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MINIREVIEW

Two-Component Signal Transduction Systems That Regulate the Temporal and Spatial Expression of *Myxococcus xanthus* Sporulation Genes

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When starved for nutrients, *Myxococcus xanthus* produces a biofilm that contains a mat of rod-shaped cells, known as peripheral rods, and aerial structures called fruiting bodies, which house thousands of dormant and stress-resistant spherical spores. Because rod-shaped cells differentiate into spherical, stress-resistant spores and spore differentiation occurs only in nascent fruiting bodies, many genes and multiple levels of regulation are required. Over the past 2 decades, many regulators of the temporal and spatial expression of *M. xanthus* sporulation genes have been uncovered. Of these sporulation gene regulators, two-component signal transduction circuits, which typically contain a histidine kinase sensor protein and a transcriptional regulator known as response regulator, are among the best characterized. In this review, we discuss prototypical two-component systems (Nla6S/Nla6 and Nla28S/Nla28) that regulate an early, preaggregation phase of sporulation gene expression during fruiting body development. We also discuss orphan response regulators (ActB and FruA) that regulate a later phase of sporulation gene expression, which begins during the aggregation stage of fruiting body development. In addition, we summarize the research on a complex two-component system (Esp) that is important for the spatial regulation of sporulation.

Myxococcus xanthus is a rod-shaped deltaproteobacterium. The natural habitat of *M. xanthus* is topsoil, where it contributes to the "earthy smell" by producing the sesquiterpene geosmin (1). In its natural environment, *M. xanthus* is a microbial predator, hunting in swarming biofilms that collectively feed on other bacteria using hydrolytic enzymes (2–4). When prey bacteria are not available, *M. xanthus* cells form a second type of biofilm that contains a mat of rod-shaped cells, known as peripheral rods, and fruiting bodies containing thousands of metabolically dormant spores (Fig. 1) (5). The spores inside fruiting bodies have thick protective coats that provide resistance to environmental stresses and allow them to survive until nutrients for growth become available, an event which triggers spore germination and, eventually, the formation of swarms that engage in group feeding.

The process of spore formation in *M. xanthus*, and in related species known collectively as the myxobacteria, is fundamentally different from endospore formation in *Bacillus* sp., which is the best-characterized model of bacterial sporulation (6). For example, *M. xanthus* sporulation is a process by which one cell type (a rod-shaped metabolically active cell) differentiates into another cell type (a spherical spore that is dormant and stress resistant). In contrast, *Bacillus* sporulation is not a true cell differentiation: an asymmetric cell division event yields a relatively large mother cell and a smaller cell that eventually becomes a dormant spore. In addition, a *Bacillus* spore develops inside a mother cell, which protects it from the environment, whereas a developing *M. xanthus* spore is directly exposed to the environment. As a consequence, *M. xanthus* must have a mechanism for maintaining the integrity of the cell envelope as it is being reorganized during spore differentiation.

An interesting feature of *M. xanthus* sporulation is its spatial restriction to nascent fruiting bodies; the peripheral rods that surround these structures fail to differentiate into spores. Hence, sporulation in *M. xanthus* is under strict temporal and spatial control. Indeed, it was shown that expression of certain genetic loci that are important for sporulation is spatially localized to the aggregates of cells that develop into fruiting bodies (7, 8).

Abbreviations used in this article are as follows: TCS, two-component system; DHp domain, dimerization and histidine phosphorylation domain; CA domain, catalytic and ATP binding domain; EBP, enhancer binding protein; CRP, cyclic AMP receptor protein; and FNR, fumarate-nitrate reduction.

REGULATORS OF SPORULATION

A number of developmental signals that regulate the formation of spores inside nascent fruiting bodies have been identified, including the intracellular starvation signal (p)ppGpp (9–12), an extracellular cell density reporter known as A-signal (13–16), and a contact-stimulated cell-cell signal known as C-signal (17–22). For more detailed discussions of *M. xanthus* developmental signals, see reviews in references 23–26, and 27.

Signal transduction proteins, which are abundant in *M. xanthus* (28–30), have also been implicated in the regulation of sporulation genes (31, 32). Of these sporulation regulators, two-component signal transduction proteins are perhaps the best-characterized group.

