Intra- and Interprotein Phosphorylation between Two-hybrid Histidine Kinases Controls *Myxococcus xanthus* Developmental Progression

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**Background:** Histidine-aspartate (His-Asp) phosphorelay proteins can be components of complex signaling systems.

**Results:** Two hybrid histidine kinases, EspA and EspC, form a signaling complex; EspA phosphorylates both receiver modules to regulate proteolytic turnover of a regulatory protein.

**Conclusion:** Two hybrid histidine kinases are integrated by inter- and intra-histidine aspartate phosphate transfer.

**Significance:** A novel His-Asp phosphorelay signal transduction mechanism was identified.

Histidine-aspartate phosphorelay signaling systems are used to couple stimuli to cellular responses. A hallmark feature is the highly modular signal transmission modules that can form both simple “two-component” systems and sophisticated multicomponent systems that integrate stimuli over time and space to generate coordinated and fine-tuned responses. The deltaproteobacterium *Myxococcus xanthus* contains a large repertoire of signaling proteins, many of which regulate its multicellular developmental program. Here, we assign an orphan hybrid histidine protein kinase, EspC, to the Esp signaling system that negatively regulates progression through the *M. xanthus* developmental program. The Esp signal system consists of the hybrid histidine protein kinase, EspA, two serine/threonine protein kinases, and a putative transport protein. We demonstrate that EspC is an essential component of this system because Δ*espA*, Δ*espC*, and Δ*espA ΔespC* double mutants share an identical developmental phenotype. Neither substitution of the phosphoaccepting histidine residue nor deletion of the entire catalytic ATPase domain in EspC produces an *in vivo* mutant developmental phenotype. In contrast, substitution of the receiver phosphoaccepting residue yields the null phenotype. Although the EspC histidine kinase can efficiently autophosphorylate *in vitro*, it does not act as a phosphodonor to its own receiver domain. Our *in vitro* and *in vivo* analyses suggest the phosphodonor is instead the EspA histidine kinase. We propose EspA and EspC participate in a novel hybrid histidine protein kinase signaling mechanism involving both inter- and intraprotein phosphate transfer. The output of this signaling system appears to be the combined phosphorylated state of the EspA and EspC receiver modules. This system regulates the proteolytic turnover of MrpC, an important regulator of the developmental program.

Signal transmission via reversible histidine-aspartate (His-Asp) phosphorelay (often termed two-component signal transduction) is a widely used mechanism for coupling specific stimuli to appropriate cellular responses (1). Genes encoding these proteins can be identified in nearly all bacteria, certain eukaryotic microorganisms, plants, and archaea (2). Members of this signal transduction family can be identified by the presence of highly conserved histidine kinase (HK)⁴ or receiver (REC) signal transmission modules. The HK module consists of a dimerization/histidine phosphotransfer (DHp, also known as HisKA) domain and a catalytic ATP hydrolysis (CA, also known as HATPase_c) domain (3). HK modules are dimeric and interact through the DHp domain; autoprophosphorylation (HK→P) occurs by CA-dependent transfer of the γ-phosphoryl group of ATP to an invariant histidine residue in the DHp domain (4). The single “switch” domain REC module catalyzes transfer of a phosphoryl group from an HK→P, or sometimes from a small molecule phosphodonor, onto an invariant aspartate residue within itself (5, 6). REC phosphorylation is thought to shift the equilibrium between inactive and active conformations. Most HK modules also display phosphate activity toward the cognate REC→P, and a phosphate motif (E/D)XX(N/T) has been identified adjacent to the phosphoaccepting His residue in the DHp domain (7). The CA domain also modulates phosphate activity (8, 9).

In the paradigm two-component system, the HK and REC modules are organized into two separate proteins as follows: a sensor histidine protein kinase (HPK) containing an N-terminal stimulus-sensing domain that modulates activity of the associated HK module, and a response regulator protein (RR) in

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⁴The abbreviations used are: HK, histidine kinase region; HPK, histidine protein kinase; HyHPK, hybrid histidine protein kinase; REC, receiver; RR, response regulator; DHp, dimerization and histidine phosphotransfer domain; CA, catalytic ATPase; FHA, forhead associated domain, PAS, Per Arnt Sim sensing domain; MASE1, membrane-associated sensing domain; 1β, inclusion body; aa, amino acid; LSB, Laemmli sample buffer.
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which the N-terminal REC domain modulates the activity of an associated effector domain. HPK stimulus-sensing domains are highly variable (10), whereas RR effector domains are most often promoter-binding elements but also include enzymatic or protein-interaction output domains (11). Finally, some RRs, termed “stand alone” receivers lack an associated output domain and mediate signal output by directly interacting with target proteins.

A variation of the simple two-component signal pathway involves hybrid histidine protein kinases (HyHPK) in which one (or sometimes more) REC domain is fused to the HK at the C terminus. Typically, HyHPKs participate in a four-step phosphorelay in which the phosphoryl group is passed from the HyHPK to a histidine phosphotransferase protein and then to an output-activating RR (12). Multistep phosphorelay systems are proposed to provide additional regulatory sites for fine-tuning signal output. HyHPKs can also function in the absence of histidine phosphotransferase proteins. In these cases, the fused REC domain(s) have been shown to modulate signal transmission to a cognate RR by functioning to inactivate the associated kinase (13) and/or to provide sites for alternative kinase phosphorylation presumably leading to signal integration (14). Thus, HyHPKs provide an excellent example of how the highly modular nature of His-Asp phosphorelays can be exploited to generate complex signaling systems.

HyHPKs represent only 20% of the HKs in bacteria but 90% of the HKs in eukaryotes (2, 15). In general, these more complex signaling systems are favored in organisms that regulate complex behaviors. Perhaps the best example can be observed among the Myxobacteria, a group within the Deltaproteobacteria, which favor a “social” (multicellular) life cycle. Several of the developing myxobacterial species (16–19) encode a large repertoire of signal transduction genes (20–24). Myxococcus xanthus, the best studied of the Myxobacteria, contains 272 His-Asp phosphorelay family homologs (24). Only 29% of the 251 (nonchemosensory related) His-Asp phosphorelay genes are encoded together as HPK-RR pairs (i.e. two-component systems); instead, 58% are genetically orphan, and the remaining 16% encoded in complex gene clusters (24) are indicative of nonlinear complex signaling pathways (9). One of the challenges in analysis of M. xanthus signaling pathways is to assign orphan His-Asp phosphorelay proteins to signaling pathways and to define the wiring networks.

That M. xanthus requires sophisticated signaling systems is perhaps no surprise given its complex life cycle. Under nutrient-limited conditions, the cells enter a complex developmental pathway involving at least three distinct cell fates. Some cells are induced to aggregate into mounds containing ~10^6 cells and, exclusively inside these mounds, are triggered to differentiate into quiescent, environmentally resistant spores (25). Other cells instead lyse during development apparently via programmed cell death (26–28). Finally, a minor proportion of the developing population differentiate into a persistor-like state termed peripheral rods (29, 30). Formation of fruiting bodies (31–34), sporulation (34, 35), and possibly programmed cell death (28) are regulated by MrpC, a transcriptional regulator that is up-regulated during development and is subject to both negative (33, 36) and positive (31, 34) regulation.

