CrdS and CrdA Comprise a Two-Component System That Is Cooperatively Regulated by the Che3 Chemosensory System in *Myxococcus xanthus*

Jonathan W. Willett and John R. Kirby
Department of Microbiology, University of Iowa, Iowa City, Iowa, USA

**ABSTRACT** *Myxococcus xanthus* serves as a model organism for development and complex signal transduction. Regulation of developmental aggregation and sporulation is controlled, in part, by the Che3 chemosensory system. The Che3 pathway consists of homologs to two methyl-accepting chemotaxis proteins (MCPs), CheA, CheW, CheB, and CheR but not CheY. Instead, the output for Che3 is the NtrC homolog CrdA, which functions to regulate developmental gene expression. In this paper we have identified an additional kinase, CrdS, which directly regulates the phosphorylation state of CrdA. Both epistasis and output for Che3 is the NtrC homolog CrdA, which functions to regulate developmental gene expression. In this paper we have identified and characterized a homolog of NtrB, designated CrdS, capable of specifically phosphorylating the NtrC homolog CrdA in *M. xanthus*. We provide kinetic data for CrdS autophosphorylation and demonstrate specificity for phosphoryl transfer from CrdS to CrdA. We further demonstrate that CheA3 destabilizes phosphorylated CrdA (CrdA~P), indicating that CheA3 likely acts as a phosphatase. Both CrdS and CheA3 control developmental progression by regulating the phosphorylation state of CrdA~P in the cell. These results support a model in which a classical two-component system and a chemosensory system act synergistically to control the activity of the response regulator CrdA.

**IMPORTANCE** While phosphorylation-mediated signal transduction is well understood in prototypical chemosensory and two-component systems (TCS), chemosensory regulation of alternative cellular functions (ACF) has not been clearly defined. The Che3 system in *Myxococcus xanthus* is a member of the ACF class of chemosensory systems and regulates development via the transcription factor CrdA (chemosensory regulator of development) (K. Wuichet and I. B. Zhulin, Sci. Signal. 3:ra50, 2010; J. R. Kirby and D. R. Zusman, Proc. Natl. Acad. Sci. U. S. A. 100:2008–2013, 2003). We have identified and characterized a homolog of NtrB, designated CrdS, capable of specifically phosphorylating the NtrC homolog CrdA in *M. xanthus*. Additionally, we demonstrate that the CrdSA two-component system is negatively regulated by CheA3, the central processor within the Che3 system of *M. xanthus*. To our knowledge, this study provides the first example of an ACF chemosensory system regulating a prototypical two-component system and extends our understanding of complex regulation of developmental signaling pathways.

**Two-component signal transduction systems are found throughout all domains of life and function to couple environmental stimuli to the appropriate cellular response. In bacteria, prototypical two-component systems (TCS) are composed of a histidine kinase (HK) and a response regulator (RR). Regulation of the output is governed by a five-step process: (i) the HK sensor domain detects an environmental signal; (ii) the ligand-bound HK undergoes a conformational change which affects autophosphorylation at a conserved histidine residue; (iii) the phosphorylated kinase interacts with an RR and transfers the phosphoryl group onto a conserved aspartate residue; (iv) the phosphorylated response regulator generates the output, which typically involves DNA binding to affect gene expression; and (v) the response regulator is dephosphorylated. Ultimately, transmission of phosphoryl groups from the HK to its cognate RR is highly specific (2, 3). Although bona fide cross-regulation has been shown for some TCS, such as Nar in *Escherichia coli* (4), cross talk does not appear to be prevalent in vivo given that these systems have evolved effective methods of insulation for signal transduction (5). RR dephosphorylation usually results from a combination of the inherent auto-dephosphorylation rate of the RR and phosphatase activity of the cognate HK. Phosphatase activity enables the HK to regulate the levels of phosphorylated RR within the cell and appears to play a critical role in limiting cross talk (6). However, while some histidine kinases have been shown to possess phosphatase activity, such as EnvZ and NarX (7, 8), there is limited experimental evidence regarding HK phosphatase activity. Furthermore, some kinases, such as *E. coli* CheA, do not possess phosphatase activity but instead rely on a dedicated phosphatase, CheZ, to limit CheY~P (RR) levels (9).

