation. MecA acts as a general buffering protein for development, acting by two distinct mechanisms to regulate inappropriate transitions to energy-intensive pathways.

Keywords

MecA; sporulation; biofilms; bimodal expression; Spo0A

Introduction

Bacillus subtilis expresses a variety of global life-style responses to stress, including competence for transformation, sporulation and biofilm formation. Because the developmental pathways are energy-intensive and both competence and sporulation cause the cessation of growth, high frequencies of transition would be disadvantageous when nutrients are plentiful. Consequently, mechanisms to minimize such inappropriate transitions are expected to increase fitness. However, it is plausible that even in the absence of stress these transition rates would be nonzero, providing a clonal population with a sub-population of cells insured in advance against sudden adversity (Dubnau & Losick, 2006, Veening *et*

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al., 2008, Losick & Desplan, 2008). Thus, rare competent or sporulating cells are present even in rapidly growing cultures. These considerations imply that selective pressure has approximated an optimal rate of transition for each adaptive pathway under conditions of plenty. In contrast, when nutrients are scarce and *B. subtilis* exits from exponential growth, the transition rates increase and cells choose from among these developmental adaptations, each of which has the potential to guarantee survival of at least a portion of the population. These transition rate increases occur in an orderly and coordinated manner using pathway-specific mechanisms. In other words, the transition rates are not fixed, but are adjusted by regulatory mechanisms to reflect changing circumstances.

Competence refers to a gene expression state in which cells can take up and integrate environmental DNA. The expression of competence genes provides a well-studied example of bimodal gene expression in bacteria (Maamar *et al.*, 2007, Suel *et al.*, 2007, Leisner *et al.*, 2007). These genes are expressed in a minor fraction of the cells in a clonal population and the transition rate adjustments described above are well illustrated by the formation of competent cells. The expression of competence genes requires the transcription factor ComK, which is also an auto-activator, acting positively and directly at its own promoter (van Sinderen *et al.*, 1995). ComK is expressed bimodally because of two additional factors acting in concert with this positive auto-regulation; stochastic variability (noise) in the basal amount of ComK per cell and the existence of a nonlinear response to ComK that permits only those cells above a threshold to auto-activate P*comK*. During exponential growth, MecA lowers the probability that such variation will result in competence by selectively targeting ComK molecules for degradation by a complex of the AAA^+ protein ClpC and the serine protease ClpP (Turgay *et al.*, 1998). As a result, the auto-regulatory loop is rarely activated, transition probability is low and very few competent cells are present in growing cultures. As cells exit from exponential growth, the anti-adaptor ComS is synthesized in response to a quorum-sensing mechanism that measures population density. ComS competitively releases ComK from binding to MecA (Prepiak & Dubnau, 2007), reducing its rate of degradation and permitting stochastic fluctuations in the basal level transcription of *comK* to be manifested as a marked increase in the probability of transitions to competence. Thus, MecA acts to dampen the likelihood of transitions to competence particularly during exponential growth. In several respects, sporulation and early biofilm gene expression follow the pattern described for competence. Both exhibit low but non-zero probabilities of transition in growing cells and increased transition rates as cultures enter stationary phase.

The progression of *B. subtilis* to a biofilm-producing state is largely governed by the transcriptional regulator SinR (Branda *et al.*, 2006) (Fig. 1A). SinR directly represses the *eps* operon, which encodes synthesis of biofilm-associated extracellular polysaccharide, as well as the *yqxM-sipW-tasA* operon, which encodes a protein component of the extracellular matrix (Branda *et al.*, 2004, Branda et al., 2006). The activity of SinR is regulated not by a change in its amount, but rather by the selective production of SinI, which binds directly to SinR, preventing it from inhibiting the transcription of its target promoters (Bai *et al.*, 1993). When SinI overcomes the repression due to SinR, matrix can be produced and a biofilm community can develop (Kearns *et al.*, 2005).

Because Spo0A~P is required not only for biofilm formation, but for competence (Hahn *et al.*, 1995) and for the expression of sporulation genes (Molle *et al.*, 2003) (Fig. 1A), this response regulator protein is the ultimate controller of all three developmental pathways. *sinI* is transcribed from two SigA-dependent promoters, one of which is activated by the binding of phosphorylated Spo0A (Gaur *et al.*, 1988, Shafikhani *et al.*, 2002). The master regulator Spo0A~P therefore lies upstream of *sinI* in biofilm development and activates *eps* by indirectly down-regulating SinR. AbrB, another regulator of biofilm formation (Hamon

et al., 2004), represses *sinI* by binding directly to its promoter (Shafikhani et al., 2002) and may also regulate *eps* by direct binding (Murray *et al.*, 2009), although the major regulation of eps transcription is certainly mediated by the SinI-SinR pathway. *abrB* transcription is down-regulated by Spo0A~P (Strauch *et al*., 1990) and AbrB activity is inhibited by the AbbA protein, which requires Spo0A~P for its production (Banse *et al.*, 2008).

Spo0A activation is dependent on phosphorylation largely through a signal transduction cascade, known as the phosphorelay (Burbulys *et al.*, 1991). Genes regulated by Spo0A exhibit a hierarchical response to the concentration of Spo0A~P; *sinI* and *abrB* have high affinity promoters whereas sporulation genes require higher levels of Spo0A~P for their activation (Fujita *et al.*, 2005). Although like competence *sinI* is expressed bimodally, the mechanism by which only certain cells are selected for *sinI* expression is not clear but may be related to kinetic heterogeneity in the production of Spo0A~P because a strain that is *spo0A*-deficient expresses little *sinI* whereas the presence of a constitutively active Spo0A results in enhanced expression of *sinI* in all cells, suggesting that the availability of Spo0A~P is normally limiting for transitions to *eps* expression and biofilm formation (Chai *et al*., 2008).

We report here that inactivation of *mecA* causes an increased expression of *eps* and of sporulation genes, while over-expression of *mecA* has the reverse effect, decreasing *eps* and spore gene expression. Also, when *mecA* is inactivated, the fraction of cells expressing these genes increases, even during growth. A mutant form of Spo0A that does not require phosphorylation bypasses the inhibition of *eps* and spore gene transcription by MecA. We have found that MecA binds directly to Spo0A but does not target this protein for degradation as it does ComK. We propose that by preventing Spo0A from acting as a transcription factor, either by preventing its phosphorylation or by acting on Spo0A~P, MecA dampens transitions to biofilm development and spore formation, thus preventing inappropriate expression of these pathways and acting as a general buffer for developmental transitions in *B. subtilis*.

Results

MecA is a negative regulator of *eps* expression and of sporulation

This study initiated from two separate observations on *mecA* mutant strains growing on solid media. These observations were made using a laboratory strain IS75. The first such observation was that *mecA* colonies presented a rough-textured appearance. Because over-production of the biofilm-associated exopolysaccharide encoded by the *eps* operon yields similar appearing colonies, we constructed a *mecA::erm eps::tet eps-lacZ* strain (BD3980). This strain exhibited smoother colonies, like those of the wild-type, suggesting that the rough colony phenotype was indeed due to over-expression of *eps*. To further characterize the role of MecA in *eps* expression, we examined an *eps-lacZ* transcriptional fusion in *mecA*$^{+}$ (BD4498) and *mecA* (BD4538) backgrounds and in a strain carrying a multi-copy plasmid (p*KD93*) that over-expresses *mecA* (BD4644) from a constitutive promoter on the vector (Kong *et al.*, 1993). (Over-expression of MecA in this strain in documented below (Fig. 6)). This experiment was done in a complex medium (LB) because *mecA* strains do not grow in the medium commonly used to study biofilm formation (not shown). The *mecA* strain showed increased expression of *eps-lacZ* throughout growth. In the experiment shown in Fig. 1B *eps-lacZ* expression in the *mecA* strain is elevated about 4-fold throughout growth. In three additional experiments in which samples were taken at T_1, the *mecA* strain showed an average increase over the wild-type strain of 5.7-fold. Expression of *eps-lacZ* was nearly eliminated in the p*KD93* background (Fig. 1B). In MsGG medium, which is customarily used to study biofilm formation, the over-expression of MecA markedly

decreased the increase in *eps-lacZ* expression that took place at T_0. We conclude that MecA is a negative regulator of *eps*, confirming the inference from colony morphology.

The second observation was that the over-expression of *mecA* from a multicopy plasmid resulted in translucent colonies, typical of strains with an early block in spore formation. This phenotype was confirmed by spore counts in sporulation medium (DSM). In a typical experiment, after 24 and 48 hours growth, a strain that carried p*KD93* (PP493) exhibited sporulation frequencies of 1.5×10^{-5} and 4.9×10^{-5} respectively, while at these times, an isogenic strain which did not over-express *mecA* (BD630) achieved sporulation frequencies of 0.32 and 0.66. The appearance of the PP493 colonies suggested that the MecA-induced block in spore formation occurred at an early stage. To test this inference, the effect of MecA on early spore gene expression was determined by monitoring light output from a fusion of the *spoIIG* promoter to firefly luciferase. We have shown elsewhere that this *luc* reporter may be used in *B. subtilis* as a real-time reflection of the rate of transcription from a given promoter (N. Mirouze, P. Prepiak and D. Dubnau, submitted). For this experiment, luminometry and OD_{600} measurements were made in a temperature-controlled plate reader on cultures growing in the presence of luciferin. Fig. 1C shows that *mecA* over-expression prevented *spoIIG* transcription. Conversely, the inactivation of *mecA* resulted in a reproducible increase in *spoIIG* expression, manifested only after the culture had entered stationary phase. Expression of a *spoIIE-lacZ* fusion was also inhibited in the presence of p*KD93*, as shown below in Fig. 5. These results demonstrate that MecA limits the expression of early Spo0A-dependent sporulation genes.