Progression through the developmental program is negatively regulated by several atypical His-Asp phosphorelay proteins (9, 37–39). The Esp (early sporulation) signaling system is an unusually complex multicomponent system consisting of two serine/threonine kinases (PktA5 and PktB8), a putative oligopeptide transport protein (EspB), and a HyHPK (EspA).

Based on genetic and biochemical analyses, EspA autophosphorylates and donates a phosphoryl group to its associated receiver to delay developmental progression by preventing accumulation of MrpC (36). It is thought that the delay of development is released by modulation of EspA activity via the upstream signaling module consisting of EspB, PktA5, and PktB8 (40, 41). However, both the exact output and the signals modulating this system are unknown.

In this study, we demonstrate that EspC, a genetic orphan HyHPK, is an essential component of the Esp signaling system. Using a combination of genetic and in vitro biochemical approaches, we demonstrate that EspA and EspC function in a novel HyHPK mechanism involving inter- and intra-HyHPK phosphotransfer. The target of this sophisticated signaling system is the regulated proteolysis of a crucial developmental regulatory protein, MrpC. This signaling system provides an important example of the inherent adaptability and plasticity of the histidine-aspartic acid phosphorelay proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Bacterial strains and plasmids used in this study are listed in Table 1. M. xanthus strains were grown under vegetative conditions on casitone yeast extract medium broth or agar plates as described previously (36, 42). Plates were supplemented with 100 μg ml⁻¹ kanamycin, where necessary. Escherichia coli cells were grown under standard laboratory conditions in lysogeny broth media (43) supplemented with 50 μg ml⁻¹ kanamycin where necessary. M. xanthus development was induced under submerged culture conditions in either a 16- or 0.5-ml format, as described previously (36, 42). Briefly, exponentially growing cells were diluted to an optical density of 0.035 and the rich media were replaced by MMC starvation buffer (10 mM MOPS, pH 7.6, 4 mM MgSO₄, 2 mM CaCl₂) to induce development. To enumerate developmental spores, cells were harvested from 1 well of 24-well plates in triplicate, heated to 50 °C for 60 min, sonicated (output 3, 30% duty), and then enumerated using a Helber bacterial counting chamber (Hawksey, UK).

**Construction of M. xanthus Mutant Strains**—In-frame deletions and site-specific point mutations were generated by homologous recombination of the relevant suicide plasmids followed by galK-mediated counter-selection on galactose (44), as described previously in detail (42). All strains were confirmed by sequence analyses of the relevant region (~1000 bp surrounding the desired substitution/deletion). Specific plasmids used to generate the respective strains are listed in supplemental Table S1. With the exception of pAS036 (see below), all inserts for the mutagenesis plasmids were generated accord-

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**TABLE 1**

| Strains/plasmid | Genotype | Source |
|-----------------|----------|--------|
| *M. xanthus* strains | | |
| DZ2 | Wild type | 67 |
| DZ4227 | DZ2 ΔespA | 37 |
| PH1008 | DZ2 espA<sub>1607A</sub> | 36 |
| PH1009 | DZ2 espA<sub>2806A</sub> | 36 |
| PH1029 | PH1009 espA<sub>1607A</sub> | This study |
| PH1044 | DZ2 ΔespC | This study |
| PH1026 | DZ2 espC<sub>1604A</sub> | This study |
| PH1027 | DZ2 espC<sub>1721A</sub> | This study |
| PH1028 | PH1026 espC<sub>1721A</sub> | This study |
| PH1047 | DZ4227 ΔespC | This study |
| PH1032 | DZ2 espC<sub>2CA</sub> | This study |
| PH1033 | PH1027 espC<sub>2CA</sub> | This study |
| PH1034 | DZ2 espC<sub>1604V</sub> | This study |
| PH1035 | PH1027 espC<sub>1604V</sub> | This study |
| PH1036 | DZ2 espC<sub>1604E</sub> | This study |
| PH1037 | PH1027 espC<sub>1604E</sub> | This study |
| PH1038 | DZ2 espC<sub>1604R</sub> | This study |
| PH1039 | PH1027 espC<sub>1604R</sub> | This study |
| PH1040 | DZ2 espC<sub>1604C</sub> | This study |
| PH1041 | PH1027 espC<sub>1604C</sub> | This study |
| PH1042 | DZ2 espC<sub>1602A</sub> | This study |
| PH1043 | PH1027 espC<sub>1602A</sub> | This study |
| PH1045 | DZ2 espA<sub>1411N</sub> | This study |
| *E. coli* strains | | |
| Top10 | F<sup>−</sup> endA1 recA1 galE15 galK16 uspG rpsL32 ΔlacY743ΔΦ10lacΔAm15 araD139Δ[ara, leu]7697 mcrA D(mrr-hsdRMS-mcrBC) λ | Invitrogen |
| BL21ADE3 | F<sup>−</sup> ompT gal dcm lon hsdS<sub>de</sub> (r<sub>6</sub>− m<sub>1</sub>− λ) (DE3 lacI lacUV5-T7 gene 1 lacI sam7 m<sub>i</sub>5)] | Novagen |

**Mutagenesis plasmids**

| pBl114 | Suicide plasmid with Km<sup>a</sup> and galK | 68 |
| pET24a+ | T7-promotor, His<sub>6</sub> tag (C-terminal), Km<sup>a</sup> | Novagen |
| pBS131 | pBi114 espC<sub>Δcodons 44–797</sub> | This study |
| pPH150 | pBi114 espA<sub>4407A</sub> | 36 |
| pAso01 | pBi114 espC<sub>Δ1606A</sub> | This study |
| pAso04 | pBi114 espC<sub>Δ774A</sub> | This study |
| pAso29 | pBi114 espC<sub>ΔCA</sub> (Δcodons 529–674) | This study |
| pAso31 | pBi114 espC<sub>Δ260V</sub> | This study |
| pAso30 | pBi114 espC<sub>Δ460E</sub> | This study |
| pAso32 | pBi114 espC<sub>Δ460R</sub> | This study |
| pAso33 | pBi114 espC<sub>Δ460C</sub> | This study |
| pAso34 | pBi114 espC<sub>Δ460A</sub> | This study |
| pAso36 | pBi114 espA<sub>Δ1411N</sub> | This study |

**Overexpression plasmids**

| pAso02 | pET24a+ espC<sub>ΔAMAX1</sub> (aa 311–833) | This study |
| pBS122 | pET24a+ espC<sub>ΔREC</sub> (aa 690–819) | This study |
| pAso19 | pET24a+ espC<sub>ΔREC</sub> (aa 690–819) | This study |
| pBS121 | pET24a+ espC<sub>ΔREC</sub> (aa 451–679) | This study |
| pAso21 | pET24a+ espC<sub>ΔH1</sub> (aa 451–679) | This study |
| pAso22 | pET24a+ espA<sub>H1</sub> (aa 390–646) | This study |
| pAso23 | pET24a+ espA<sub>ΔH1</sub> (aa 390–646) | This study |

To generate them are listed in Table S2 and supplemental Table S2. For pBS131 (∆espC), the insert was cloned into the XhoI/BamHI sites of pBl114. For all other mutagenesis plasmids, the insert was cloned into the EcoRI/BamHI sites of pBl114. Plasmid pAso36 (espA<sub>Δ4411D</sub>) was generated using a “one-step directed mutagenesis” PCR protocol (45) with outward-facing mutagenesis primers (supplemental Table S2) and pAso35 as a template. pAso35 contains codons 240–571 of espA cloned into pBl114. All plasmids were sequenced to confirm the absence of PCR-generated errors.