In general, more complex signal transduction pathways occur as a result of the modular nature of signal transduction proteins. Complex signaling pathways include multistep phosphorylations and branched pathways. Branched pathways can consist of many
HKs working together to regulate the phosphorylation state of one target RR. Several complex signaling pathways have been shown to regulate cellular processes such as division in *Caulobacter crescentus* and sporulation in *Bacillus subtilis* (10, 11). Multiple sensory inputs allow cells to send diverse signals into critical signal transduction pathways. *Myxococcus xanthus* utilizes many signaling proteins (the genome contains 264 TCS and 61 chemosensory system proteins) which affect development and thus serves as an excellent model for studying complex signal transduction pathways (12). The developmental program of *M. xanthus* requires both intra- and intercellular signaling mechanisms for the coordination of motility to produce multicellular fruiting bodies filled with myxospores (10).

Previously, we demonstrated that the *M. xanthus* Che3 system is required for proper regulation of developmental gene expression, which affects entry into aggregation and sporulation. Encoded within the che3 gene cluster are two membrane-bound methyl-accepting chemotaxis proteins (MCPs), one hybrid CheA histidine kinase, one CheW coupling protein, one CheB methyl-esterase, and one CheR methyltransferase homolog. The gene cluster does not encode a CheY response regulator protein but instead contains a transcription factor, designated CrdA. The results from that study demonstrated that the Che3 chemosensory system utilizes homologs for chemotaxis to regulate alternative cellular functions distinct from motility (13–15). Mutations within the *M. xanthus* che3 operon lead to defective timing of development: a mutation in cheA3 resulted in premature aggregation, while disruption of crdA delayed entry into development. Yet, relatively little is known about alternative cellular function (ACF) chemosensory systems, with some notable exceptions, including the similarly named Che3 pathway in *Rhodospirillum centenum*, which regulates cyst formation (16), and the Wsp chemosensory system in *Pseudomonas aeruginosa*, which regulates c-di-GMP production involved in biofilm formation (17).

In this study we have identified an additional regulator of *M. xanthus* CrdA, designated CrdS (Mxan_5184), a homolog of the NtrB class of kinases. Our genetic and biochemical data indicate that CrdS is an active kinase involved in the regulation of CrdA. In vitro reconstruction of the CrdS-CrdA signaling cascade demonstrates that CrdS is a kinase that specifically functions to regulate phosphorylated CrdA (CrdA-P) levels. We provide additional evidence that CrdS displays a kinetic preference for CrdA and does not phosphorylate other NtrC-like activators encoded within the *M. xanthus* genome. Epistasis analysis further demonstrates that CrdA is the most likely target for CrdS in vivo. Our model for the architecture for the *M. xanthus* CrdSA/Che3 pathways resembles those phosphorylases governing sporulation in *B. subtilis*, quorum sensing in *Vibrio* species, and nitrate regulation in *E. coli*, where multiple kinases act synergistically to control the level of phosphorylation of a target response regulator.

**RESULTS**

Identification of an *M. xanthus* NtrB kinase homolog, CrdS. Previous data indicated that in addition to CheA3, another unidentified kinase could serve as a sensory input for CrdA (18). Given that TCS cognate kinase and response regulator pairs are frequently encoded within the same operon or are located in relatively close proximity on the genome, we examined the genomes of other members of the *Myxococcales* order for additional kinases that cooccur with crdA. CrdS (Mxan_5184) was identified as a likely kinase for CrdA after observing the gene neighborhoods surrounding crdA and cooccurrence of other accessory genes, including the kinase gene crdS (Fig. 1A). The distribution of crdS, crdA, and crdB appears to be conserved in the *Myxococcales*, similarly to many other signal transduction pathways conserved throughout the order (19). One of the best examples highlighting crdSAB conservation is found in *Anaeromyxobacter* sp. strain FW109-5, which otherwise lacks the majority of the genes found within the che3 cluster (Fig. 1B). Three additional open reading frames (ORFs) within the putative crdS operon are also present in most members of the *Myxococcales* order (Fig. 1A). Interestingly, only *Stigmatella aurantia* and *M. xanthus* possess the additional chemosensory genes found within the Che3 system (including cheA3, cheB3, and cheR3). The most likely conclusion is that the che3 chemosensory gene cluster resulted from an insertion relative to the common ancestor for this clade (Fig. 1B).