MecA biases the fraction of cells entering the biofilm and sporulation pathways

Prior studies had shown that *eps* is expressed in only a few percent of the cells in a given population (Chai et al., 2008). To determine whether *mecA* affected the proportion of cells expressing *eps*, we examined mutant strains by fluorescence microscopy, using a fusion of the *eps* promoter to cyan fluorescent protein (*eps-cfp*). These strains, *eps-cfp* (BDBD4621), *eps-cfp mecA::erm* (BD4642) and *eps-cfp* p*KD93* (BD4643), were grown in LB to T_1 and samples were prepared from each for fluorescence microscopy. In the wild-type background (Fig. 2A), 3.9% of the cells expressed *eps-cfp*, confirming the published results (Chai et al., 2008). This was increased in the *mecA* strain (Fig. 2B) to 35.6% and in the p*KD93* strain (Fig. 2C) we were unable to find any cells expressing *eps*, among approximately 5,000 cells examined. These results show that MecA acts to limit the number of *eps*-expressing cells, but is not the only factor responsible for this limitation, because even in its absence expression did not occur in all the cells. The 9-fold increase in the fraction of expressing cells in the *mecA* strain is approximately consistent with the 5.7-fold increase in *eps-lacZ* noted above.

A similar experiment was carried out with a fusion of the *spoIIE* promoter to GFP using cultures growing in DSM until T_{-1}, well before the normal onset of sporulation. In the wild-type strain, occasional fluorescent cells were observed. For example, in the field shown in Fig. 2D, which was selected to include a fluorescent cell, one such cell is visible among about 274 cells. The frequency of such cells is below 0.1%. In the *mecA* null strain field (Fig. 2E), 9 fluorescent cells are evident among 389 cells. In a strain over-expressing *mecA* from a multicopy plasmid, no fluorescent cells were detected among several thousand examined (not shown). Corresponding results were obtained by measuring the frequency of spores among cells during logarithmic growth in DSM. In the wild-type strain, the measured frequency of heat resistant spores was 1.4×10^{-7}. In the *mecA* strain the frequency was 5×10^{-3}, while no spores were detected in the p*KD93* strain. As with competence and *eps* expression, it appears that MecA limits rare transitions to sporulation in growing cultures.

To explore the reasons for these two seemingly unrelated MecA-associated phenotypes, affecting *eps* and early spore gene expression, we carried out a series of epistasis experiments employing mutations in regulatory genes known to affect biofilm and spore development.

MecA regulates *eps* expression largely through the SinI/SinR pathway

To investigate where *mecA* acts in the *eps* regulatory pathway, we sought to determine if the effect of *mecA* over-expression on *eps-lacZ* expression could be bypassed by inactivation of *sinR*, a direct repressor of *eps* (Kearns et al., 2005). As expected, the *sinR::cat* strain (BD4544) showed elevated *eps* expression, approximately 28-fold higher than the amount seen in the wild-type strain (BD4498) (Fig. 3A). Although the p*KD93* strain (BD4644) was greatly reduced in the expression of *eps*, the p*KD93 sinR::cat* strain (BD4549) expressed about 40% as much *eps-lacZ* as the *sinR::cat* strain, demonstrating substantial bypass of the *mecA* over-expression effect by inactivation of *sinR* and suggesting that MecA exerts much of its effect on *eps* expression upstream of *sinR*.

We next determined whether MecA affected the expression of *sinI*, which encodes an antagonist of SinR (Bai et al., 1993). Western blots, using antiserum raised against SinI, were performed on extracts of the wild-type, *mecA* and p*KD93* strains. Fig. 4A shows that there is less SinI in the p*KD93* strain, and more in the *mecA* strain than in the wild-type. These data led us to suspect that MecA decreases *eps* expression at least in part by depressing the level of SinI. Fig. 4B shows that similar effects were observed with a *sinI-lacZ* translational fusion reporter strain, showing that MecA down-regulates the expression of *sinI-lacZ.*

AbrB, like SinR, is a negative regulator of *eps* (Hamon et al., 2004, Chu *et al.*, 2008, Winkelman *et al.*, 2009) and has recently been reported to bind directly to the promoter of the *eps* operon (Murray et al., 2009). AbrB also appears to repress *sinI* (Shafikhani & Leighton, 2004). As expected from the published results, the *abrB* mutation raised expression of *eps-lacZ* in the *mecA*$^+$ background about 3.7-fold, a far less dramatic effect than seen with the *sinR* knockout (Fig. 3A). To test whether the MecA effect on *eps* could be bypassed by inactivation of *abrB* as it was by the loss of *sinR* function, an *eps-lacZ abrB* p*KD93* strain (BD5113) was tested (Fig. 3A). Unlike inactivation of *sinR*, the *abrB* mutation was not able to raise the low level of *eps-lacZ* expression due to MecA overproduction. However, the inactivation of both *abrB* and *sinR* (BD4624) bypassed the p*KD93* phenotype to a level nearly twice that achieved by inactivation of *sinR* alone in the p*KD93* background. Interestingly, the level of expression in the p*KD93 sinR abrB* strain was about half the level in the *sinR abrB* strain, suggesting that MecA can limit *eps* transcription by another pathway, not involving SinR or AbrB.

Taken together, these data suggest that MecA exerts its negative effect on *eps* most importantly by potentiating the activity of the major repressor SinR. We also conclude from the data in Fig. 4 that the MecA effect on SinR activity is likely due to a negative effect on the production rather than the activity of SinI.

Spo0A is a potential target for the regulation of *eps* by MecA

Because both *abrB* and *sinI* are controlled by the transcription factor Spo0A~P, a simple hypothesis is that the MecA effect is exerted through this response regulator. This would also explain the ability of MecA to limit sporulation, which has an absolute requirement for Spo0A~P.

To further explore the involvement of Spo0A and hence the plausibility of our hypothesis that MecA acts on *eps* expression via Spo0A, we carried out additional epistasis

experiments. Fig. 3B shows that as expected, *eps* expression was totally dependent on *spo0A* (BD4928) and that inactivation of *mecA* does not bypass the dependence of *eps* expression on *spo0A*; a double *spo0A mecA* mutant (BD5589) fails to express *eps-lacZ*. We then determined whether inactivation of *abrB* and *sinR*, singly or together, could bypass the depressing effect of *spo0A* inactivation on the expression of *eps* as they did for over-expression of MecA. Inactivation of either *sinR* (BD4929) or *abrB* (4930) bypassed the *spo0A* requirement to a level somewhat in excess of the wild-type level, but much below the level achieved by the *sinR* mutant, suggesting that without Spo0A neither repressor was present at a concentration sufficient to limit *eps* transcription. Indeed, inactivation of both repressors (BD4931) restored *eps* expression in the *spo0A* background to approximately the level exhibited by the *sinR* mutant. It is important to recognize that in the *spo0A* knockout mutant, AbrB accumulates and exerts a depressing influence on *eps* expression in excess of that in the wild-type situation. Clearly, SinR is the major repressor of *eps* transcription (Kearns et al., 2005).

Fig. 3A demonstrated that inactivation of both *sinR* and *abrB* partially bypassed the negative effect of MecA on *eps* expression. Fig. 3B demonstrates further that eliminating repression by both SinR and AbrB is sufficient to restore substantial *eps* expression when Spo0A is absent. In total, these results enhance the plausibility of the hypothesis that MecA may be regulating *eps* by interfering with Spo0A.

However, the experiments presented so far do not determine whether MecA limits the amount of Spo0A protein in the cell, the phosphorylation of Spo0A or the activity of Spo0A~P as a transcription factor.

The MecA inhibition of *spoIIE* and sinI *expression* is bypassed by the *sad67* mutation

To further investigate the role of MecA, we utilized the *sad67* mutation that makes this Spo0A factor independent of phosphorylation for its activity and removes 19 amino acid residues from the receiver domain of this transcription factor (Ireton *et al.*, 1993). For the first experiment we used a construct in which the Sad67 protein was expressed from the IPTG-inducible P*spac* promoter in a strain that over-expressed *mecA* (BD4628) and we determined the amount of SinI in the cell by Western blotting. Fig. 5A shows that two hours after induction, bypass of MecA overproduction by Sad67 took place. In contrast, induction of the wild-type *spo0A* under control of the P*spac* promoter (BD4692) did not bypass MecA overproduction (Fig. 5B, C).