**Bioinformatic Analyses—Orthologs for EspA and EspC**

In the publicly available *Myxococcales* genomic sequences were identified using reciprocal BlastP analysis as described by Huntley et al. (18). Briefly, the protein sequence of either EspA or EspC was used as query in BlastP analysis against the protein database from the following: *Stigmatella aurantiaca* DW4/3-1 (accession number CP002271) (18); *Myxococcus fulvus* HW-1 (accession CP002830) (46); *Coralloccocus coralloides* DSM2259 (accession CP003389) (19); *Anaeromycobacter dehalogenans* 2CP-C (accession CP000251.1) (47); *Haliangium ochraceum* SMP-2 (accession CP001804.1) (48); *Sorangium cellulosum* Soce 56 (accession AM746676) (17), and *Plesiocystis pacifica* SIR-1 (accession ABCS0000000). The protein with the best hit (highest bit score) was then used as a query in a second BlastP analysis against the protein database from *M. xanthus* DK1622 (accession CP000113) (16). If the highest scoring match was identical to the protein sequence originally used for the first BlastP analysis, the two proteins were considered orthologs.

**Construction of Protein Overproduction Plasmids—Protein**

Overproduction plasmids and the primer sequences used to generate them are listed in Table S1 and supplemental Table S2, respectively. Overproduction plasmids pAso22 (EspA<sub>H1</sub>–His<sub>6</sub>) and pAso23 (EspA<sub>ΔH1</sub>–His<sub>6</sub>) encode the DHp and CA domains (collectively referred to as the kinase region (HK)) of espA with a C-terminal hexahistidine (His<sub>6</sub>) affinity tag.
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pAS022 and pAS023 were constructed by PCR amplifying the espA (MXAN_0931) codons 390–646 from DZ2 or PH1008 genomic DNA, respectively, and cloned into the EcoRI site of pET24a+. The resulting plasmids were restricted with SacI; overhangs were blunted with T4 DNA polymerase (Fermentas) and religated to bring the His6 affinity tag in-frame with the espAHK fragment. Overproduction plasmids pBS121 (EspC_HK-His6) and pAS021 (EspC_HK_H461A-His6) encode the HK of EspC and kinase-inactive point mutant with C-terminal His6 affinity tags and were constructed by PCR-amplifying espC (MXAN_6855) codons 451–679 from DZ2 or PH1026 genomic DNA, respectively. The resulting fragments were cloned into the EcoRI and XhoI sites of pET24a+. Overproduction plasmids pBS122 (EspC_REC-His6) and pAS019 (EspC_REC_D749A-His6) encode the REC of EspC with a C-terminal His6 affinity tag and were constructed by PCR-amplifying espC (MXAN_6855) codons 690–819 from DZ2 or PH1027 genomic DNA, respectively. The resulting fragments were cloned into the EcoRI and XhoI sites of pET24a+. All constructs were sequenced to confirm absence of PCR-generated errors.

Overproduction and Purification of Recombinant Proteins—EspA_{HK}-His6 or EspC_{HK}-His6 (and the respective point mutants) were each produced as inclusion bodies (IBs) from 1 liter of *E. coli* BL21 (DE3) cultures by induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. To isolate IBs, the respective cell cultures were resuspended in 25 ml of TND buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT) and lysed by passage through a French press (SLM-AMINCO/Spectronic) three times at ~18,000 p.s.i. IBs were collected by centrifugation at 4600 × g for 30 min at 4 °C, resuspended in 25 ml of TND buffer, and treated by French press as above. To remove contaminating proteins, IBs were pelleted as described above and resuspended in 20 ml of TND buffer. Guanidine HCl was added to a final concentration of 6 M, and the solution was incubated for 2 h at RT with rotation. Purified IBs were pelleted as above, washed in TND buffer, and then resuspended in 15 ml of TND buffer. Guanidine HCl was added to a final concentration of 6 M, and the IB protein was solubilized by vortexing and then clarified by centrifugation at 100,000 × g for 30 min at RT. The solution was diluted with TND buffer to achieve a guanidine HCl final concentration of 2 M and a protein concentration of ~0.1 M as determined by the absorbance at 280 nm (ε_{280} (EspA_{HK}-His6) = 1490; ε_{280} (EspC_{HK}-His6) = 2980). The solution was then dialyzed in TGND buffer (50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 150 mM NaCl, 1 mM DTT) for 2 h at 4 °C, two times, followed by dialysis in fresh buffer overnight. The resulting protein preparation was clarified by centrifugation at 17,000 × g for 5 min at 4 °C and concentrated using a 10 molecular weight cut-off Amicon Ultra column (Millipore). The refolded proteins were stored at −20 °C for further assays.

EspC_{REC}-His6 (and its respective point mutant) were overproduced as soluble proteins in *E. coli* strain BL21 (DE3) by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C. For purification of the recombinant proteins, the cell cultures were resuspended in lysis buffer (50 mM HEPES, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and lysed by French press as described above. The lysate was clarified by centrifugation at 100,000 × g for 1 h at 4 °C, and the supernatant was subjected to purification by nickel affinity chromatography at 4 °C (ÄKTA purifier, GE Healthcare) using a 1-ml His trap FF1 nickel affinity column (Amersham Biosciences). The proteins were eluted using a 30-ml linear gradient of 20–500 mM imidazole in lysis buffer. A portion of each eluted fraction was analyzed by SDS-PAGE. Elution fractions containing the peak levels of purified protein were pooled, dialyzed, and stored as described above.

Radiolabeled In Vitro Autophosphorylation and Phospho-transfer Assays—*In vitro* autophosphorylation of 10 μM kinase was carried out in phosphorylation buffer (TGND buffer, 5 mM MgCl2, 50 mM KCl) supplemented with 0.5 mM ATP and 1.7 μM [γ-32P]ATP (222 TBq mmol−1; Hartmann Analytic, Braunschweig) for intervals between 0 and 60 min. At each time point, 10 μl samples were withdrawn, quenched with 2 × LSB (125 mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 10% β-mercaptoethanol, 0.02% (v/v) bromphenol blue), and analyzed as described previously (36), except 15% polyacrylamide gels were used to resolve radiolabeled proteins. The gels were exposed to a Storage Phosphor Screen (GE Healthcare) overnight and analyzed using a Storm™ 860 imaging system (Amersham Biosciences). 32P-Labeled HK signal intensities from three independent experiments were quantified by ImageQuant (GE Healthcare) with local average background correction and manual background subtraction. The average intensity and associated standard deviation were calculated using Microsoft Excel.