**Phenotypic analysis shows CrdA is epistatic to both CrdS and CheA3.** The observation that the crdSAB genes display similar occurrences and similar gene neighborhoods within the *Myxococcales* clade suggests that the corresponding proteins function together and with similar roles. Because CrdS has high homology to NtrB histidine kinases, we hypothesized that CrdS is the cognate kinase for the NtrC-like activator CrdA. To test this hypothesis, we generated mutations in *crdS* and analyzed the mutants for defects in timing of development. The phenotypes for *crdS*, *crdA*, and *cheA3* mutant cells were compared for their capacity to aggregate and sporulate on 1.5% agar starvation (CF) medium (Fig. 2). Based on previously characterized NtrB-NtrC kinase regulator pairs (20), we expected that the *crdS* and *crdA* mutants would exhibit similar phenotypes. Both the *crdS* and *crdA* mutant cells displayed a significant delay for entry into development (aggregation foci were apparent at 72 h), while the *cheA3* mutant cells aggregate prematurely relative to the parent. Epistasis analysis allowed us to determine that the *crdA* mutation is epistatic to both the *cheA3* mutation and overexpression of *crdS* (see below). Both the *crdA cheA3* and *crdA cheR3* mutants display delays in development, similar to the phenotype observed for the *crdA* mutant. It is worth noting that the *crdS cheA3* mutant is also delayed during development, indicating that the *crdS* mutation is dominant to the *cheA3* mutation.

Because the *crdS* and *crdA* mutants are delayed for entry into development, we predicted that overexpression of *crdS* or *crdA* in the wild-type parent background would lead to premature development. In addition, we predicted that the overexpression of *cheA3* would delay development. To test these possibilities, we generated constructs to express *crdS*, *crdA*, or *cheA3* under control of the constitutively active promoter for pilA following integration at the ectopic Mx8 phage attachment site (*attB8*) (21–23). Western blots using anti-T7 antibodies confirmed that CrdS, CrdA, and CheA3 are produced under the conditions of our assays for each strain containing these constructs (data not shown). As predicted, the *PpiA-crdS* and *PpiA-crdA* mutant cells displayed a premature phenotype, whereas the *PpiA-cheA3* strain displayed a delay in development. Based on the observed phenotypes for the *PpiA-crdS* and *crdA* mutant cells, we were able to assess epistasis between the *PpiA-crdS* and *crdA* mutations. If CrdA is the cognate response regulator for CrdS, then the *crdA* mutation should be epistatic to *PpiA-crdS* expression in regard to the timing of development. To test this possibility, we constructed a double mutant containing the *PpiA-crdS* construct in the *crdA* mutant back-
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(2) a FG-GAP protein, and (3) a protein containing an SBP_bac5 domain. White arrows represent the crdSAB conservation but lack the S. aurantiaca (orange) homologs. Homologs were identified by BLAST (41). Striped arrows indicate conserved homologs. The dots between bacter sp. Fw109-5.

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Addition of a chemosensory module in proximity to a prototypical TCS in Myxococcus xanthus. (A) A 16S rRNA gene phylogenetic tree of members of the Myxococcales order was generated using DNASTAR MegAlign. Arrows indicate the gene orientation of the crdS (red), crdA (blue), crdB (green), and cheA (orange) homologs. Homologs were identified by BLAST (41). Striped arrows indicate conserved homologs. The dots between crdA and crdB in M. xanthus and S. aurantiaca DW4/3-1 indicate an insertion. Only M. xanthus and S. aurantiaca DW4/3-1 contain homologs of the che3 system. Other organisms maintain crdSAB conservation but lack the che3 system. (B) Illustration of the crdSAB regions in Anaeromyxobacter sp. Fw109-5 and M. xanthus. Sequences present in M. xanthus and S. aurantiaca suggest the che3 cluster was obtained by insertion between the crdB and crdF homologs, relative to those shown for Anaeromyxobacter sp. Fw109-5. Anaeromyxobacter Fw109-5 is closely related to M. xanthus but lacks the che3 cluster. Numbered ORFs encode (1) a penicillin binding protein, (2) a FG-GAP protein, and (3) a protein containing an SBP_bac5 domain. White arrows represent the M. xanthus che3 cluster described previously (18).