A similar experiment was carried out to test the ability of the Sad67 protein to bypass the over-production of MecA with *spoIIE-lacZ* as the reporter (Fig. 5D). As expected, MecA overproduction completely prevents the expression of *spoIIE-lacZ* in a strain carrying p*KD93* (PP487), as it did for *spoIIG-lacZ* (Fig. 1C). When the P*spac-sad67* construct was induced by the addition of IPTG in a strain carrying the *mecA* over-expressing plasmid (PP488), *spoIIE-lacZ* was strongly expressed, overcoming the inhibition by MecA (Fig. 5D). Even the leaky expression of *sad67* from the uninduced P*spac* promoter was sufficient to achieve a delayed, intermediate level of *spoIIE-lacZ* expression. The induced expression in the presence of p*KD93* was about two-thirds of that achieved in an isogenic strain lacking p*KD93* (PP485), which was unaffected by the addition of IPTG. The *sad67* bypass of the ability of p*KD93* to inhibit expression of both *spoIIE* and *sinI* is consistent with the hypothesis that MecA interferes with the phosphorylation of Spo0A, although it can also be explained by a failure of this mutant protein to interact with MecA (see below).

ClpC participates in the regulation of *eps*

MecA controls the cellular levels of the competence transcription factor ComK by regulated proteolysis, acting as an adaptor to target ComK for degradation by the ClpC/ClpP protease (Turgay *et al.*, 1997). Because the known functions of MecA and ClpC are so intimately connected, it appeared possible that ClpC would also be involved in the regulation of *eps* expression. Specifically, MecA may target Spo0A for degradation by ClpC/ClpP. The *sad67* bypass experiments might then be explained as a failure of MecA to target the Sad67 protein, which lacks 19 residues from its receiver domain. Fig. 3C shows that the inactivation of *clpC* indeed causes increased expression of *eps* (in BD4580), indicating that ClpC does play a negative role in the regulation of *eps*. However, the level of *eps-lacZ* expression in the *clpC::tet* strain is nearly 4-fold lower than that reached in a *mecA* loss-of-function mutant (compare Figs. 3C with 3B and 1B).

The over-expression of MecA down-regulates *comK* transcription even when *clpC* is inactivated, because MecA binding is sufficient to prevent ComK from interacting with the *comK* promoter even when degradation of ComK by ClpC/ClpP cannot occur (Kong & Dubnau, 1994). In the present case, ClpC inactivation *prevented mecA* over-expression from down-regulating *eps* transcription to the level of the p*KD93 clpC*$^{+}$ strain (Fig. 3C). This dramatic difference and the lesser effect of *clpC* compared to *mecA* inactivation hint that ClpC and MecA may act differently in regulating the expression of *eps* and *comK*.

MecA does not target Spo0A for degradation, inhibit the phosphorelay or dephosphorylate Spo0A~P

Despite the difference just noted, it seemed possible that MecA was targeting Spo0A for degradation. As noted above, if MecA did this by direct binding, the Sad67 bypass might be due to a failure of MecA to bind to this mutant protein. More generally, if MecA were involved in the degradation of Spo0A, we would expect more of this protein to be present in a *mecA*-deficient strain and less in the p*KD93* strain. We therefore compared the amounts of Spo0A and MecA by Western blotting during growth in DSM of *mecA*$^{+}$(BD2149), *mecA* (BD2148) and p*KD93* (PP493) strains (Fig. 6). Panel B shows that the p*KD93* strain accumulates excess MecA compared to the wild-type strain as expected and that the amounts of MecA accumulated in each of these two strains did not vary markedly as the cultures entered stationary phase. Panel A shows that very little Spo0A was detectable just before T_0. Importantly, in sample 1, which was taken just before T_0 and in sample 2, taken just after T_0, the amounts of Spo0A in the three strains were similar. Thereafter (sample 3, 4 and 5), Spo0A did accumulate to a lesser extent in the p*KD93* strain than in the wild-type. In the *mecA* strain, excess Spo0A was detected in sample 5. We have done a similar experiment in LB and again found no noticeable differences in the amounts of Spo0A among these three strains at T_{-1} and at 30 minutes after T_0 (not shown). We interpret these results as follows. When a culture growing in DSM enters stationary phase, Spo0A becomes phosphorylated, thereby enhancing transcription from the SigH-dependent *spo0A* promoter (Strauch *et al.*, 1992, Fujita & Sadaie, 1998). We suggest that MecA limits the phosphorylation or the activity of Spo0A~P, and as a result, different amounts of Spo0A protein are present in the mutant and wild-type strains only after T_0. Note that the absence of MecA has an effect on the amount of Spo0A only in sample 5, between T_2 and T_3. This is consistent with the results in Fig. 1C, which show an increased transcription of *spoIIG* in a *mecA* strain only after about T_1.

As another test of the hypothesis that MecA may target Spo0A for degradation or otherwise decrease its stability, we added puromycin (200 μg/ml) just after T_1 to Δ*mecA* (BD2149) and pKD93 (PP493) strains and collected samples at intervals for Western blotting to detect decay of the Spo0A signal. During 40 minutes incubation in the presence of puromycin,

little decay was discernable in either strain (Fig. 6D). The PP493 samples were also probed using anti-MecA antiserum, and decay was readily detected, with an estimated half-life between 10 and 15 minutes, showing that the puromycin was working. This experiment demonstrates that Spo0A is quite stable under these conditions, even in a MecA over-expressing strain, making it quite unlikely that the latter protein modulates the decay of Spo0A.

Finally, to further pursue the issue of Spo0A degradation, we used an *in vitro* degradation assay to test whether or not MecA directly targets Spo0A for degradation by ClpC/ClpP. Fig. 7B shows that in the presence of MecA, ClpC, ClpP and ATP, ComK is targeted for degradation as expected (Turgay et al., 1997). Also as expected, when ComK was omitted, MecA itself was degraded (Fig. 7A) but only in the presence of ATP. These controls demonstrate that the protein preparations were active, behaving as expected. In contrast, no degradation of either His-tagged (Fig. 7A) or untagged versions of Spo0A (Fig. 7C) was observed. These preparations of Spo0A were active in binding to radiolabeled *sinI* and *abrB* promoter fragments (not shown). Taken together, our *in vivo* (Fig. 6) and *in vitro* (Fig. 7) results suggest strongly that MecA does not exert its negative effect by targeting Spo0A for degradation, nor does it otherwise affect the total amount of Spo0A protein in the cell.

We next determined whether the addition of MecA would interfere with the phosphorylation of Spo0A in the presence of ^{32}P-γ-ATP and the His-tagged phosphorelay components KinA, Spo0F and Spo0B. As shown in Fig. 7D, the addition of MecA-His_6, even at concentrations up to 120 μM, did not decrease the yield of Spo0A~P. We conclude that MecA by itself neither interferes with the phosphorelay nor causes the dephosphorylation of Spo0A~P in the presence of the phosphorelay components.

MecA and Spo0A interact directly

The evidence presented so far suggests that MecA directly or indirectly interferes with the activity of Spo0A~P as a transcription factor. To test whether MecA binds directly to Spo0A or to the other phosphorelay proteins, we utilized surface plasmon resonance (SPR). MecA-His_6 was immobilized to the surface of a CM5 chip using primary amine chemistry. Various concentrations of KinA, Spo0F, Spo0B or Spo0A were passed over the chip surface (Fig. 8A). With all of these proteins except Spo0A, the results were negative (not shown). This failure of MecA to interact with KinA, Spo0F or Spo0B is consistent with its failure to inhibit the phosphorelay reaction *in vitro* (Fig. 7D). In contrast, Spo0A exhibited a dose-dependent binding reaction with immobilized MecA within the low micromolar range of Spo0A concentrations. Similar results were obtained when anti-Spo0A antibodies that had been previously immobilized on the CM5 surface were used to capture Spo0A and MecA was then injected over the chip surface (not shown). When heat-denatured Spo0A was used, no appreciable binding was observed and the presence of bovine serum albumin injected together with the Spo0A did not inhibit the binding reaction, demonstrating the specificity of the interaction. As a final verification, non-His-tagged MecA prepared from an intein construct was used with similar results (not shown).

MecA is a two-domain protein (Persuh *et al.*, 1999). Its N-terminal domain (NTD) establishes the major contacts when binding to ComK or ComS, whereas the C-terminal domain (CTD) contacts ClpC. We used SPR to determine which domain of MecA contacted Spo0A. For this, we expressed and purified each of the domains as His-tagged proteins and coupled them to CM5 surfaces. Fig. 8B shows that the NTD and CTD of MecA can bind separately to Spo0A. In this experiment equal masses of the full-length, NTD and CTD proteins were immobilized. Because the relative masses of MecA:CTD:NTD are 1:0.6:0.5 (Persuh et al., 1999), the immobilized molar amounts of the two domains were about 2-fold larger than that of the full-length MecA. These results suggest that although Spo0A binds to

the individual domains, these interaction affinities are somewhat lower than to full-length MecA. Importantly, because the major contacts between MecA and ComK are with the NTD of ComK, it is evident that ComK and Spo0A bind differently to MecA.