Phosphotransfer reactions were performed by first autophosphorylating 15 μM of either EspA_{HK}-His6 or EspC_{HK}-His6 (or respective point mutants) for 30 min. 6.5 μl of the HK~P were then added to 3.5 μl of either phosphorylation buffer, 28 μM EspC_{REC}-His6 or EspC_{REC_D749A}-His6, leading to an equivalent 10 μM final concentration for each reaction partner. The reactions were quenched after 2 min and analyzed as above, except 20% polyacrylamide gels were used.

Immunoblot Analysis—Protein lysates for immunoblot analyses were prepared from developmental cells grown in 16 ml of submerged culture format. At the indicated time points, the MMC buffer was removed, and the cells were resuspended in 1 ml of ice-cold MMC buffer incubated with an equivalent volume of 26% ice-cold trichloroacetic acid for 15 min on ice, pelleted at 17,000 × g for 5 min at 4 °C, and washed twice with acetone. The pellet was resuspended in 1 × clear LSB (62.5 mM Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS) and heated for 1 min at 94 °C. The protein concentration of each sample was determined using a BCA protein assay kit (Thermo Scientific) according to manufacturer’s instructions. Samples were diluted in 2 × LSB to 2 μg μl−1 and stored at −20 °C.

For immunoblot analysis, samples containing 20 μg of protein were resolved by denaturing SDS-PAGE in 8% (anti-EspA and -EspC immunoblot) or in 12% (anti-MrpC immunoblots) polyacrylamide gels. Proteins were transferred to a PVDF membrane using a semi-dry transfer apparatus. Western blot analyses were performed using anti-EspC (supplemental Methods), anti-EspA (41), or anti-MrpC (26) primary antibodies at dilutions of 1:200, 1:1000, or 1:2000, respectively. Goat α-rabbit IgG conjugated to horseradish peroxidase (HRP) secondary anti-
body (Pierce) was used at a dilution of 1:20,000, and signals were detected with enhanced chemiluminescence substrate (Pierce) followed by exposure to autoradiography film (Thermo Scientific) or detected by LAS 4000 (Fujifilm Life Science). Representative immunoblot patterns are shown, but similar patterns were obtained from at least two biological replicates.

In Vivo MrpC Turnover Assays—Wild type (DZ2) or ΔespA ΔespC mutant (PH1047) strains were developed in 16 ml of submerged culture format for 9 h and treated with 34 μg ml⁻¹ chloramphenicol to block de novo protein synthesis (50). At 0, 10, 20, or 30 min after addition of chloramphenicol, the cells were harvested, pelleted at 4,600 × g for 2 min at 4 °C, resuspended in 400 μl of hot (70 °C) LSB, heated for 5 min at 99 °C, and stored at −20 °C for further analyses. 15 μl of protein samples were analyzed by immunoblot as described above. Band intensities from triplicate experiments were quantified with ImageJ 1.43U (49). The MrpC half-life in wild type cells was calculated assuming a first order kinetic degradation reaction (51–53). Briefly, each background-subtracted band intensity value was first normalized to the intensity at t = 0, and the natural log of the resulting values were plotted versus time in Microsoft Excel. The slope (k) of a linear fit of each graph was used to calculate the t½ using the equation $t_{1/2} = \ln(2)/k$ (53).

RESULTS

EspA and EspC Function in Same Signaling Pathway—espA and espC each encode orphan HxHPKs and both have a similar mutant phenotype in which progression through the developmental program is accelerated relative to wild type (37, 39). To analyze whether these proteins participated in a linear signaling pathway, we took a genetic epistasis approach. We first generated isogenic ΔespC and ΔespAΔespC strains and compared their developmental phenotypes to the wild type and ΔespA strains. The ΔespA, ΔespC, and ΔespAΔespC strains each yielded an identical developmental phenotype; aggregation centers were visible between 12 and 24 h after induction of starvation, which was ~12 h earlier than wild type (Fig. 1A). Likewise, all three mutants produce heat- and sonication-resistant spores ~12 h earlier than the wild type (Fig. 1A). These genetic data suggest that EspA and EspC function in the same signaling pathway to regulate developmental progression.

The observation that ΔespAΔespC double mutant phenocopied the single mutants could arise from one of two possibilities. 1) EspA regulates developmental progression, but EspC is necessary for EspA production (or vice versa). 2) EspA and EspC act together to regulate developmental progression. To distinguish between these two possibilities, we examined EspA and EspC production patterns by immunoblot analysis of cell lysates prepared from wild type, ΔespA, or ΔespC strains. The anti-EspC immunosera generated in this study could specifically detect an ~89-kDa band (similar to the predicted EspC molecular mass of ~88 kDa) in the wild type, but not ΔespC, lysates (Fig. 1B). EspC was not detected at t = 0, but began to accumulate at ~12 h, peaked at ~30 h, and then began to decrease, similar to the EspA accumulation pattern (Fig. 1B). EspC and EspA were present in the ΔespA and ΔespC strains, respectively, and accumulated earlier in the respective backgrounds than in the wild type (Fig. 1B). Together, these data suggest that EspA and EspC act together to regulate developmental progression. Additionally, this dual activity might, directly or indirectly, repress the accumulation of both proteins.

EspA and EspC share 48 and 40% identity in their HK and REC regions, respectively (Fig. 2). These two proteins are not paralogs, because at least three proteins (encoded by Mxan_0195, Mxan_2386, and Mxan_0095) can be identified that display a higher degree of conservation to EspA than does EspC. Orthologs to EspA and EspC can be identified in M. fulvus (LILAB_04045 and LILAB_12195, respectively), C. coralloides (COCOR_00882 and COCOR_07432, respectively), and M. fulvus (LILAB_04045 and LILAB_12195, respectively), but orthologs for neither protein could be identified in A. dehagogenans, H. ochraceum, P. pacifica, or S. cellullosum. Thus, if EspA was identified in a genome, then EspC was always additionally present, consistent with a single Esp signaling module in which both proteins act together.

espC<sub>Δ759A</sub> but Not espC<sub>ΔH461A</sub> Mutants Effect Developmental Progression—We have previously demonstrated that both EspA autophosphorylation and phosphotransfer to its associated receiver domain are necessary for appropriate developmental progression; alanine substitution of the kinase autophosphorylation site (H407A) or receiver phosphoaccepting site (D696A) leads to the null mutant phenotype (36) (Fig. 3A). Consistently, we observed an identical phenotype if both residues in espA were mutated (espA<sub>H407A,D696A</sub>) (Fig. 3A). To determine whether EspC kinase activity is similarly necessary for regulation of developmental progression, we likewise generated an alanine substitution of the predicted autophosphorylation site...
(His-461) in EspC (espC\textsubscript{H461A}). Surprisingly, this mutant exhibited the wild type phenotype with respect to timing of aggregation and production of heat- and sonication-resistant spores (Fig. 3A). In contrast, alanine substitution of the conserved phosphoaccepting residue (Asp-749) in the receiver domain of EspC (espC\textsubscript{D749A}) resulted in the ΔespC phenotype (Fig. 3A). An identical mutant phenotype was also observed in a strain in which both EspC His-461 and Asp-749 were substituted with alanine (espC\textsubscript{H461A,D749A}) (Fig. 3A).