To determine if phosphorylation is required for CrdA activity, we replaced the putative site of phosphorylation, aspartate 53, with either an alanine or a glutamate. To assess activity, we complemented the crdA mutant with the Ppila-crdA, Ppila-crdA(D53A), or Ppila-crdA(D53E) construct (Fig. 2). While the wild-type copy of crdA could complement the mutant, the Ppila-crdA(D53A) construct was unable to restore development. In contrast, development was restored for the Ppila-crdA(D53E) construct, consistent with previous observations that D-to-E replacements can mimic phosphorylated response regulators (24). Together, the data indicate that phosphorylation of CrdA is required for regulation of development.

CrdSsoluble is capable of autophosphorylation in vitro. The genetic analyses described above indicate that CrdS provides input for the CrdA response regulator in vivo. The most likely mechanism for regulation of CrdA is by phosphorylation. To investigate if CrdS is capable of autophosphorylation using ATP, we purified a soluble form of CrdS, designated CrdSsoluble (Fig. 3A), in which the N-terminal region is replaced with a His tag. The CrdSsoluble construct expresses amino acid (aa) residues 346 to 578 and results in a 26.7-kDa protein. Attempts to purify full-length CrdS were not successful, likely due to the fact that it is predicted to contain two transmembrane regions flanking a putative periplasmic sensor domain (Fig. 3A). Multiple kinases lacking N-terminal input domains have been successfully characterized, such as DivJ, NarX, DesK, and EnvZ (25–27).

Purified CrdSsoluble is active and capable of autophosphorylation in the presence of excess ATP, as determined by the presence of a band corresponding to radiolabeled CrdS (Fig. 3B). Fifty percent maximal phosphorylation is reached in 8.1 minutes, with maximal phosphorylation occurring within 30 minutes (Fig. 3C). The phosphorylated form of CrdSsoluble is very stable, exhibiting a half-life (t1/2) of 122.6 ± 23.5 h (Table 1). We further analyzed the kinetics of CrdSsoluble autophosphorylation and determined its Km for ATP to be 24.5 ± 4.9 μM (Fig. 3D; also see Fig. S2 in the supplemental material). A Km of approximately 25 μM is similar to those of HKs found in other organisms, such as WalK, KinA, and NarQ (26, 28–30). These data allowed us to determine the Vmax for CrdSsoluble autophosphorylation to be 0.73 ± 0.04 μM ATP min⁻¹.

CrdS displays a kinetic preference for phosphotransfer to CrdA. Laub and Goulian have demonstrated that TCS cognate kinase regulator pairs display kinetic preference for phosphorylation in vitro (4). Additionally, Laub et al. have shown that the in vitro results for kinetic specificity typically translate to in vivo preference (3). The main criterion for demonstrating specificity in vitro is the time scale for the phosphotransfer reaction. Nonspecific phosphotransfer between HKs and RRs is observed only following extensive incubation times (2).
**FIG 2** crdS, cheA3, and crdA mutants display altered timings of development. Developmental assays were performed as described in Materials and Methods. Phenotypic assays were conducted by spotting 10 μl of cells at 250 Klett units on starvation (CF) media. Images were acquired at 50× magnification at 18, 24, and 48 hours (left to right). Developmental progression is indicated by the presence of aggregates or opaque fruiting bodies containing 10^5 to 10^6 cells. The constitutively active pilA promoter (PpilA) was used to express crdS from the ectopic Mx8 phage attachment site. Premature or delayed phenotypes are relative to the wild-type parent.