We further addressed this issue by determining if a ComK-derived peptide can bind to MecA if the latter is already bound to Spo0A. For this we used K17, a 17mer peptide (CHRVPKRQEFMLYPKEER) derived from the sequence of ComK that contains its recognition sequence (underlined) for binding to MecA (Prepiak & Dubnau, 2007). This peptide and full-length ComK exhibit indistinguishable binding affinities to MecA in SPR experiments showing that all the important contacts for MecA binding are established by K17. Fig. 9 shows an SPR experiment in which a low concentration (2.4 μM) of Spo0A (see Fig. 8A) was continuously injected over a MecA surface. As the reaction approached saturation, K17 (11 μM) was co-injected. If K17 competed for binding with Spo0A, displacing it, we would detect a decreased response when it was injected because its mass is about 8% that of monomeric Spo0A. Instead, an additional response was detected, which approached a new, higher equilibrium. When buffer was then injected, the response quickly decreased to about the level of the initial Spo0A response and then continued to decline, suggesting that the off-rate for the MecA-K17 complex may be higher than that of the MecA-Spo0A complex. We conclude that K17 and Spo0A can bind at the same time to MecA and that Spo0A therefore does not obscure the ComK recognition sequence when it binds and that Spo0A binding does not depend solely on contacts with this sequence. Obviously, these results do not prove that full length ComK and Spo0A can bind simultaneously to MecA, but only that they probably use different recognition sequences. This result is consistent with the absence of a sequence within Spo0A similar to the FMLYPK motif in ComK for binding to MecA.

Both domains of MecA are needed to inhibit Spo0A~P

The interaction of both the NTD and CTD of MecA with Spo0A raises the question whether both domains are needed for the inhibitory effect of MecA on the activity of Spo0A~P. We approached this question by expressing the individual domains from a multi-copy plasmid and measuring sporulation frequencies. The over-expression plasmids were described previously (Persuh et al., 1999). The NTD-over-expressing strain (BD2142) exhibited a sporulation frequency of 0.72 normalized to the wild-type strain (IS75). The normalized sporulation frequency of the CTD-over-expressing strain (PP559) was 0.76. As expected, the average normalized frequency of two different pKD93-containing strains (PP493 and PP560) was low; 4.1×10^{-5}. The inability of the MecA NTD alone to inhibit spore formation stands in stark contrast to the inhibition of competence exhibited by the same NTD-over-expressing construct (Persuh et al., 1999), once again suggesting that MecA interacts differently with Spo0A and ComK. It has been established that inactivation of *clpC* prevents sporulation (Gerth *et al.*, 1998, Msadek *et al.*, 1998, Pan *et al.*, 2001). Because the CTD of MecA binds to ClpC (Persuh et al., 1999), it is conceivable that the sequestration of ClpC by MecA contributes to the negative effect of MecA on sporulation. However, the inability of the MecA-CTD to inhibit spore formation argues against this possibility, as does our observation that the over-production of the CTD does not decrease the expression of *spoIIG-luc* (not shown).

Discussion

MecA *and the regulation of* eps and sporulation

We have shown that MecA is a pleiotropic regulator of bacterial development. Besides its role in the regulation of competence through the targeting of ComK for degradation by ClpC/ClpP, we have now shown that *mecA* mutant cells transition to sporulation and to *eps*

expression more frequently than the wild-type. The most important conclusion from this study is that MecA can interfere with sporulation and *eps* expression by its direct interaction with Spo0A.

A series of epistasis experiments and the bypass of the MecA overproduction phenotype by expression of the Sad67 mutant form of Spo0A strongly suggested that the negative effects of MecA on *eps* expression and on spore formation are exerted via Spo0A (Figs. 3–5). The failure of MecA to impact the amount of Spo0A protein in growing cells (Fig. 6) and the failure of *spo0A* expression from the P*spac* promoter to bypass p*KD93* (Fig. 5) both show that MecA does not act on the synthesis of Spo0A and is therefore likely to act post-translationally. Consistent with this, the SPR results show that MecA binds directly to Spo0A (Fig. 8). Finally, both *in vitro* and *in vivo* data 21 suggest that MecA does not increase the rate of degradation of Spo0A (Figs. 6, 7). Fig. 7D shows that MecA by itself does not prevent phosphoryl flux through the phosphorelay. This may seem to contradict the *sad67* results, but we suspect that this bypass of pKD93 is due to a failure of MecA to bind effectively to the Sad67 protein, which contains a 19-residue deletion.

The most likely explanation for the inhibiting effect of MecA seems to be that it prevents Spo0A~P from acting as a transcription factor (Fig. 10). It may do this by preventing it from binding to DNA, similarly to the action of RapH, RapF and RapC with another response regulator, ComA (Smits *et al.*, 2007, Core & Perego, 2003, Bongiorni *et al.*, 2005), or by binding to Spo0A~P on the DNA, preventing it from interacting with RNA polymerase as TorI does with the response regulator TorR (Ansaldi *et al.*, 2004). Other possibilities exist, but appear less likely. For example, MecA may potentiate the activity of Spo0A~P as a substrate for Spo0E or some other phosphatase.

Although it appears that MecA does not target Spo0A for degradation by ClpC/ClpP, a *clpC* knockout strain does over-express *eps* (Fig. 3C). We did not attempt to explore equivalent effects on spore expression because a *clpC* loss-of-function mutation prevents sporulation (Gerth et al., 1998, Msadek et al., 1998, Pan et al., 2001). As noted above, the *in vivo* behavior observed for the present system differs from that of ComK regulation. ClpC is not needed for the down-regulation of *comK* when *mecA* is over-expressed, because binding of ComK by excess MecA is sufficient to prevent it from binding to P*comK* even in the absence of ClpC (Turgay et al., 1997). In contrast, when p*KD93* is present a *clpC* mutant still over-expresses *eps* (Fig. 3C). The failure of *clpC* inactivation to yield as high a level of *eps* transcription as does inactivation of *mecA* (Fig. 3) also differentiates this system from competence regulation and leads to the hypothesis that MecA and ClpC regulate *eps* transcription using distinct pathways. Recent work with the SlrR protein suggests a candidate mechanism for the effect of ClpC (Chai *et al.*, 2010b). SlrR binds to SinR, and its over-expression titrates this repressor, resulting in over-expression of *eps*. Interestingly, SlrR accumulates in a *clpC* mutant and in such a mutant more robust biofilms are formed, consistent with the results of Fig. 3C (Chai *et al.*, 2010a).

Interactions of MecA with its partners

ComK and ComS compete for binding to MecA, interacting with overlapping binding sites (Prepiak & Dubnau, 2007). Binding to this site on MecA leads to the degradation of either substrate by ClpC/ClpP. The SPR results in Fig. 9 suggest strongly that Spo0A binding to MecA does not require the ComK/ComS site on MecA. Because MecA-ClpC does not target Spo0A for degradation, we suspect that distinct binding sites for ComK and Spo0A may facilitate different outcomes; either degradation or inactivation as a transcription factor, but this hypothesis remains to be proved. It has been shown that the small protein Spx also binds to MecA, increasing its affinity for ComK (Nakano *et al.*, 2002a). Spx and ComK can bind together to MecA, and therefore interact with different surfaces on this versatile protein. We

do not know if the Spx and Spo0A binding sites are distinct. Defining these sites and their interactions and the identification of additional binding partners for MecA will be an important task for the future.

The biological roles of MecA

MecA controls premature transitions to the competent state by directing the degradation of ComK and also controls transitions to *eps* expression and spore formation by a non-degradative mechanism. In addition, it has been reported that MecA regulates motility by read-through from a ComK-dependent promoter, but also by an uncharacterized ComK-independent mechanism (Liu & Zuber, 1998, Rashid *et al.*, 1996).

By interacting with Spo0A, MecA acts at a critical point to regulate several developmental adaptations, all bimodally expressed. We propose that MecA acts as a buffer during exponential growth, ensuring that most cells thriving in a nutrient-rich environment do not commit valuable resources to an unnecessary physiological adaptation. Nevertheless, we suggest that noise in the accumulation of MecA, or of a pathway-specific effector protein, provides a bet-hedging mechanism, ensuring that a few cells escape and enter one or another of these pathways.

In contrast to this situation obtaining during growth, cells entering stationary phase may undergo programmed changes that alter the barrier imposed by MecA. These represent deterministic mechanisms that are superimposed on stochastic systems of decision-making. For example, the MecA buffer system for ComK is overcome because the quorum-sensing pathway leads to the production of ComS and also because the mean basal expression of ComK is adjusted upward and then downward as a culture arrives in stationary phase (Leisner et al., 2007, Maamar et al., 2007).

Is there a ComS equivalent that alters the buffering effect of MecA on Spo0A? Perhaps the programmed activation of the phosphorelay is sufficient, so that when the concentration of Spo0A~P in a given cell exceeds a threshold set by the MecA concentration, the excess Spo0A~P initiates biofilm and spore formation. The kinetics of *spoIIG-luc* expression in the *mecA* strain hints that more than this passive mechanism may be at play. The timing of the initial rise in *spoIIG* expression is not perturbed in the *mecA* mutant, suggesting that in the wild-type strain, neither the increase in Spo0A~P that occurs at this time nor its activity, are limited by MecA. In contrast, the later rise in *spoIIG* expression is augmented in the mutant, suggesting that MecA does limit this increase. We suggest that during exponential growth there is enough MecA to lower the probability of transition to sporulation. When the phosphorelay becomes active, the increased amount of Spo0A~P overwhelms the MecA barrier and spore formation initiates in many cells. Later during this process, the MecA barrier may be enhanced, limiting the activity of Spo0A~P. The nature of this enhancement is completely unknown.