The prototypical TCS contains a sensor histidine kinase protein and a response regulator protein (33). Histidine kinases typically contain a sensor input domain and a transmitter domain

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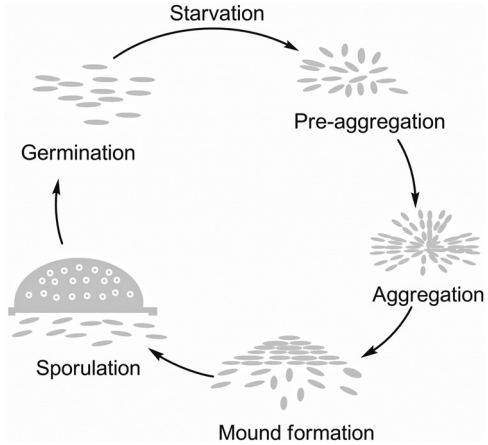

FIG 1 The *Myxococcus xanthus* life cycle. *M. xanthus* is a microbial predator that obtains nutrients by forming swarming biofilms and collectively feeding on other bacteria. When prey bacteria are not available to provide nutrients, cells undergo a multicellular developmental cycle that culminates with the formation of spore-filled fruiting bodies. The stages of development include preaggregation (1 to 5 h poststarvation), aggregation and mound formation (6 to 18 h poststarvation), and sporulation (24 to 120 h poststarvation). Sporulation occurs inside the dome-shaped mounds and is the process by which rod-shaped cells are converted into spherical, stress-resistant spores. The availability of nutrients, which presumably are provided by prey bacteria, triggers spore germination and, eventually, the formation of swarms that engage in group feeding.

(34). The sensor input domain is often a transmembrane domain for detecting extracellular signals and has high sequence variability. In contrast, the transmitter domain is a conserved cytoplasmic domain containing the DHp domain and the CA domain. When a signal is detected by the sensor domain of the histidine kinase, the CA domain transfers a phosphate from ATP to the conserved histidine residue within the DHp domain. The phosphoryl group is then transferred from the histidine kinase to a conserved aspartate residue in the receiver domain of the response regulator (35). Phosphorylation of the response regulator's receiver domain causes a conformational change in its effector domain, which is often a DNA binding domain that allows the response regulator to modulate transcription (36).

In addition to classical TCSs, histidine kinases and response regulators are used to create more-complex signal transduction systems (33). One common variant of the TCS is the phosphorelay system. In such a system, the phosphoryl group is passed from a hybrid histidine kinase, which contains a transmitter domain and a receiver domain, to a histidine phosphotransferase protein and then to a response regulator. TCSs are also frequently organized as branched systems in which one histidine kinase interacts with multiple response regulators or vice versa. In addition, many histidine kinases are bifunctional and can phosphorylate or dephosphorylate their cognate response regulators based on the stimuli (37).

In this review, we discuss prototypical TCSs that are important for an early, preaggregation phase of sporulation gene expression and orphan response regulators that are important for a later, aggregation phase of sporulation gene expression. We also discuss the Esp system, which is a complex TCS that helps ensure that sporulation is spatially localized in nascent fruiting bodies.