To determine whether the generated point mutations adversely affect the protein accumulation patterns of either EspA or EspC, we analyzed developmental cell lysates generated from the respective strains by anti-EspC and anti-EspA immunoblot analysis. Neither EspC nor EspA protein accumulation was reduced in the espC\textsubscript{H461A}, espC\textsubscript{D749A} or espC\textsubscript{H461A,D749A} strains (Fig. 3B). Thus, the phenotypes observed are due to disruption of the activity of the mutated proteins rather than to reduction of protein stability for EspA or EspC. The espC mutants that displayed the early developmental phenotype (espC\textsubscript{D749A} and espC\textsubscript{H461A,D749A}) also displayed earlier and more rapid accumulation of both EspA and EspC; peak protein levels were detected ~12 h earlier than in the wild type (Fig. 3B). The espC\textsubscript{H461A} strain, which does not display a mutant phenotype, may cause slightly earlier production of both EspCH461A and EspA. However, both the rate of accumulation and time point of peak production matched that of the wild type (Fig. 3B). Together, these results suggest that although phosphorylation of the EspC receiver is necessary for appropriate developmental regulation, the phosphoryl group is not donated from EspC kinase.

EspC Autophosphorylates on His-461 in Vitro—To ascertain whether EspC is capable of autophosphorylation in vitro, we next cloned, overproduced, and purified a recombinant version of the EspC-predicted histidine kinase region (aa 451–679) fused at the C terminus with a hexahistidine affinity tag (EspCHK-His\textsubscript{6}). We also generated a phosphorylation-deficient H461A version of this protein (EspCH\textsubscript{H461A}-His\textsubscript{6}). As we were unable to find conditions for soluble overproduction, both proteins were refolded from inclusion bodies. To examine whether these two proteins could autophosphorylate, 10 μM of either EspC\textsubscript{H461A}-His\textsubscript{6} or EspCH\textsubscript{H461A}-His\textsubscript{6} was incubated in the presence of [γ-\textsuperscript{32}P]ATP for 60 min and resolved by electrophoresis.

**FIGURE 2.** Domain organization of the EspA and EspC hybrid histidine kinases. Percent amino acid identity for the indicated domains is represented by shaded areas. The invariant histidine (H) residues in the dimerization and histidine phosphotransfer domains (DHp) and invariant aspartate (D) residues in the receiver (REC) domains are indicated. FHA, forhead-associated domain; MASE1, predicted integral membrane sensory domain.

**FIGURE 3.** Phosphorylation of EspC receiver domain, but not EspC autophosphorylation, is required for regulation of development. A, developmental phenotypes of espA and espC signal transmission point mutants. Wild type (wt; D22), ΔespA (D222427), espA\textsubscript{H407A} (PH1008), espA\textsubscript{D696A} (PH1009), espA\textsubscript{H407A,D696A} (PH1029), ΔespC (PH1044), espC\textsubscript{H461A} (PH1026), espC\textsubscript{H461A,D749A} (PH1027), and espC\textsubscript{H461A,D749A} (PH1028) strains were developed under submerged culture in 24-well culture dishes, and pictures were recorded at the indicated hours of development. Heat- and sonication-resistant spores were isolated at the indicated time points and displayed as the percent of wild type spores at 72 h. Spore numbers are the average and associated standard deviation of three biological replicates. Scale bar, 0.5 mm; nd, not determined. B, EspC and EspA developmental protein accumulation patterns. Anti-EspC (left panel) and anti-EspA (right panel) immunoblot analyses of cell lysates were prepared from the indicated strains developed for the indicated hours under 16 ml of submerged culture format.
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FIGURE 4. EspC kinase region autophosphorylates in vitro. A, in vitro autophosphorylation of EspA<sub>HK</sub>-His<sub>6</sub>, EspC<sub>HK</sub>-His<sub>6</sub>, EspA<sub>HK</sub> <sup>H407A</sup>-His<sub>6</sub>, and EspC<sub>HK</sub> <sup>H407A</sup>-His<sub>6</sub>. 10 µM of each recombinant protein was incubated in the presence of [γ-<sup>32</sup>P]ATP for 60 min, quenched, and resolved by SDS-PAGE, and the radioactivity was detected by exposure to a PhosphorImager screen. A radiolabeled band of each protein was subsequently detected by Coomassie blue stain. B, relative in vitro autophosphorylation rates of EspC and EspA kinase regions. 10 µM EspA<sub>HK</sub>-His<sub>6</sub> or EspC<sub>HK</sub>-His<sub>6</sub> was incubated in the presence of [γ-<sup>32</sup>P]ATP at 30 min. We next added the kinase to either buffer alone or to an equimolar (10 µM) concentration of either EspC<sub>HK</sub>-His<sub>6</sub> or EspA<sub>HK</sub>-His<sub>6</sub>, and allowed phosphotransfer reactions to proceed for 2 min. As expected, if either EspC<sub>HK</sub>-His<sub>6</sub> or EspA<sub>HK</sub>-His<sub>6</sub> was first autophosphorylated and subsequently incubated only with buffer, the <sup>32</sup>P signal was readily detected on the wild type, but not on the mutated, kinase. C, quantification of EspC (dashed line) and EspA (solid line) HK autophosphorylation rates in B. Relative signal intensities of the bands from three independent time courses were quantified and normalized to the maximal signal intensity of each protein. The average relative signal intensity and associated standard deviation from each time point was plotted.

and radioactive incorporation onto either protein was analyzed by exposure to a phospho-imager screen. A radiolabeled band corresponding to EspC<sub>HK</sub>-His<sub>6</sub>, but not EspC<sub>HK</sub> <sup>H461A</sup>-His<sub>6</sub>, could be detected (Fig. 4A), suggesting that the EspC kinase region is capable of in vitro autophosphorylation on His-461.