**FIG 3** In vitro phosphorylation of CrdS. (A) Domain structure of CrdS, CheA3, and CrdA. CheA3 is a hybrid kinase containing the HPT, HK_CA, and CheW binding domains along with a C-terminal receiver (REC) domain. CrdA is homologous to NtrC of *E. coli* and contains the N-terminal REC domain, a central Sigma_54 activation domain, and a C-terminal HTH_8 DNA binding motif. CrdS is a histidine kinase with two transmembrane-spanning regions and HAMP, DHp, and HK_CA domains. The CrdS soluble construct expresses a constitutively active form of CrdS lacking the N-terminal membrane-spanning regions and HAMP, DHp, and HK_CA domains. The CrdS soluble construct and CrdA, CrdS, and CheA3 are drawn to scale. (B) Autoradiograph showing CrdS soluble autophosphorylation. Five micromolar CrdS soluble was incubated with excess [γ-^32^P]ATP, and aliquots were removed at the indicated time points (in minutes). Samples were resolved by SDS-PAGE and quantified following exposure to a phosphor screen. (C) Kinetics of CrdS soluble autophosphorylation. Pixel intensity versus time was used to generate the curve showing CrdS soluble autophosphorylation rates. Arbitrary pixel units were converted to percent CrdS~32P. Maximal phosphorylation was reached within 30 minutes. (D) Km determination of CrdS soluble. The graph depicts the velocity for CrdS soluble phosphorylation in number of μM per minute versus μM ATP substrate concentration. Rates were determined as indicated in Materials and Methods. The Km of CrdS soluble was determined to be 24.5 ± 4.9 μM ATP, with a corresponding Vmax of 0.73 ± 0.04 μM of CrdS~32P per minute.
In order to test the possibility that CrdS has a kinetic preference for CrdA, we performed in vitro phosphotransfer time course assays. CrdS\textsubscript{soluble} was allowed to autophosphorylate for 30 minutes (maximally labeled) and subsequently added to equal molar amounts of purified CrdA (see Fig. S3 in the supplemental material). The proteins were fractionated by SDS-PAGE and analyzed for incorporation of label using a phosphorimager. Within 5 s, phosphorylated CrdS\textsubscript{soluble} was no longer detectable, indicating a high rate of turnover for CrdS\textsubscript{soluble}~P in the presence of CrdA (Fig. 4A). The calculated half-life for CrdS\textsubscript{soluble}~P in the presence of CrdA is less than 1 s, indicating that the CrdS-CrdA interaction is highly specific (Table 1). In addition, CrdA~P also displays high turnover, such that CrdA~P was nearly undetectable within 30 s of incubation with the maximally labeled CrdS\textsubscript{soluble}~P (Fig. 4A). The observation that rapid turnover for the phosphorylated RR occurs in the presence of the HK is an indication that the kinase may also possess phosphatase activity (8). The time scale for CrdS-CrdA phosphotransfer is similar to those for previously determined HK–RR cognate pairs (3, 28, 31).

Previous work to define HK–RR specificity has utilized organisms with relatively few TCS, such as E. coli and C. crescentus. Because M. xanthus possesses 27 NtrC-like activator (NAL) proteins with domain structures similar to that of CrdA (12, 32), we tested the possibility for CrdS phosphotransfer to other NLAs. We first generated a sequence homology tree of all 27 NtrC-like proteins (see Fig. S1 in the supplemental material). Based on this tree, we chose two NtrC-like activators, designated NtrC\textsubscript{1189} (Mxan\textsubscript{1189}) and NtrC\textsubscript{4261} (Mxan\textsubscript{4261}), which both display high sequence homology to CrdA. These NLAs were purified and incubated with labeled CrdS\textsubscript{soluble}~P to assay for in vitro phosphotransfer activity, similar to those experiments performed with CrdA. No significant loss of CrdS\textsubscript{soluble}~P phosphorylation to NtrC\textsubscript{1189} or NtrC\textsubscript{4261} was observed over a 10-min time scale, indicating a lack of specificity between CrdS\textsubscript{soluble} and the alternative NLAs (see Fig. S4 and S5 in the supplemental material). We also determined the half-life of CrdS\textsubscript{soluble}~P in the presence of either NtrC\textsubscript{1189} or NtrC\textsubscript{4261} and observed changes that were not significant compared to the reaction mixture containing CrdA (Table 1). Although NtrC\textsubscript{1189} and NtrC\textsubscript{4261} induced a 50-fold decrease in the half-life of CrdS\textsubscript{soluble}~P (to about 2 h), CrdA induced a 1 million-fold decrease (to about 1 s). Thus, CrdS\textsubscript{soluble} displays a kinetic preference for phosphotransfer to CrdA in vitro.