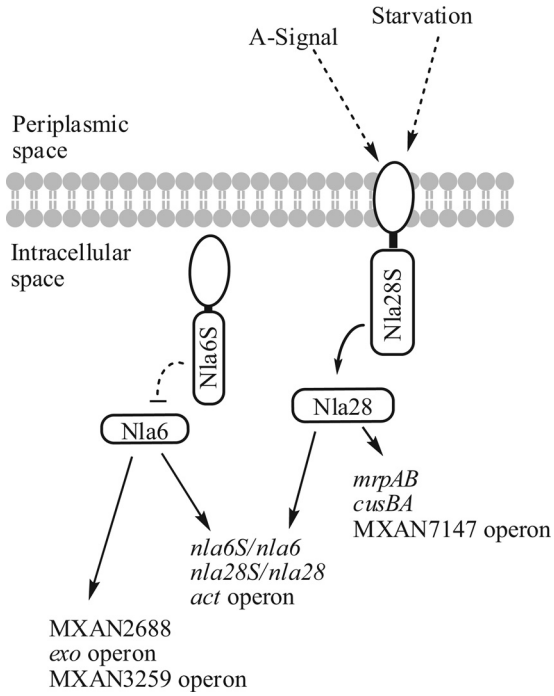

FIG 2 Diagram of the Nla6S/Nla6 and Nla28S/Nla28 two-component systems. Nla6S/Nla6 and Nla28S/Nla28 are early-acting two-component systems that directly regulate expression of sporulation genes. Nla6S and Nla28S are predicted to be cytoplasmic and membrane-bound histidine kinase sensors, respectively (28). The signal detected by Nla6S is unknown; however, it has been proposed that the cell density reporter known as A-signal or nutrient levels (starvation) may be the activating signal for Nla28S (67). It is believed that Nla6S modulates the activity of the Nla6 response regulator via dephosphorylation, whereas Nla28S modulates the activity of the Nla28 response regulator via phosphorylation (38, 67). Nla6 is a transcriptional activator that regulates genetic loci such as *exo*, MXAN2688, and MXAN3259 that are important for spore differentiation and spore stress resistance (59). Nla28 is a transcriptional activator that regulates genetic loci such as *cusBA* and MXAN7147, which are primarily involved in spore stress resistance (Li et al., unpublished). Nla6 and Nla28 also modulate expression of other transcriptional regulators that are important for sporulation: Nla6 modulates expression of *actB* and *nla28*, and Nla28 modulates expression of *actB*, *mrpAB*, and *nla6* (43) (Li et al., unpublished). In addition, Nla6 and Nla28 are involved in autoregulation (43).

TWO-COMPONENT SYSTEM PROTEINS THAT REGULATE *M. XANTHUS* SPORULATION

Early regulators of sporulation. (i) Nla6S/Nla6. Nla6S (S for histidine kinase sensor) and Nla6 form an early-acting two-component system that begins functioning about 1 h after starvation initiates fruiting body development (Fig. 1 and 2). Nla6S is a cytoplasmic protein that has a remarkable property; it lacks many of the conserved sequence motifs of typical histidine kinases but still has all the *in vitro* properties of histidine kinases (38). In particular, an alignment of the C-terminal transmitter region of Nla6S with those of known histidine kinases uncovered a putative DHp domain; however, no CA domain was identified (28, 38–40). A closer look at the predicted secondary structure of the C-terminal transmitter region of Nla6S revealed characteristics of a functional CA domain. Moreover, biochemical analyses indicate that both the DHp and CA domains in the Nla6S transmitter region are functional (38). In subsequent phylogenetic analyses, potential orthologs of Nla6S were found, but only in the sequenced, fruiting

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members of the *Cystobacterineae* suborder of the myxobacteria (38). Taken together, these data indicate that Nla6S is the prototype for a new family of histidine kinases thus far found only in fruiting *Cystobacterineae*.

Nla6 is a member of the EBP family of response regulators (41–43), which work with the σ^{54} protein to activate transcription at σ^{54} promoter elements. In particular, σ^{54} directs RNA polymerase to conserved sequences in the −24 and −12 regions of target promoters (44) and EBPs, which bind to tandem repeat sequences located upstream of the −24 and −12 regions (45–47), use the energy from ATP hydrolysis to help σ^{54}-RNA polymerase form an open promoter complex, and initiate transcription (48–50).

Since the *nla6S* histidine kinase and *nla6* response regulator genes are cotranscribed (28, 43) and two-component system partners are often regulated in such a manner, it has been assumed that Nla6S and Nla6 form a two-component pair. When this idea was tested experimentally, however, no *in vitro* transfer of a phosphoryl group from Nla6S to Nla6 was detected (38). Perhaps an additional component is required to facilitate the *in vitro* phosphotransfer from Nla6S to Nla6. Alternatively, the primary function of Nla6S may be to dephosphorylate Nla6; some proteins with similarity to histidine kinases, such as RedE in the Red signal transduction system of *M. xanthus* and CheA3 in the Crd/Che3 signal transduction system of *M. xanthus*, function as phosphatases (37, 51–53). This dephosphorylation activity, known as transmitter phosphatase activity, is mediated by a conserved D/EXXT/N motif found immediately adjacent to the phosphoaccepting histidine residue in the DHp domain (54, 55). Indeed, Nla6S has a DXXN motif immediately adjacent to the putative phosphate-accepting histidine residue in its DHp domain, suggesting that Nla6S could act as an Nla6 phosphatase. Thus, Nla6 may be phosphorylated by another histidine kinase or a small-molecule phosphate donor such as acetyl phosphate *in vivo* (56–58) and Nla6S may regulate Nla6 activity via dephosphorylation.