The control of energy-intensive stress response pathways by MecA-dependent mechanisms is complex, varied and delicately balanced; cells faced with environmental stress enter developmental pathways at high rates when appropriate and at rates that maximize fitness and survival of a shared genotype. When times are good, MecA serves to dampen but not eliminate the expression of these developmental pathways, which relies on stochastic fluctuations in the levels of regulatory proteins. MecA appears to be an important hub protein in the *B. subtilis* regulatory network for development.

EXPERIMENTAL PROCEDURES

Microbiological procedures

The *B. subtilis* strains used are all derivatives of strain 168 and are listed in Table 1. Bacteria were grown in liquid competence minimal medium (Albano *et al.*, 1987), in Luria-Bertani (LB) medium or in DSM (Schaeffer *et al.*, 1965). When required for selection, the media were supplemented with chloramphenicol, erythromycin or kanamycin (each at 5 μg/ml), phleomycin (2 μg/ml) or spectinomycin (100 μg/ml). *B. subtilis* competent cells were prepared as described previously (Albano et al., 1987). *E. coli* DH5α (Invitrogen) was used for cloning. Strain construction was by transformation or by transduction with bacteriophage PBS1. Sporulation was measured after growth for 24 and 48 hours at 37° C in DSM medium by plating for viable counts before and after heating the cells at 80° C for 30 min.

β-galactosidase and luciferase assays

β-galactosidase assays were carried out as previously described (Albano et al., 1987), modified for use in a plate reader. For the detection of luciferase activity, strains were first grown in DSM medium to an optical density at 600 nm (OD_{600}) of 2. Cells were then centrifuged and resuspended in fresh DSM, adjusting all the cultures to an OD_{600} of 2. These pre-cultures were then diluted 20 fold in fresh DSM and 200 μl was distributed in each of two wells in a 96-well black plate (Corning). 10 μl of luciferin were added to each well to reach a final concentration of 1.5 mg/ml (4.7 mM). The cultures were incubated at 37°C with agitation in a PerkinElmer Envision™ 2104 Multilabel Reader equipped with an enhanced sensitivity photomultiplier for luminometry. The temperature of the clear plastic lid was maintained at 38°C to avoid condensation. Relative Luminescence Unit (RLU) and OD_{600} were measured at 1.5 min intervals.

Western blot analysis

Preparation of whole cell extracts and Western blotting was carried out by standard methods. Protein extracts were made by pelleting 1 ml of cells, washing in STM (50 mM NaCl, 25% sucrose, 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$), lysing by sonication, followed by mixing with 5X glycerol sample buffer (0.225 M Tris-HCl pH 6.8, 50% glycerol, 5% SDS, 0.05% Bromophenol Blue, 1% *β*-mercaptoethanol). Proteins were resolved by SDS-PAGE (12% Tris-tricine) (Schagger & von Jagow, 1987) at a constant amperage (25 mA) and then transferred to nitrocellulose membranes for 1 hour at 12 V in a semi-dry transfer apparatus (Bio-Rad). Transferred proteins were detected using appropriate antibodies, all raised in rabbits and used with the following dilutions: 1:500 for anti-SinI, 1:2,000 for anti-MecA and 1:5,000 for anti-Spo0A and anti-ComK, followed by secondary anti-rabbit antibodies conjugated to horseradish peroxidase (Zymed #401315, 1:10,000). Secondary antibodies were detected using enhanced chemiluminescence ECL+ (Amersham). The anti-SinI antiserum was kind gift from D. Kearns.

Microscopy

Cultures were grown in LB or DSM until the indicated times and samples were attached to poly-L-lysine-coated slides, mounted in Slowfade (Molecular Probes). Either staining with propidium iodide (10 μg/ml) or DIC imaging was used to visualize the cell bodies. Microscopy was performed with an upright Nikon Eclipse 90i microscope equipped with an Orca-ER Digital Camera (Hamamatsu), and a Nikon TIRF 1.45 NA Plan Neo-Fluor 100 X oil immersion objective. Velocity software (Improvision) was used for image acquisition and processing. Two images fluorescence images for CFP and PI were captured for each field of cells. Appropriate Semrock optical filter sets were used for each fluorophore.

Protein Purification

Spo0A protein purification was performed as previously described by Muchová et al (Muchova *et al.*, 2004). Briefly, four one liter cultures of the *E. coli* strain PP494, which carries *spo0A* cloned into the pET26b(+) vector (Novagene), were grown in LB with 30 μg/μl kanamycin until the cultures reached an OD_{600} of 0.6. IPTG (1 mM final concentration) was added and the cultures were incubated at 30°C for 4.5 hours. PP494 was a kind gift from I. Barak. The cells were harvested by centrifugation and resuspended in Buffer A (250 mM NaCl, 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM DTT, 1 mM PMSF). The cells were lysed with an EmulsiFlex-C5 (Avestin) disruptor and centrifuged for 20 minutes at 25,000 rpm. The supernatant was collected and loaded onto a pre-equilibrated 5 ml Heparin Hi Trap column for FPLC. Protein fractions were eluted with a gradient of Buffer A (up to 1M NaCl). Fractions with Spo0A were collected, pooled, concentrated to 1 mg/ml, and stored at −80°C until use.

MecA-His_6 was purified as described previously (Turgay et al., 1997). One liter cultures of *E. coli* M15 harboring pQE60-*mecA* were grown in LB with Ampicillin (100 μg/ml) at 37° C until OD_{600} = 500 – 800, at which time expression of the protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After three hours of induction, cells were harvested by centrifugation at 4° C for 10 min at 5,000 rpm. The pellets were resuspended in cold lysis buffer (25 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM imidazole) and centrifuged again using the same conditions. The pellets were resuspended in 25 ml cold lysis buffer and treated with one tablet of protease inhibitor cocktail (Roche #1873580). Cells were broken by passage through a French Press three times at 1,200 psi. Cell debris was pelleted by centrifugation at 4° C for 35 min at 20,000 rpm. The supernatants were mixed with 1 ml of Ni^{2+} resin (Qiagen), equilibrated in lysis buffer in a 50 ml conical tube and then diluted with lysis buffer to approximately 45 ml. Incubation with the nickel beads was continued for one hour on a rotary shaker at 4° C to facilitate binding. The mixture was then poured into a column (BioRad), allowed to flow through and washed extensively (~50 column volumes) with wash buffer (25 mM HEPES, pH 7.5, 500 mM NaCl, 25 mM imidazole). The protein was then eluted with 75 mM imidazole. Total protein was assayed with the BioRad protein determination reagent and purity was determined by 12% SDS-PAGE and Coomassie blue staining. Protein samples were dialyzed into storage buffer (25 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, 0.5 mM DTT). Protein concentrations were determined by dilution into 7.4 M guanidinium hydrochloride, 50 mM Na phosphate, pH 7.5 using extinction coefficients at 280 nm determined from amino acid compositions (http:://au.expasy.org/tools/protparam.html).

For the purification of ClpC and ClpP, one liter cultures of *E. coli* M15 harboring plasmids that express either *clpC* or *clpP* fused to self-cleaving intein tags. The *clpC*-intein construct was a gift from M. Nakano (Nakano *et al.*, 2002b). The strains were grown in LB with ampicillin (100 μg/ml) at 37° C until OD_{600} = 500 – 800, at which time expression of the protein was induced with 4 mM IPTG. These constructs, kind gifts from M. Nakano, were under IPTG-inducible control (IMPACT system, New England Biolabs). Following addition of IPTG, cultures were incubated overnight with shaking at 15° C. Cells were harvested by centrifugation at 4° C for 10 min at 5,000 rpm. The pellet was resuspended in cold column buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 1mM EDTA) and centrifuged again under the same conditions. The pellet was then resuspended in 25 ml cold column buffer. The cells were broken by passing through a French Press three times at 1,200 psi. Cell debris was pelleted by centrifuging at 4° C for 35 min at 20,000 rpm. The supernatants were mixed with 0.5 ml of chitin resin (New England Biolabs), equilibrated in column buffer in a 50 ml conical tube and then diluted with column buffer to approximately 45 ml. The protein fraction with chitin beads was incubated on a rotary shaker for 1 hour at 4° C to facilitate binding. The mixture was poured into a column (BioRad), allowed to flow through and was

washed extensively (~50 column volumes) with column buffer. Cleavage of the intein tag was induced by quickly flushing the column with 3 column volumes of cleavage buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, 50 mM DTT), closing the column and leaving it overnight at 4 C. The flow-through was collected the following day. Protein concentrations were determined by BioRad protein assay and protein purity was determined using 12% SDS-PAGE and Coomassie blue staining. Protein samples were dialyzed into storage buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$). Protein concentration was determined spectrophotometrically as described above.

SPR experiments

A Biacore 2000 instrument was used for all experiments. MecA-His_6 was covalently coupled to the surface of CM5 sensor chips using amine-coupling chemistry. Solutions containing the indicated concentrations of the analyte proteins in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) were passed over the sensor chip surface at a constant flow rate 20 μl/min and at 25° C. The response of the same solutions on a mock-coupled surface was subtracted from all sensograms.