To compare the relative activities of the EspC and EspA kinase domains, we generated comparable constructs to produce EspA<sub>HK</sub>-His<sub>6</sub> (aa 390–646) or the kinase-deficient EspA<sub>HK</sub> <sup>H407A</sup>-His<sub>6</sub>. Although these two recombinant proteins could be overproduced as soluble proteins, they were denatured and refolded as for the EspC kinase constructs. As observed previously for a GST-Espa<sub>HK</sub> construct (36), EspA<sub>HK</sub>-His<sub>6</sub> but not EspA<sub>HK</sub> <sup>H407A</sup>-His<sub>6</sub> could be labeled by <sup>32</sup>P (Fig. 4A). To compare EspA<sub>HK</sub>-His<sub>6</sub> and EspC<sub>HK</sub>-His<sub>6</sub>, activity in vitro, 10 µM of the respective proteins was incubated in the presence of [γ-<sup>32</sup>P]ATP for intervals between ~0 and 60 min (Fig. 4, B and C). In the case of EspC<sub>HK</sub>-His<sub>6</sub>, <sup>32</sup>P incorporation could be detected after less than 1 min, and the signal continued to accumulate for 30 min, after which it began to decrease. EspA<sub>HK</sub>-His<sub>6</sub> exhibited slower accumulation of the <sup>32</sup>P label that began to saturate between 30 and 60 min of incubation (Fig. 4, B and C). Together, these results indicate that the EspC kinase region is capable of efficient autophosphorylation in vitro with greater activity than that of EspA, despite the observation that the kinase activity is apparently not required during development in vivo.

EspA<sub>HK</sub> but Not EspC<sub>HK</sub> Can Transfer a Phosphoryl Group to EspC<sub>REC</sub> in Vitro—To test our hypothesis that EspA, and not EspC, acts as the phosphodonor to EspC<sub>REC</sub>, we examined phosphotransfer between EspA<sub>HK</sub> or EspC<sub>HK</sub> to the EspC receiver domain in vitro. For this purpose, the EspC receiver domain (aa 690–819), or the putative phosphoaccepting deficient version was each overproduced with C-terminal hexahistidine affinity tags (EspC<sub>REC</sub>-His<sub>6</sub> or EspC<sub>REC</sub> <sup>D749A</sup>-His<sub>6</sub>, respectively). To first examine whether EspA<sub>HK</sub> could phosphorylate its own receiver in vitro, we allowed for kinase autophosphorylation by incubating EspC<sub>HK</sub>-His<sub>6</sub> (or the EspC<sub>HK</sub> <sup>H461A</sup>-His<sub>6</sub> control) with [γ-<sup>32</sup>P]ATP for 30 min. We next added the kinase to either buffer alone or to an equimolar (10 µM) concentration of either EspC<sub>REC</sub>-His<sub>6</sub> or EspC<sub>REC</sub> <sup>D749A</sup>-His<sub>6</sub> and allowed phosphotransfer reactions to proceed for 2 min. As expected, if either EspC<sub>HK</sub>-His<sub>6</sub> or EspC<sub>HK</sub> <sup>H461A</sup>-His<sub>6</sub> was first autophosphorylated and subsequently incubated only with buffer, the <sup>32</sup>P signal was readily detected on the wild type, but not on the mutated, kinase (Fig. 5, A and B, lanes 1 and 2). If the kinase was instead added to either EspC<sub>REC</sub>-His<sub>6</sub> or EspC<sub>REC</sub> <sup>D749A</sup>-His<sub>6</sub>, no significant change in signal incorporation on EspC<sub>HK</sub>-His<sub>6</sub> could be observed, and no significant <sup>32</sup>P label could be detected on either receiver construct (Fig. 5, A and B, lanes 3–5). These results suggest EspC<sub>HK</sub>-His<sub>6</sub> does not act as an efficient phosphodonor for its own receiver in vitro.

We next performed this experiment substituting EspA<sub>HK</sub> for that of EspC<sub>HK</sub>. As expected, if either EspA<sub>HK</sub>-His<sub>6</sub> or EspA<sub>HK</sub> <sup>H407A</sup>-His<sub>6</sub> was first autophosphorylated and subsequently incubated only with buffer, the <sup>32</sup>P signal was readily detected on the wild type but not on the mutated kinase (Fig. 5, A and B, lanes 6 and 7). In contrast, if EspC<sub>REC</sub>-His<sub>6</sub> was substituted for the buffer, the signal intensity on EspA<sub>HK</sub>-His<sub>6</sub> was reduced to 16 ± 5, and 8 ± 1% of the original kinase signal could be clearly detected on EspC<sub>REC</sub>-His<sub>6</sub> (Fig. 5, A and B, lane 8). These effects were not observed if the kinase was instead added to EspC<sub>REC</sub> <sup>D749A</sup>-His<sub>6</sub> (Fig. 5, A and B, lane 10) or if EspA<sub>HK</sub> <sup>H407A</sup>-His<sub>6</sub> was added instead of the functional HK (Fig. 5, A and B, lane 9). These results indicate that the EspA kinase region can efficiently phosphorylate the receiver of EspC.
on its predicted phosphoaccepting residue, Asp-749, although under equivalent conditions EspC cannot.

Disruption of a Phosphatase Motif in EspA but Not EspC Delays Development—Our results so far suggested that EspC kinase activity is not necessary for regulation of developmental progression. However, many kinases also display phosphatase activity that is not necessarily disrupted by substitution of the phosphoaccepting histidine residue (9, 54, 55). We next set out to investigate whether EspC may function exclusively as a phosphatase in this system, by generating phosphatase motif (462EXXN465) substitutions that correspond to those producing reduced phosphatase activity in other bifunctional kinases (7, 56). Strains producing EspCN465Y displayed a phenotype indistinguishable from wild type with respect to fruiting body formation and sporulation (Fig. 6A), although the levels of EspCN465Y were slightly reduced relative to that of EspC (Fig. 6B). As expected, if the D749A mutation was combined with the N465Y mutation in EspC (espC<sub>N465Y,D749A</sub>), the early developmental phenotype was observed, and the double mutant protein levels increased to a similar intensity of EspCD749A (Fig. 6B). Several additional substitutions in the putative phosphatase motif corresponding to substitutions producing reduced phosphatase activity in other bifunctional kinases (espC<sub>E462A</sub>, espC<sub>N465E</sub>, espC<sub>N465R</sub>, or espC<sub>N465C</sub>) displayed a developmental phenotype indistinguishable from wild type (supplemental Fig. S1A). If these mutations were combined with D749A, all double mutants displayed a phenotype indistinguishable from the espC<sub>D749A</sub> early developmental phenotype (supplemental Fig. S1A). The single mutants exhibited reduced protein levels relative to the wild type, which were increased in the double mutants (supplemental Fig. S1B).
Residues necessary for phosphatase activity have also previously been identified in the CA domain of some kinases, and CA activity has been shown to stimulate phosphatase activity (8, 9, 57). Therefore, we additionally deleted the entire EspC CA domain (aa 529–674) alone or in combination with the D749A mutation. The espC_{ΔCA} mutant displayed a phenotype indistinguishable from wild type with respect to fruiting body formation and sporulation, although the espC_{ΔCA,D749A} displayed the early developmental phenotype (Fig. 6A). EspC_{ΔCA} and EspC_{ΔCA,D749A} could be detected by anti-EspC immunoblot at a molecular mass of ~65 kDa (predicted molecular mass, 73 kDa) (Fig. 6B) and at reduced levels relative to wild type. The reduced intensity of these two mutants relative to EspC in the respective background strains is likely due to loss of some of the antibody epitopes in this protein; anti-EspC antisera were generated against EspC aa 311–833 such that the EspC_{ΔCA} mutants lack 28% of the antigen.