Attempts to inactive *nla6S* have thus far been unsuccessful. However, the function of Nla6S has been inferred from studies of Nla6. A mutation in *nla6* slightly delays but does not inhibit the formation of the tightly packed cell aggregates that become fruiting bodies. In contrast, its effect on the sporulation process is dramatic, reducing the number of viable, stress-resistant spores 500-fold compared to the level seen with the wild type (42). Recent work indicated that the *nla6* mutation affects the process by which rod-shaped cells inside nascent fruiting bodies differentiate into spherical spores and the spores' acquisition of stress resistance (59). These findings led to the proposal that the primary developmental function of the Nla6s/Nla6 TCS is to regulate production of stress-resistant spores.

To understand how the Nla6S/Nla6 TCS regulates sporulation, potential developmental targets of Nla6 were identified using its 10-bp tandem repeat binding site and the *M. xanthus* genome sequence (28, 43, 59). Nineteen operons containing 67 genes and 21 single genes were tagged as potential Nla6 targets using this strategy. Most of the 24 genes that have been experimentally confirmed to be Nla6 targets fall into two functional categories: (i) transcriptional regulators of sporulation such as *nla28S-nla28* and *actB* and (ii) genes that are important for spore differentiation and stress resistance, among which the *exo* operon is the best studied (8, 60–62). The *exo* operon contains nine genes (*exoA* to *exoI*), and most of those genes are known to be important for the export of spore coat polysaccharide, spore differentiation, and spore stress resistance (60, 61, 63). Other Nla6 targets that are important for spore differentiation and stress resistance include the MXAN3259 locus. The MXAN3259 gene encodes a putative member of the polysaccharide deacetylase family of enzymes, which are known to be involved in sporulation in bacteria such as *Bacillus subtilis* and *Streptococcus pneumoniae* (64, 65). The predicted functions of the unconfirmed targets of Nla6 include transcriptional regulation/signal transduction, cell wall/membrane biogenesis, and solute transport.

On the basis of recent studies, it has been suggested that the Nla6S/Nla6 TCS may be a general regulator of stress-associated genes (59, 66). Presumably, the confirmed developmental targets of the Nla6S/Nla6 TCS fall into this category, since they show Nla6-dependent activation 1 h after *M. xanthus* cells encounter starvation-induced stress (59). This early activation occurs well before Nla6S/Nla6 TCS targets are predicted to function; they are important for the sporulation, which starts after about 24 h of development (after the nascent fruiting body is constructed). It was suggested that the Nla6S/Nla6-mediated activation of early genes helps prepare cells for spore differentiation later in development (59).

(ii) Nla28S/Nla28. Nla28S (S for histidine kinase sensor) and Nla28 form a second two-component system that functions in the early stages (1 h poststarvation) of fruiting body development (Fig. 1 and 2). Nla28S and Nla28 were first tagged as potential two-component system partners based on DNA sequence and expression data indicating that *nla28S* and *nla28* genes are cotranscribed (28, 43). Nla28S is a transmembrane histidine kinase. *In vitro* studies showed that Nla28S is a functional histidine kinase with K_m and k_{cat} values comparable to those of other well-studied histidine kinases and that it specifically transfers a phosphoryl group to Nla28 (67), which is a member of the EBP family of response regulators (28, 43).

Two pieces of evidence suggest that Nla28S/Nla28 is an early-functioning two-component system. First, the *nla28S* and *nla28* genes are expressed shortly after starvation initiates development (59, 67). Second, a mutation in *nla28* starts to affect developmental gene expression patterns 1 h poststarvation (43). The early signal to which the Nla28S kinase responds has yet to be identified. However, one candidate is A-signal, which is an early-acting cell density reporter (13, 14, 16). In particular, expression of *nla28S* increases when exogenous A-signal is added to A-signal-deficient cells (67) and, since expression of the *nla28S-nla28* operon is autoregulated (43), it is possible that Nla28S is involved in the detection of A-signal.

Because *nla28S* is expressed at the onset of development, it is also possible that Nla28S monitors nutrient levels. Indeed, the developmental phenotypes of the *nla28S* mutant are different on media containing different levels of nutrients (67). While no significant defect in aggregation or sporulation efficiency was observed when the Δ*nla28S* strain developed on stringent-starvation agar containing no added nutrients, aggregation was delayed and the sporulation efficiency was reduced when the strain was placed on slow-starvation agar containing low levels of essential nutrients. Nutrient level-dependent developmental phenotypes were previously observed in an analysis of the *asgD* mutant (68). It was suggested that AsgD, a putative hybrid histidine kinase, is involved in detecting nutrient levels and in perceiving starvation. It was also suggested that nutrient levels must be relatively low for the *asgD* mutant cells to detect starvation; cells are unable to detect starva-

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tion on agar containing low levels of nutrients, even though the nutrient levels are too low to sustain growth, but are capable of detecting starvation when no nutrients are added. Perhaps Nla28S has a nutrient-sensing function similar to that of AsgD, which would be consistent with developmental phenotypes of the *nla28S* mutant.