In vitro degradation assay

In vitro degradation assays were based on published procedures (Turgay et al., 1998) and contained the necessary components for degradation; ClpC, ClpP and ATP, the adaptor protein MecA and a substrate, either ComK or Spo0A. ClpC, ClpP and ComK were added at 1.5 μM, MecA was added at 0.5 μM unless otherwise indicated and Spo0A was added at 0.3 μM. An ATP regeneration system was used containing 10 mM ATP, pH 7, 0.033 mg/ml creatine phosphokinase and 1.6 mM creatine phosphate. A preincubation step with ADP (5 mM) was employed to allow for assembly of the proteolytic complex. The reaction components were mixed in Buffer A (100 mM KCl, 25 mM MOPS, pH 7, 5 mM $MgCl_2$) in the following order: ClpC, MecA, ComK or Spo0A, ClpP, ADP. After addition of ADP the reactions were incubated at 30° C for 30 min after which the reactions were moved to 37° C and the ATP and regenerating system were added. Reactions were incubated for one hour at 37° C. Samples of 45 μl were collected and the reaction was stopped by adding glycerol samples buffer (see Western blot protocol). Western blotting was used to detect ComK, Spo0A or MecA as described above.

Phosphorelay reaction

Phosphorylation reactions were done as described in Burbulys at. al. (1991) Reaction mixtures (total 20 μl) contained 1 μM KinA-His_6, 0.2 μM Spo0F-His_6, 0.2 μM Spo0B-His_6, 1 μM Spo0A and 0–120 μM MecA-His_6. The reactions were initiated by addition of ATP, incubated 1 hour at 25°C and stopped by addition of SDS Sample buffer. Just before loading, bovine serum albumin was added to equalize the total protein loaded per lane and the samples were immediately loaded on a 16% SDS polyacrylamide gel. Gels were exposed using a Phosphorimager Screen, which was scanned using a *Typhoon*™ scanner (GE Healthcare).

Acknowledgments

We thank all members of our lab and the Neiditch lab as well as R. Grau, K. Muchova and I Barak for useful advice and discussions. We also thank M. Nakano and D. Kearns for the kind gifts of strains and anti-sera and Miguel Dias for constructing the *eps-cfp* fusion. This work was supported by NIH grant GM057720.

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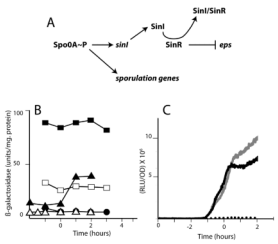

Fig. 1.

(A) Scheme showing the role of Spo0A~P as a master upstream regulator of both *eps* expression and transcription of sporulation genes. Spo0A~P activates a promoter in front of *sinI* (Shafikhani et al., 2002). The *eps* operon is directly repressed by SinR (Kearns et al., 2005). Generally, repression due to SinR is lifted when it is sequestered by the protein SinI (Bai et al., 1993). (B) *eps-lacZ* expression in *mecA* and p*KD93* strains. Wild-type (BD4498, empty squares), *mecA::erm* (BD4538, filled squares) and p*KD93* (BD4644, filled circles) strains were grown in LB and β-galactosidase activities were determined at the indicated times. The triangles show results for strains growing in MsGG medium; wild-type (BD4498, closed triangles) and pKD93 (BD4644, open triangles). (C) *spoIIG-luc* expression in *mecA* and p*KD93* strains. Wild-type (PP533, black line), *mecA* (PP551, gray line) and p*KD93* (PP565, dotted line) strains were grown in DSM in a plate reader and growth (OD at 600 nm) and light output was measured every 1.5 minutes. For both panels, “Time” is given as hours before and after the transition to stationary phase.

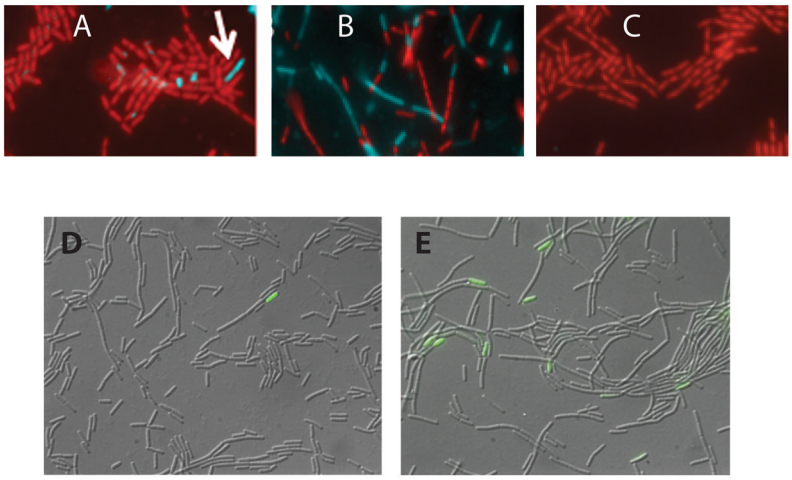

Fig. 2.

Heterogeneous expression of *eps-cfp* and *spoIIE-gfp* in *mecA* and p*KD93* strains. Strains were grown in LB and sampled at T_1. Panels A, B and C present typical fields from the wild-type (BD4621), *mecA::erm* (BD4642) *and pKD93* (BD4643) strains respectively. Cell bodies were stained with propidium iodide and pseudocolored red. CFP fluorescence was pseudocolored cyan and overlayed on the propidium iodide channel. For panels D and E, a strain expressing *spoIIE-gfp* was grown in DSM to T_{-1}. Images showing GFP fluorescence were overlayed on DIC images. Panels D and E show images from strains with the wild-type (PP480) and *mecA::erm* (PP479) backgrounds respectively.

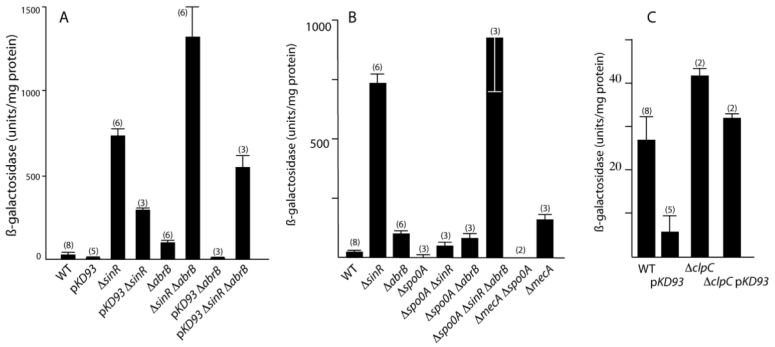

Fig. 3.

Effects of *sinR*, *abrB* and *clpC* inactivation on *eps-lacZ* expression in wild-type (BD4498) and p*KD93* (BD4643) backgrounds. Strains carrying the indicated mutations, were grown in LB and samples taken for β-galactosidase determination at T_1. (A) The following strains, all carrying *eps-lacZ*, were used for this experiment: BD4498 (wild-type), BD4644 (p*KD93*), BD4544 (*sinR::cat*), BD4549 (*sinR::cat* p*KD93*), BD4623 (*abrB::cat*), BD4615 (*sinR::kan abrB::cat*), BD5113 (*abrB::cat* p*KD93*), BD4624 (*abrB::cat sinR::kan* p*KD93*). (B) The following strains carrying *eps-lacZ* were used for this experiment: wild-type (BD4498), *sinR::cat* (BD4544), *abrB::cat* (BD4623), *spo0A::*kan (BD4928), *spo0A::kan sinR::*cat (BD4929), *spo0A::kan abrB::cat eps-lacZ* (BD4930), *spo0A::kan abrB::cat sinR::kan* (BD4931), *spo0A::kan mecA::*erm (BD5589), *mecA::erm* (BD4538). (C) The following strains were used for this experiment: wild-type (BD4498), p*KD93* (BD4644), *clpC::tet* (BD4580), *clpC::tet* p*KD93* (BD5114). The presence of the *pKD93* plasmid indicates that *mecA* is over-expressed. The numbers in parentheses refer to the number of independent measurements for each strain and the whiskers show standard deviations.

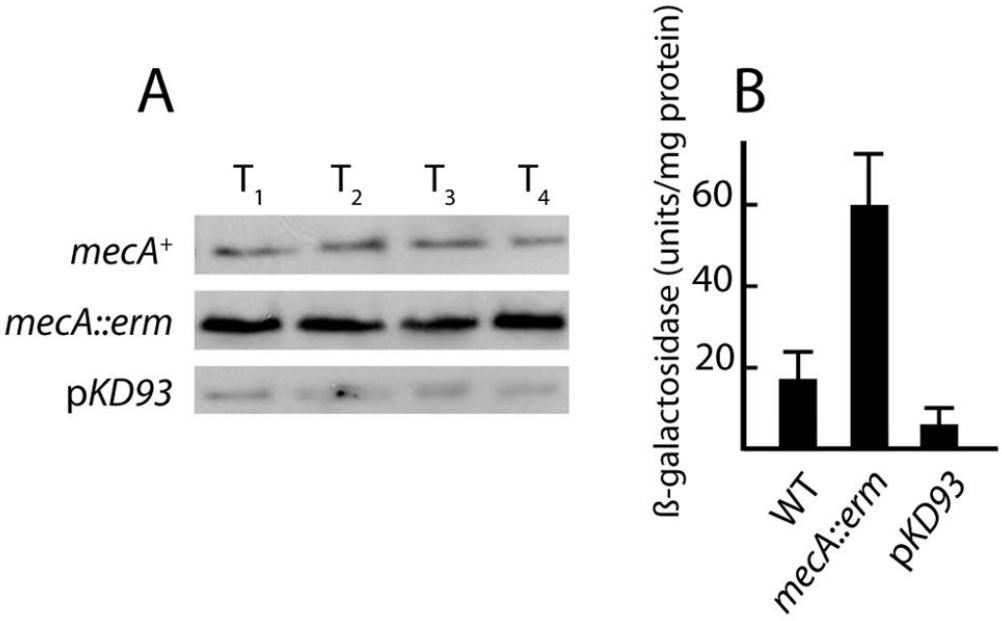

Fig. 4.