To determine whether disruption of the putative phosphatase motif in EspA (408-EXXN411) would produce the delayed developmental phenotype consistent with hyper-phosphorylation of EspA and/or EspC receivers preventing developmental progression, we next analyzed the phenotype of an espA_{N411D} mutant. This mutant began to aggregate between 30 and 36 h of development (~6 h later than the wild type) and produced 66% of wild type spores at 72 h of development.

Together, these results suggest that the putative phosphatase motif in EspA, but not EspC, is important for appropriate regulation of developmental progression. Finally, the observation that the EspC_{ΔCA} mutants display a wild type phenotype further confirms that EspC kinase activity is not necessary for in vivo control of the developmental progression.

EspA/C System Regulates the Protein Stability of Transcription Factor MrpC—We have previously demonstrated that EspA regulates the accumulation of the key developmental regulator MrpC, because in a ΔespA mutant MrpC protein, but not mrpC transcript, is produced earlier than in the wild type (36). Because our results suggested that EspA and EspC function together, we next examined whether EspC is also involved in regulating MrpC accumulation during development. We used anti-MrpC immunoblot analysis to compare the production of MrpC in the various espC mutants to the wild type. In wild type cells, MrpC could be detected immediately after induction of development (t = 0), decreased between 0 and 12 h, and thereafter gradually increased over at least 36 h of development (Fig. 7A). Consistent with what was observed previously in the ΔespA mutant (36), all early developing EspC mutants (ΔespC, espC_{D749A}, and espC_{H461A,D749A}) produced MrpC more rapidly than the wild type, with MrpC levels at 18 and 24 h higher than the wild type levels (Fig. 7A). In contrast, the espC_{H461A} mutant displayed an accumulation pattern more similar to wild type, although the MrpC level is further reduced at 36 h (Fig. 7A). Taken together, these results suggest that phosphorylation of both EspA and EspC is necessary for appropriate MrpC accumulation during development.

Given that the mrpC transcriptional pattern in espA mutants matched that of wild type (36), we postulated that the Esp system either affected translation or proteolytic turnover of MrpC. If MrpC is subject to proteolytic turnover, we would predict that MrpC levels would decrease in cells in which de novo protein synthesis is blocked. To examine whether this is the case, we developed wild type cells for 9 h in submerged culture, treated the cells with 34 μg ml⁻¹ chloramphenicol, harvested cells after 0, 10, 20, or 30 min, and examined the MrpC levels by immunoblot (Fig. 7B). In the wild type strain, MrpC levels decreased steadily during the 30 min of treatment with a calculated half-life of 30 ± 8 min (Fig. 7C). To determine whether MrpC turnover was altered in the absence of EspA and EspC,
The obligatory role of EspC in this signaling system is strongly supported by the observation that the ΔespA, ΔespC, and ΔespA ΔespC mutants display identical developmental phenotypes (Fig. 1). Consistently, we observed that if an espA ortholog could be identified in a sequenced myxobacterial genome, we could also identify an espC ortholog in the same genome. Our data indicate that EspA and EspC function together via an intimate hybrid histidine kinase signaling mechanism involving both an inter- and intra-HyHPK phosphorylation. First, we determined that the EspC receiver must be phosphorylated to negatively regulate development, but that EspC kinase is not the phosphoryl donor, because inactivation of kinase autophosphorylation either by substitution of the phosphoaccepting histidine residues or deletion of the entire CA domain yields the wild type phenotype (Figs. 3 and 6). The EspC\textsubscript{HKG} is functional because it efficiently autophosphorylates \textit{in vitro} (Fig. 4). EspC\textsubscript{HKG} does not, however, efficiently donate a phosphoryl group to the EspC receiver (Fig. 5). Therefore, the phosphoryl donor must either be a small molecule donor (e.g. the intracellular acetyl-phosphate pools (5, 58) or an alternate kinase. The former is unlikely because the EspC receiver domain does not autophosphorylate in the presence of acetyl-\textsuperscript{\textsuperscript{\textsuperscript{32}}P}phosphate \textit{in vitro} (data not shown). Instead, our data suggest the phosphoryl donor is EspA kinase because of the following: 1) inactivation (EspA\textsubscript{H407A}) produces a phenotype identical to the EspC\textsubscript{D749A} mutant (Fig. 3); 2) our deletion mutant analysis indicated EspA and EspC must function together (Fig. 1); and 3)EspA\textsubscript{HKG} efficiently donates a phosphoryl group to the EspC receiver (Fig. 5).

We suggest that the simplest interpretation of our observation of identical mutant phenotypes of the ΔespA, ΔespC, ΔespA ΔespC, espA\textsubscript{D696A}, and espC\textsubscript{D749A} strains is that the output from this system is the combined phosphorylation of both EspA and EspC receiver domains (Fig. 8). Given that it has been previously observed that receiver domains can both inhibit (13) or stimulate (59) cognate kinase activities, we also considered alternate scenarios in which one of the receivers plays a phosphorylation-dependent regulatory role to modulate the kinase activity of the other. We immediately discarded scenarios in which either of the receivers inhibits EspA kinase activity, because these models predict a delayed developmental phenotype in the ΔespC mutant or in a mutant where the EspA receiver domain was deleted (espA\textsubscript{AREC}), and both of these mutants exhibit early development (this study and Ref. 36). We also discarded a model in which an EspA-phosphorylated receiver is the sole output, but EspA kinase-dependent phosphorylation of the EspC receiver stimulates the EspA kinase because EspA efficiently phosphorylates its own receiver \textit{in vitro} (36). Finally, we disfavor a model in which the EspC receiver is the sole output, and phosphorylation of the EspA receiver is necessary to stimulate phosphorylation of the EspC receiver. In this last scenario, we would predict that in espA\textsubscript{D696A} and espA\textsubscript{AREC} strains a low level of EspA kinase phosphotransfer to EspC receiver would produce an intermediate phenotype rather than the null phenotypes observed in the respective mutants (Figs. 1 and 3) (36). It has also been previously proposed that kinases can form heterodimers (60). However, we do not favor a model in which EspA and EspC

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**FIGURE 8. Model of the Esp signaling system.** Two-hybrid histidine kinases EspC (dark gray) and EspA (light gray) regulate the accumulation rate of an important developmental regulator, MrpC, to ensure appropriate and coordinated progression through the \textit{M. xanthus} developmental program. The combined phosphorylation of EspA and EspC receiver domains (REC) activates an unknown protease or protease targeting factor (\( \times \)) to stimulate MrpC turnover. EspA histidine kinase region (HK) autophosphorylates and donates a phosphoryl group to both its own and EspC receiver domains. EspA may also act as a phosphatase on EspA and or EspC REC. Autophosphorylation of EspC histidine kinase domain is not required for stimulating MrpC turnover under laboratory developmental conditions. EspA activity is controlled by a signaling module consisting of two serine/threonine kinases, PktA5 (A5) and PktB8 (B8), and a putative transport protein (EspB) predicted to reside in the cytoplasmic membrane (CM) (37, 41). PktA5 and PktB8 are thought to interact with the Forkhead associated (FHA) domain in EspA (41). Two PAS domains in EspA and one in EspC may be involved in sensing internal or membrane-associated redox stimuli (65). EspC is predicted to be anchored in the CM by putative MASE1 sensing domain of unknown function (64). The array of signaling domains may allow cell fate-specific accumulation of MrpC within the developmental program.

we performed the same experiment with the ΔespA ΔespC strain. Interestingly, in this mutant, MrpC levels did not appreciably decrease (Fig. 7B), and quantification of the immunoblot revealed that no loss in MrpC signal was detected over 30 min of chloramphenicol treatment. Together, these results demonstrate that the MrpC protein is subject to proteolytic turnover, which is not observed in the absence of the Esp system.