Mutations in the *nla28S* or *nla28* gene primarily affect the process of sporulation (42, 67), although it seems that the *nla28* mutation does cause a slight delay in the formation of tightly packed aggregates of cells (42). The sporulation phenotype of the *nla28* mutation has been characterized in detail, and it has little (if any) effect on the cell shape change associated with sporulation, but it causes the number of viable, stress-resistant spores to be reduced about 50-fold (42). This, together with the sporulation defect of the Δ*nla28S* strain on slow-starvation agar, led to the proposal that the Nla28S/Nla28 TCS regulates the acquisition of spore stress resistance properties. In contrast, its early-function counterpart, the Nla6S/Nla6 TCS, appears to regulate the shape change associated with sporulation and the spores' acquisition of stress resistance.

To date, 12 developmentally regulated operons containing 38 genes have been confirmed as direct Nla28 targets using the expression profiles of *nla28* mutant cells and *in vitro* promoter binding assays (43) (T. Li, D. Lemon, K. Murphy, and A. Garza, unpublished data). In addition, about 60 single genes or operons have been tagged as putative Nla28 targets using the 8-bp direct repeat binding site of Nla28 and the *M. xanthus* genome sequence (Li et al., unpublished).

Several of the confirmed Nla28 target operons contain genes for TCS proteins. These include the *nla6S-nla6* operon and the operon containing *actB*, which is an EBP-type response regulator that functions downstream of *nla6S-nla6* and *nla28S-nla28*. It also includes the operon containing *mrpA* and *mrpB*, which code for a putative histidine kinase and an EBP-type response regulator, respectively (69, 70). MrpB is thought to be a direct regulator of the *mrpC* gene. MrpC appears to be a member of the CRP/FNR family of transcription factors, and it coregulates several sporulation genes with the FruA response regulator-like protein (70–74). FruA and ActB are described in more detail below.

Most of the confirmed targets of Nla28 were previously uncharacterized. Insertions in each of these loci produced developmental phenotypes similar to that of an *nla28* mutation (Li et al., unpublished); they caused a slight delay in the formation of tightly packed cell aggregates and a strong defect in spore stress resistance. Interestingly, the putative products of many of the genes in these Nla28 target loci have similarity to proteins involved in stress resistance in other bacteria, which is consistent with the proposed spore stress resistance function of the Nla28S/Nla28 TCS.

Like the confirmed targets of the Nla6S/Nla6 TCS, the confirmed targets of the Nla28S/Nla28 TCS are activated early in development (1 h poststarvation) and yet they are predicted to function during the late, sporulation stage of development (Li et al., unpublished). Hence, the activation of early genes by both the Nla28S/Nla28 and Nla6S/Nla6 TCSs may help prepare cells for sporulation later in development.

LATE REGULATORS OF SPORULATION

(i) ActA and ActB. ActA and ActB are encoded by genes in the *act* operon. ActA is a putative response regulator that is predicted to have a GGDEF-type effector domain, which is found in diguanylate cyclase enzymes. ActB appears to be a member of the EBP family of response regulators (75). Developmental expression of the *act* operon is subjected to multiple levels of regulation, including direct regulation by the early-functioning Nla6S/Nla6 and Nla28S/Nla28 TCSs (43, 59) (Fig. 2), direct autoregulation via ActB (43), and direct or indirect regulation by the CsgA C-signaling protein and the FruA response regulator-like protein (76). The fact that the *act* operon is the hub for a large amount of regulatory inputs suggests that it has an important developmental function. Indeed, as discussed below, ActB may be a key regulatory switch for sporulation inside a nascent fruiting body.