The amount of SinI protein and the expression of *sinI-lacZ* in *mecA* and p*KD93* strains. (A) Immunoblot using anti-SinI antiserum on extracts of wild-type (BD4498), *mecA::erm* (BD4538) and p*KD93* (BD4644) strains grown in LB to the indicated times. Equal amounts of total protein were loaded on each lane. (B) β-galactosidase activities produced from *sinI-lacZ* in wild-type (BD4555), *mecA::erm* (BD4568) and p*KD93* (BD4557) strains grown in LB to T_1. The data for panels A and B were derived from three independent measurements. The presence of the *pKD93* plasmid indicates that *mecA* is over-expressed. The whiskers show standard deviations.

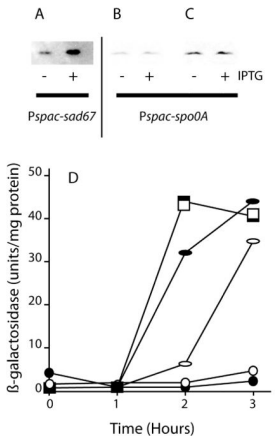

Fig. 5.

Bypass of p*KD93* inhibition by *sad67*. Strains carrying p*KD93* and *Pspac-sad67* (BD4628) (A) or p*KD93* and *Pspac-spo0A* (BD4692) (B and C) were grown in LB until 1 hour before T_0. The cultures were split and IPTG (1 mM) was added to half of each culture. Growth was continued for two hours and samples were collected for Western blotting with anti-SinI antiserum. The two lanes in panel C are identical to those in B except that they have been enhanced to more clearly show the absence of induction. The vertical line is an indication that panel A is from a different gel than panels B and C. For both P*spac* strains, induction of Spo0A and the Sad67 protein was verified by stripping the gels and probing with Spo0A antiserum (not shown). Panel D shows bypass of *spoIIE-lacZ* expression in the presence of p*KD93* in cultures growing in DSM. The solid and empty symbols show results from cultures incubated with and without IPTG, respectively. The squares show results for a *Pspac-sad67 spoIIE-lacZ* strain (PP485). Only one line is shown for the empty and filled squares. The circles show *spoIIE-lacZ* expression for a *spoIIE-lacZ* strain carrying p*KD93* (PP487). The ellipses show expression in a P*spac-sad67 spoIIE-lacZ* p*KD93* strain. Time zero is defined as the time when IPTG (1 mM) was added (at T_0).

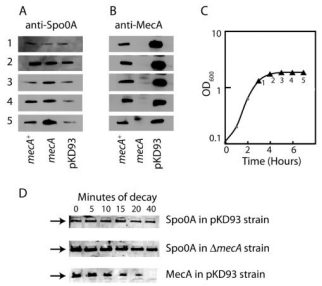

Fig. 6.

Detection of Spo0A protein by immunoblotting in wild-type (BD2149), *mecA::erm* (BD2148) and p*KD93* (PP493) strains. The cultures were grown in DSM and samples were taken at the times indicated on the growth curve (panel C) for immunoblotting with anti-Spo0A and anti-MecA antisera (panels A and B respectively. The three strains grew identically and a curve is shown only for the wild-type strain. Equal amounts of protein were loaded in each lane. (D) BD2148 and PP493 were grown in DSM to just after T1 and puromycin (200 mg/ml) was added to inhibit protein synthesis. Samples were taken for blotting with anti-Spo0A antiserum (both strains) and with anti-MecA antiserum (PP493 only) at the indicated times. The arrows show the position of the Spo0A (top two panels) and Mec (bottom panel) signals.

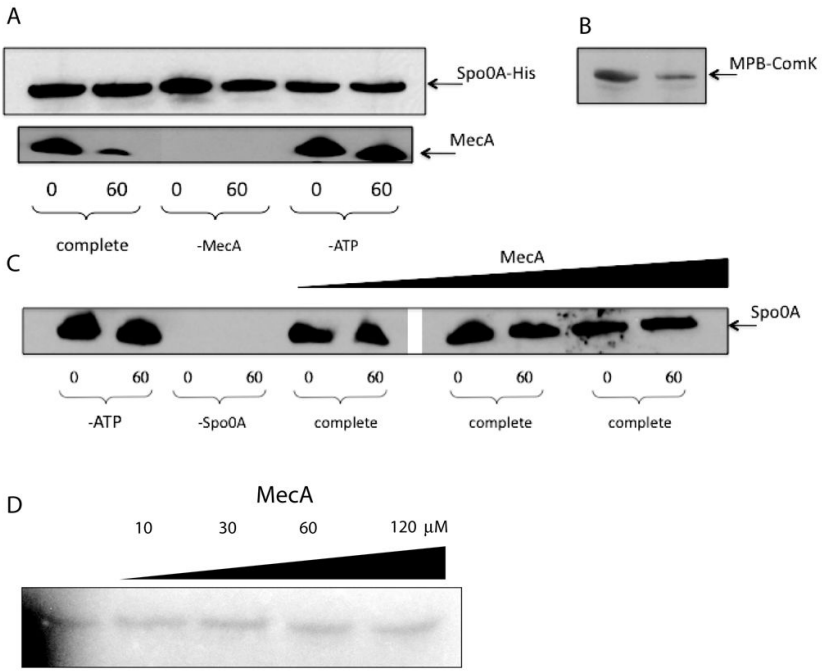

Fig. 7.

In vitro degradation assay. Spo0A-His, Spo0A, ComK-MBP and MecA were detected by immunoblotting with their cognate antisera. Samples were taken after zero and 60 minutes incubation. All incubation mixtures contained MecA, ClpC, ClpP, ATP and an ATP regenerating system unless otherwise indicated. (A) The incubation mixtures contained Spo0A-His. (B) The incubation mixture contained ComK-MBP, ClpC, ClpP, MecA and ATP. (C) Native Spo0A protein (0.3 μM) and ATP were included in these incubations, unless otherwise indicated and the concentration of MecA was varied in the 6 rightmost lanes (0.4 μM, 1.5 μM and 5 μM). Samples were run on the same gel, but an irrelevant portion was excised between lanes 6 and 7. (D) His-tagged KinA, Spo0F, Spo0B and untagged Spo0A (each at 0.2 μM) and various concentrations of MecA-His_6 were incubated in the presence of 32P-γ-ATP as described in Experimental Procedures and following the published procedures (Fujita & Losick, 2003) and (Burbulys et al., 1991). The samples were autoradiographed after resolution by SDS-PAGE. Just before loading, bovine serum albumin was added to equalize the total protein loaded per lane. The gel was deliberately underexposed to ensure that the signals were within the sensitive range of film response. In control experiments, omission of the individual phosphorelay components prevented phosphorylation of Spo0A (not shown).

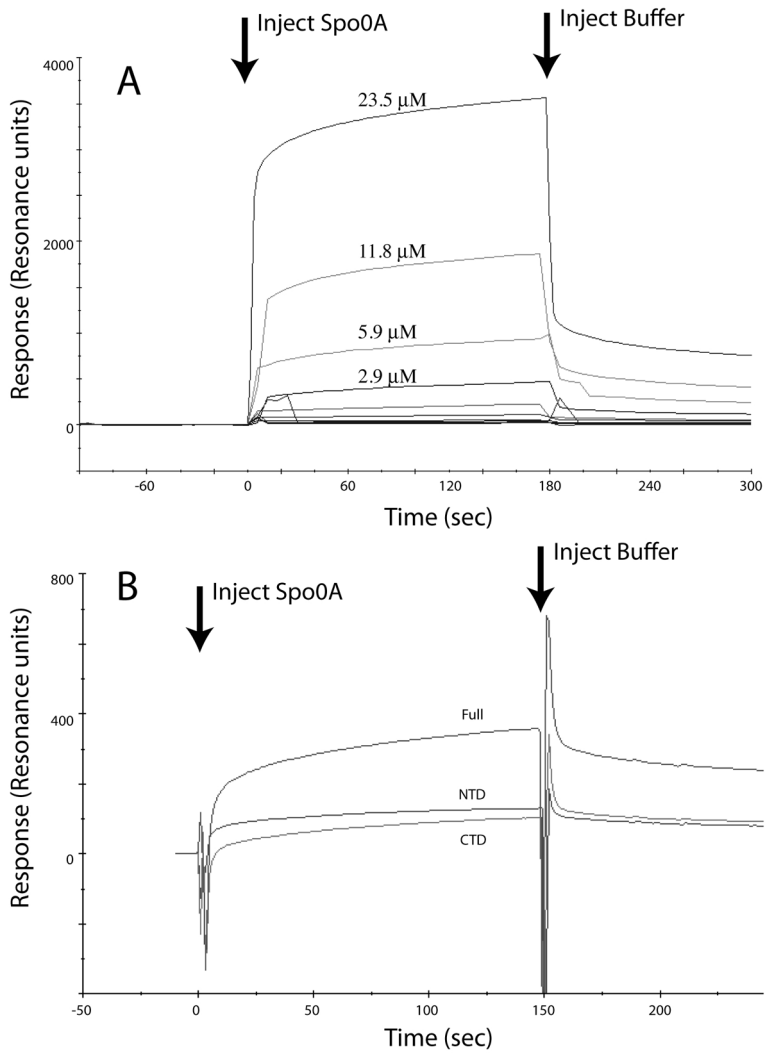

Fig. 8.