**DISCUSSION**

EspA, EspB, PktA5, and PktB8 form an unusually complex bacterial signaling system that negatively regulates progression through the elaborate \textit{M. xanthus} developmental program (Fig. 8) (36, 37, 41). In this study, we reveal an additional level of complexity in this system by demonstrating that a second HyHPK, EspC, is an obligate component of this system. Together, our data suggest a model in which EspA and EspC function together via an intimate hybrid histidine kinase signaling mechanism involving both an inter-HyHPK phosphorylation (from EspA kinase region to EspC receiver domain) and intra-HyHPK phosphorylation (from EspA kinase to its own receiver) (Fig. 8). The combined phosphorylation of EspA and EspC receiver domains (EspA\textsuperscript{A}~P/EspC~P) is necessary to stimulate turnover of an important developmental regulator, MrpC.
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form obligate heterodimers, because both EspA and EspC kinase domains are functional as individually purified proteins (Fig. 4), and we could detect EspAHK-EspC1/HK and EspCHK-EspC1/HK but not EspAHK-EspC1/HK interactions by yeast two-hybrid analysis. Thus, we favor the model in which EspA dimers interact with EspC dimers at minimum through the EspA1/HK and EspC1/EC domains.

Our data suggest no significant role for the EspC1/HK domain in control of developmental progression because neither substitution of the phosphoaccepting residue, deletion of the CA domain, nor mutation of several residues predicted to disrupt putative phosphatase activity produce an obvious mutant phenotype. Because we were unable to develop a system to test in vitro phosphorylase activity of EspC1/HK, we cannot test whether EspC1/HK displays phosphatase activity and whether these mutants indeed disrupt phosphorylase activity. However, a disruption of this motive in espA (espA411D) produced a delayed developmental phenotype (Fig. 6) that is consistent with hyperphosphorylation of EspA and/or EspC receivers. Because the EspC1/HK module is capable of efficient autophosphorylation in vitro, we hypothesize that EspC kinase activity is retained because it is necessary for modulating the Esp system under conditions not triggered in the laboratory setting.

Our data suggest that the target of the Esp signaling system is the proteolytic turnover of MrpC, a key developmental transcriptional regulator that is necessary to promote aggregation of cells into fruiting bodies (31, 61), and it may play a role in programmed cell death (26, 28). MrpC transcriptional activity is thought to be negatively regulated by a serine/threonine kinase cascade and stimulated in an MrpC isoform thought to lack the 25 N-terminal amino acids (33). We and others have previously demonstrated that premature activation (34) or accumulation (36) of MrpC leads to early accumulation of the MrpC targets and produces an early developmental phenotype. The observation that MrpC accumulates inappropriately rapidly in the ΔespA (36) and ΔespC mutants (Fig. 7) is explained by the demonstration that during early development MrpC is rapidly turned over in wild type (t0 30 min) but not in ΔespA ΔespC mutant cells. Given that the espA12696A and espC17494A point mutants exhibit the same phenotype as the deletion mutants, we propose that the EspA/P/ESP~C~P signaling state stimulates proteolytic turnover of MrpC.

We do not yet understand how EspA/P/ESP~C~P stimulates proteolytic turnover of MrpC, but there are several examples of regulated proteolysis controlled by RRAs. For example, E. coli RpoS, the master regulator of the general stress response, is targeted to the ClpXP proteosome by interaction with the phosphorylated RR, RssB (62), and the C. crescentus single domain RR, CpdR, promotes degradation of the master regulator, CtrA, by interacting with ClpXP and promoting its localization to its target (63). We favor the hypothesis that EspA/ESP~C~P receiver domains regulate MrpC proteolysis by directly binding target proteins (e.g. sequestering a targeting protein in the phosphorylated state). Alternatively, it may be that the Esp system feeds into a multistep phosphorylation cascade involving an unidentified histidine phosphotransferase protein and soluble RR output protein.

An additional output of EspA/P/ESP~C~P is the repression of EspA and EspC protein accumulation, because early developing espA or espC point mutant strains also accumulate both EspA and EspC earlier than in the wild type (Figs. 1B and 3B) (36). At least in the case of the espA16407A mutant, early EspA16407A production correlates with increased espA transcription (36), suggesting that the system somehow additionally modulates a transcriptional regulator of espA/ESP~C~P.

An intriguing possibility is that this transcriptional regulator is MrpC itself, leading to a negative feedback such that as long as EspA/ESP~C~P are phosphorylated their levels and MrpC levels remain low.

What is the advantage of this intricate signaling system? An obvious answer is that regulation of MrpC accumulation can be coupled to stimuli sensing domains of both EspA and EspC. This complex signal sensing capacity may be justified because different patterns of MrpC accumulation may mediate different developmental cell fate decisions in M. xanthus (31, 32). Perhaps different combinations of extra- and cytoplasmic signals could lead the Esp system to regulate MrpC accumulation differently in cells destined for different developmental cell fates. Both Esp proteins contain multiple sensing domains; EspC contains an integral membrane MAES1 domain of unknown function (64) and a PAS domain; EspA contains a fork-head associated (FHA) domain and two PAS domains (Fig. 2). PAS domains have been implicated in binding of various diverse ligands, mediating protein interactions, protein localization, and signal transduction (65). FHA domains are protein interaction modules that recognize phosphothreonine-containing proteins (66), and the EspA FHA domain is very likely involved in binding to the kinases PtkA5 and PktB8 that modulate EspA activity (41). We propose that each of these sensing domains could contribute to modulation of the level of EspA/P/ESP~C~P-generating cell-specific regulation of MrpC accumulation in response to temporal, spatial, and condition-specific signals. In the case of EspC, the sensing domains may regulate EspC kinase activity in response to signals not present under laboratory conditions and/or by simply regulating accessibility of EspC receiver to EspA kinase.

In summary, this study reveals a novel signaling mechanism involving two orphan HyHPKs that participate in an obligate inter- and intraphosphotransfer such that the signaling output is a combined phosphorylation of the two receiver domains. This system represents an excellent example of the inherent plasticity of the His-Asp signaling family, and in particular, it highlights HyHPKs as important players in signal integration.

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