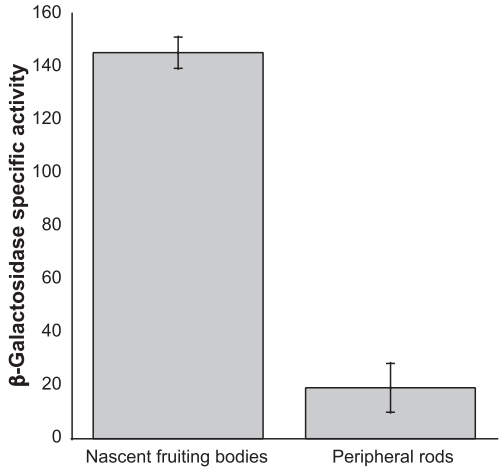

FIG 3 Expression of an *actB*::*lacZ* fusion in fruiting body cells and peripheral rods. Cells were harvested at 24 h of development, and the fruiting body cells and peripheral rods were separated by differential centrifugation as previously described (98). Mean β-galactosidase-specific activities from three independent replicates are shown (Garza, unpublished). Error bars are standard deviations of the means.

Mutational analyses indicate that *actA* and *actB* are required for sporulation inside nascent fruiting bodies but not for the formation of aggregates of cells (75). This finding and the fact that *actA* and *actB* are cotranscribed suggest that both proteins are required for sporulation and that they work in the same signal transduction pathway (75). Little else is known about the function of *actA*; however, *actB* function has been studied in some detail.

Gronewold and Kaiser showed that developmental gene expression patterns go awry in an *actB* deletion mutant only after aggregation is initiated (77), which is consistent with the idea that ActB is a sporulation regulator. Recently, we used differential centrifugation to examine *actB* expression in two developmental cell types: cells in nascent fruiting bodies, which eventually differentiate spores, and the peripheral rods, which fail to differentiate into spores (A. Garza, unpublished data). As shown in Fig. 3, expression of the *actB* gene is about 5-fold to 7-fold higher in cells isolated from nascent fruiting bodies than in the peripheral rods that surround the nascent fruiting bodies. This finding, together with the observed phenotypes of the *actB* mutant strain, suggest that ActB is a key regulatory switch that activates expression of sporulation genes inside nascent fruiting bodies and, in doing so, helps ensure that spore differentiation is spatially restricted to this structure. It is worth noting that ActB positively autoregulates the *act* operon and that such regulation is thought to be important for switch-like functions, which dramatically boost expression of key

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transcription factors (78, 79). The histidine kinase sensor that activates ActB via phosphorylation and presumably triggers ActB-mediated expression of sporulation genes in nascent fruiting bodies has yet to be identified.

It was proposed that ActB triggers sporulation inside nascent fruiting bodies by directly regulating the *csgA* gene (75). This proposal was based on two pieces of data: an *actB* deletion reduces expression of the *csgA* gene and the CsgA protein, which is crucial for C-signaling (80, 81), and relatively high levels of C-signal are required to induce sporulation (82, 83). However, we failed to find a good match to the putative ActB binding site (43) or a good match to the σ^{54} promoter consensus sequence when we searched 1,000 bp of DNA upstream of the *csgA* gene. Thus, it seems unlikely that ActB directly regulates developmental expression of *csgA*. To date, the only direct ActB targets that have been identified are the *act* operon itself and the MXAN4899 gene (43), which encodes an EBP-type transcriptional regulator that is primarily involved in sporulation (84, 85). Clearly, additional developmental targets of ActB must be identified to better understand how it regulates sporulation inside developing fruiting bodies.

(ii) FruA. FruA has similarity to response regulators (86, 87); however, FruA lacks residues that are important for phosphorylation of other response regulators, a histidine kinase that phosphorylates FruA *in vitro* has yet to be identified, and several lines of evidence indicate that the *in vitro* DNA binding affinity of FruA is not altered by phosphorylation (73). Hence, it is unclear whether FruA is part of a TCS and whether phosphorylation is required for its *in vivo* activity.

FruA is part of the C-signaling network and is important for both aggregation and sporulation (86, 87). For a discussion of FruA's roles in aggregation and in the C-signaling network, see previous reviews in references 24, 88, 89, and 32; they will not be discussed in detail here. The results of several studies indicate that FruA plays a direct role in the regulation of sporulation. For example, FruA directly regulates the *exo* operon (90), which is important for the export of spore coat polysaccharide (61). As mentioned above, the *exo* operon is also a direct target of the Nla6S/Nla6 TCS (59). In addition, the *dev* operon and the *fmgBC* operon, which play important but undefined roles in sporulation, are coregulated by FruA and CRP-like transcription factor MrpC (72, 91).