MecA binds directly to Spo0A. (A) MecA-His_6 was immobilized on a CM5 chip surface and Spo0A at the indicated concentrations was passed over the chip surface. (B) As with panel A, except that full length MecA, and twice the molar concentration each of the NTD and CTD of MecA were immobilized on separate surfaces. Spo0A (3 μM) was passed over the chip surfaces. For both panels, values from a mock-coupled chip surface without MecA-His_6 were subtracted.

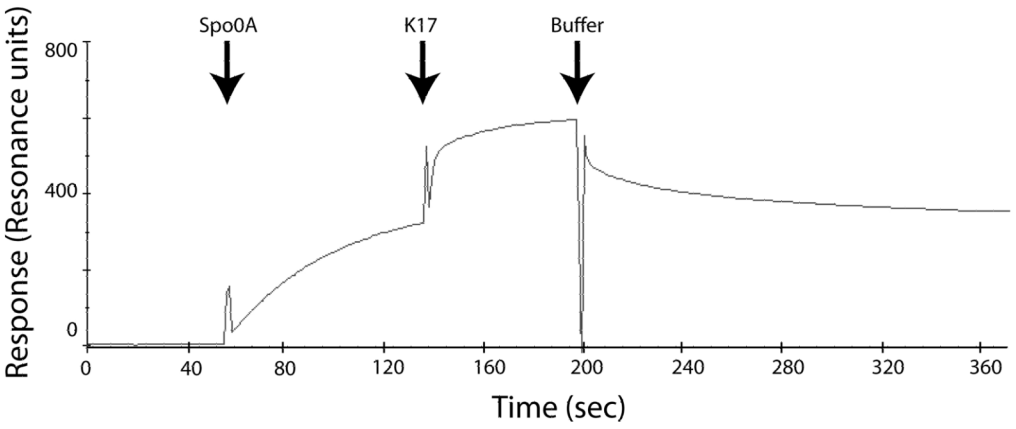

Fig. 9.

K17 and Spo0A bind simultaneously to MecA. MecA-His_6 was immobilized on the surface of a CM5 chip. At the indicated time, Spo0A (2.4 μM) was injected. At a later time K17 peptide at a concentration of 11 μM was co-injected with Spo0A as indicated, followed about 70 seconds later by protein-free buffer.

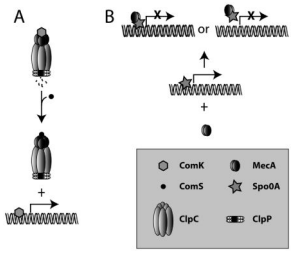

Fig. 10.

Two modes of developmental regulation by MecA. In the case of competence (A), MecA binds to the transcriptional activator ComK and targets it for degradation by a complex of MecA, ClpC and ClpP (Turgay et al., 1998). These proteins exist as dimers, hexamers and double heptamer rings respectively. When quorum-sensing results in the synthesis of ComS, this small protein competes with ComK for binding to MecA (Prepiak & Dubnau, 2007). Free ComK can then bind to competence promoters. As shown here, MecA can also interact with Spo0A (B) either preventing it from binding to its target promoters or from acting as a transcription factor once bound.

Table 1

Strains

Strain	Genotype[1]	Source
***B. subtilis* strains:**		
BD630	*his leu met*	Lab strain
BD1512	*comG-lacZ (erm)*	
BD2091	*mecA::erm*	(Kong et al., 1993)
BD2142	*amyE::spo0A-lacZ (cat)* p*MecA-NTD*	(Persuh et al., 1999)
BD2148	*mecA::spc comK::kan spo0A-lacZ (cat)*	Lab strain
BD2149	*comK::kan spo0A-lacZ (cat)*	
BD2200	*comG-lacZ (erm)* p*KD93 (Phl)*	Lab strain
BD3980	*mecA::erm epsG::cat eps-lacZ::tet*	This work
BD4498	*eps-lacZ (tet)*	D. Kearns
BD4538	*eps-lacZ (tet) mecA::erm*	This work
BD4544	*eps-lacZ (tet) sinR::cat*	This work
BD4549	*eps-lacZ (tet)* p*KD93 (phl)*[2] *sinR::cat*	This work
BD4555	*sinI-lacZ (cat)*	I. Smith
BD4557	*sinI-lacZ (cat)* p*KD93 (phl)*	This work
BF4568	*sinI-lacZ (cat) mecA::erm*	This work
BD4580	*eps-lacZ (cat) clpC::tet*	This work
BD4615	*eps-lacZ (tet) abrB::cat sinR::kan*	This work
BD4621	*eps-cfp (cat)*	M. Dias
BD4623	*eps-lacZ (tet) abrB::cat*	This work
BD4624	*eps-lacZ (tet) abrB::cat sinR::kan* p*KD93 (phl)*	This work
BD4626	*spo0A::kan amyE::Pspac-sad67* (*cat*)	(Ireton et al., 1993)
BD4628	*spo0A::kan amyE::*P*spac-sad67* p*KD93 (phl)*	This work
BD4642	*eps-cfp (cat) mecA::erm*	This work
BD4643	*eps-cfp (cat)* p*KD93 (phl)*	This work
BD4644	*eps-lacZ (tet)* p*KD93 (phl)*	This work
BD4692	P*spac-spo0A* (*cat*)[4] p*KD93 (phl)*	This work
BD4928	*eps-lacZ (tet) spo0A::kan*	This work
BD4929	*eps-lacZ (tet) spo0A::kan sinR::cat*	This work
BD4930	*eps-lacZ (tet) spo0A::kan abrB::cat*	This work
BD4931	*eps-lacZ (tet) spo0A::kan abrB::cat sinR::kan*[3]	This work
BD5113	*eps-lacZ (tet)* p*KD93 (phl) abrB::cat*	This work
BD5114	*eps-lacZ (cat) clpC::tet* m*c mecA (kan)*	This work
BD5589	*eps-lacZ (tet) spo0A::kan mecA::erm*	This work
PP479	*spoIIE-gfp (spc) mecA::erm comK::kan*	(Fujita & Losick, 2003)
PP480	*spoIIE-gfp (spc) comK::kan*	(Fujita & Losick, 2003)
PP485	*thrC::spoIIE-lacZ* (*erm*) *amyE::Pspac-sad67(cat)*	(Ireton et al., 1993)
PP487	*thrC::spoIIE-lacZ* (*erm*) p*KD93 (phl)*	This work
PP488	*thrC::spoIIE-lacZ* (*erm*) *amyE::Pspac-sad67(cat)* p*KD93 (phl)*	This work

Strain	Genotype[1]	Source
PP493	*amyE::spo0A-lacZ (cat)* p*KD93 (phl)*	Lab strain
PP510	*thrC::spoIIE-lacZ* (*erm*) *amyE::Pspac-spo0A (cat)*)	(Fujita et al., 2005)
PP512	*thrC::spoIIE-lacZ* (*erm*) *amyE::Pspac-spo0A (cat)* p*KD93 (phl*)	This work
PP516	*thrC::spoIIE-lacZ* (*erm*) *amyE::Pspac-sad67(cat)*) *spo0A::kan*	This work
PP522	*thrC::spoIIE-lacZ* (*erm*) *amyE::Pspac-sad67(cat)*) *spo0A::kan* p*KD93*	This work
PP533	*spoIIG-luc (cat)*	This work
PP551	*spoIIG-luc (cat) mecA::erm comK::spc*	This work
PP559	*amyE::comK-lacZ (cat)* p*MecA-CTD*	(Persuh et al., 1999)
PP560	*amyE::comK-lacZ (cat)* p*KD93*	This work
PP565	*spoIIG-luc (cat)* p*KD93(phl)*	This work
***E. coli* strain:**		
PP494	*B. subtilis spo0A* in pET26b(+) in *E. coli* B834	(Muchova et al., 2004)

[1] All strains were constructed in the *his leu met* background of IS75, except for PP485, PP487, PP488, PP479 and PP480 which are in the prototrophic PY79 background and PP510, PP512, PPP516 and PP522 which are in a *trp phe* background.

[2] The plasmid p*KD93*, consists of pUB110, into which was inserted *mecA* under control of a constitutive promoter (Kong et al., 1993). This plasmid has been shown by Western blotting to overproduce MecA. The plasmid expressed resistance to both phleomycin (*phl*) and kanamycin (*kan*).

[3] BD4931 was constructed by congression with selection for Kan and Cat, picking up the *spo0A*, *sinR* and *abrB* markers at the same time, as verified by.

[4] P*spac-spo0A* is a Campbell-like integrant, which inactivates the native *spo0A* gene.