REGULATORS OF THE TIMING OF SPORULATION

The Esp system. The Esp TCS is required for the temporal and spatial regulation of sporulation (31, 92, 93). Esp is a well-characterized complex TCS and an excellent example of the plasticity of signal transduction networks in *M. xanthus*; the Esp TCS contains two hybrid histidine kinases and two serine/threonine protein kinases (Fig. 4). The Esp components of this TCS are encoded by the *espAB* genes, which are in one operon, and the *espC* gene, which is located at a separate locus. The *espA* and *espC* genes encode hybrid histidine kinases (cytosolic and membrane bound, respectively), and the *espB* gene encodes a putative oligopeptide transport membrane protein. Cells carrying an *espA* or *espC* deletion aggregate and sporulate earlier than the wild-type cells, whereas cells carrying an *espB* deletion aggregate and sporulate many hours after wild-type cells (92, 93). Furthermore, in contrast to wild-type cells, which sporulate in nascent fruiting bodies, sporulation of the Δ*espA* mutant and the Δ*espC* mutant can occur outside these structures (92, 93).

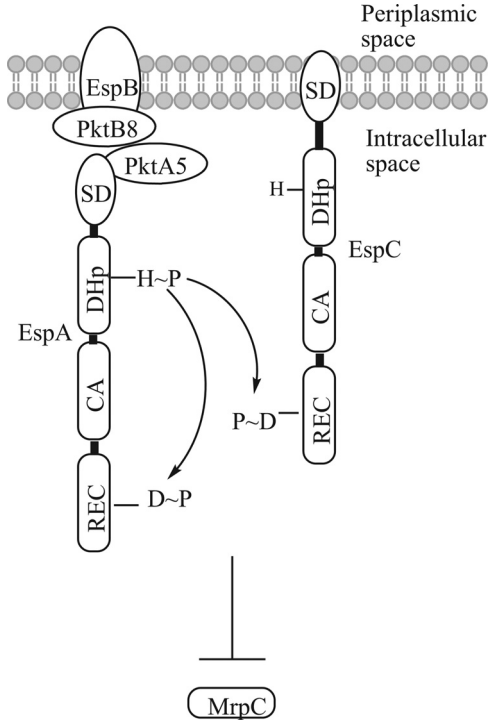

FIG 4 Diagram of the Esp two-component pathway. Esp is a complex two-component pathway that regulates the timing of sporulation (92, 93, 95). This two-component pathway consists of the following proteins: the cytoplasmic EspA and membrane-bound EspC hybrid histidine kinases, which contain a histidine kinase transmitter domain and a response regulator receiver domain, the serine/threonine protein kinases called PktA5 and PktB8, and the putative EspB oligopeptide transport membrane protein. Several lines of evidence indicate that EspA and EspC form a signaling unit; EspA performs autophosphorylation via its transmitter domain and then transfers a phosphoryl group to its receiver domain and the receiver domain of EspC (94). It has been proposed that EspB, PktA5, and PktB8 work together to modulate the activity of EspA (92, 95). It is believed that the EspAC signaling unit directly or indirectly inhibits the accumulation of MrpC, which is a positive regulator of sporulation, until the proper time in fruiting body development (97). SD, sensor domain; DHp, dimerization and histidine phosphorylation domain; CA, catalytic and ATP binding domain; REC, receiver domain.

The EspA and EspC proteins are hybrid histidine kinases that contain a histidine kinase transmitter domain and a response regulator receiver domain. Recent *in vitro* studies showed that the transmitter domain of EspA transfers a phosphoryl group to its own receiver domain (94). Furthermore, the transmitter domain of EspA, but not the transmitter domain of EspC, is capable of transferring a phosphoryl group to the receiver domain of EspC (94). This result and the similarity of the early sporulation phenotypes of the *espA* and *espC* mutants (92, 93) suggest that EspA and EspC form a signaling unit that inhibits sporulation until the proper time in fruiting body development.

In addition to the EspA and EspC hybrid histidine kinases, the Esp TCS contains serine/threonine protein kinases called PktA5 and PktB8. PktA5 and PktB8 were originally identified as potential components in the Esp TCS based on the chromosomal location of their corresponding genes: *pktA5* and *pktB8* are located immediately upstream and downstream of the *espAB* genes, respectively (95). The results of epistasis experiments, coupled with the similarity of the *pktA5* mutant, *pktB8* mutant, and *espB* mutant developmental phenotypes, led to the proposal that EspB, PktA5, and

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PktB8 work together to modulate the activity of the EspAC signaling unit (92, 95).

Two pieces of evidence support the idea that PktA5 and PktB8 interact with the EspAC signaling unit via EspA. First, *in vitro* studies indicate that PktA5 autophosphorylates on a threonine residue(s) (95) and that the sensor region of EspA contains a putative forkhead-associated domain, which is a phosphopeptide recognition domain with specificity toward phosphothreonine (96). Second, using lysates from developing cells and immunoprecipitation, interactions between EspA and both of the Pkt proteins were identified (95). Interactions between EspB, which is predicted to be membrane bound, and EspAC have not been identified; however, it has been suggested that EspB interacts with EspA indirectly via a Pkt protein and through this interaction relays environmental information (92, 95).

How does the Esp TCS influence the timing of sporulation? Higgs et al. (97) showed that MrpC protein levels, but not *mrpC* mRNA levels, increase earlier in development in the Δ*espA* mutant than in a wild-type strain. In addition, it was shown that a deletion of *espA* and *espC* affects the turnover of MrpC (94). Since MrpC is a crucial, positive regulator of sporulation, this finding suggests that the Esp TCS directly or indirectly controls the timing of MrpC accumulation and, as a consequence, the timing of sporulation in developing cells. Presumably, the Esp TCS inhibits the accumulation of MrpC and the onset of sporulation until the appropriate time in fruiting body development.

SUMMARY AND FUTURE WORK

When starved for nutrients, *M. xanthus* produces a biofilm that contains a mat of peripheral rods and aerial structures called fruiting bodies, which house thousands of dormant and stress-resistant spherical spores. Because *M. xanthus* spores represent a differentiated cell type and this cellular differentiation occurs only in nascent fruiting bodies, many genes and multiple levels of regulation are required for sporulation.

In this review, we have described two phases of induction of *M. xanthus* sporulation genes: an early phase that begins prior to the onset of aggregation and a later phase that begins after aggregation commences. We have discussed prototypical TCSs (Nla6S/Nla6 and Nla28S/Nla28) that are involved in the early phase of sporulation gene expression and orphan response regulators (ActB and FruA) that are involved in the later phase of sporulation gene expression. We have also discussed the Esp system, which is a complex TCS that regulates the timing of sporulation.

Many of the promoter/gene targets (output) of these TCS and TCS components have been identified, whereas little is known about the extracellular or intracellular signals (inputs) that modulate their activities. In fact, the only TCS for which a candidate activating signal (nutrient levels or A-signal) has been identified is Nla28S/Nla28. In the case of the orphan response regulator ActB, the histidine kinase that detects the input signal is also unknown. In the case of the response regulator-like protein FruA, it is unclear whether it has a signal-detecting histidine kinase partner.

There are a number of interesting and unanswered questions about the function of the sporulation regulators discussed here. Why do the Nla6S/Nla6 and Nla28S/Nla28 TCSs activate expression of their sporulation gene targets well before cells start to construct the structures that will eventually house the spores? It has been suggested that these TCSs help prepare cells for sporulation early in the developmental process (59). Perhaps these TCSs activate genes that make cells more stress resistant, allowing them to cope with the prolonged period (about 12 to 24 h) of starvation that precedes the formation of nascent fruiting bodies. Since only a fraction of the cells that enter development become spores, it would be interesting to know whether the Nla6S/Nla6 and Nla28S/Nla28 TCS targets are expressed only in this subpopulation of developing cells.

The Esp TCS seems to function as a negative regulatory switch, ensuring that sporulation is inhibited until nascent fruiting bodies have been built (94). Does ActB (and, presumably, its histidine kinase partner) serve as a positive regulatory switch that activates expression of sporulation genes inside nascent fruiting bodies, as suggested? Are the apparent opposing activities of the Esp TCS and ActB coordinately regulated? Perhaps the answers to these questions will help address the long-standing problem of how sporulation in *M. xanthus* is restricted to the nascent fruiting body. Of course, there are other regulators such as the contact-stimulated cell-cell signal known as C-signal that have been implicated in the spatially localized sporulation inside nascent fruiting bodies (7). The challenge for the future will be to determine the connections between the different spatial regulators of sporulation in *M. xanthus* and to connect these regulatory circuits to those functioning early in development.

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