To assess whether NtrC\textsubscript{1189} and NtrC\textsubscript{4261} were competent for phosphotransfer, we tested whether they could be labeled either by acetyl phosphate (AcP) or by their predicted cognate kinases. Both NtrC\textsubscript{1189} and NtrC\textsubscript{4261} lie within putative operons next to their predicted cognate NtrB family histidine kinases, Mxan\textsubscript{1190} and Mxan\textsubscript{4262}, respectively. We purified the soluble portions of the kinases HK\textsubscript{1190} (Mxan\textsubscript{1190}; amino acids [aa] 205 to 424) and HK\textsubscript{4262} (Mxan\textsubscript{4262}; aa 477 to 702), lacking the membrane spanning and periplasmic domains. Both kinases were capable of autophosphorylation and displayed rapid turnover of label in the presence of their cognate NtrC response regulator targets (see Fig. S5 in the supplemental material). Additionally, both NtrC\textsubscript{1189} and NtrC\textsubscript{4261} were labeled with \(^{32}\)PAcP (Fig. 4C), indicating that the HK\textsubscript{1190}, NtrC\textsubscript{1189}, HK\textsubscript{4262}, and NtrC\textsubscript{4261} proteins were competent for autophosphorylation and phosphotransfer reactions. Importantly, neither HK\textsubscript{1190} nor HK\textsubscript{4262} was capable of phosphotransferring.

### Table 1 Transfer from CrdS–P to CrdA is specific\(^a\)

| Protein(s) | Half-life (h) |
|------------|--------------|
| CrdS–P     | 122.6 ± 23.5 |
| CrdS–P and CrdA | ≤0.0001 ± 0.0001 |
| CrdS–P and NtrC\textsubscript{1189} | 2.8 ± 0.08 |
| CrdS–P and NtrC\textsubscript{4261} | 2.3 ± 0.09 |
| CrdS–P and CrdA\textsubscript{D53A} | 2.0 ± 0.2 |
| CrdS–P and CrdA\textsubscript{D53E} | 1.5 ± 0.1 |

\(^a\) CrdS–P half-life is significantly reduced in the presence of CrdA. Calculations of half-lives are detailed in Materials and Methods. Values given are the means ± standard errors. CrdS\textsubscript{soluble} was incubated with excess ATP and added to equal volumes of target protein at a final concentration of 5 μM. The decrease in the CrdS\textsubscript{soluble}~P half-life indicates the velocity for each reaction and reveals phosphotransfer fidelity.

![FIG 4](mbio.asm.org/FIG 4) CrdS phosphorylation of CrdA in vitro. (A) Phosphotransfer between CrdS\textsubscript{soluble}~P and CrdA. Loss of phosphorylated CrdS indicates rapid phosphotransfer to CrdA. Complete transfer occurs within 5 seconds, as indicated by the loss of the CrdS\textsubscript{soluble}~P band and the appearance of the CrdA~P band. (B) CrdS(H371) and CrdA(D53) are required for phosphotransfer. All reaction mixtures contain excess total ATP and 0.3 μM \(\gamma^{32}\)PATP in kinase buffer and were incubated for 30 minutes, fractionated by SDS-PAGE, and visualized by autoradiography as described. Lane 1 contains CrdS(H371A), which is unable to autophosphorylate. Lane 2 contains phosphorylated CrdS\textsubscript{soluble}. Lane 3 contains CrdS\textsubscript{soluble} incubated with CrdA for 10 minutes, leading to complete loss of CrdS. CrdS\textsubscript{soluble}~P is unable to transfer phosphoryl groups to CrdA\textsubscript{D53A} (lane 4) or CrdA\textsubscript{D53E} (lane 5). (C) CrdA is phosphorylated by acetyl phosphate (AcP). Radiolabeled AcP was generated as described in Materials and Methods. When CrdA is incubated with \[^{32}\]PAcP, CrdA~32P is formed (lane 1). When the conserved residue, D53, of CrdA is mutated [to CrdA\textsubscript{D53A} or CrdA\textsubscript{D53E}] or when wild-type CrdA is incubated with excess unlabeled AcP (Sigma), no labeling is apparent (lanes 2 to 4). The alternative target proteins, NtrC\textsubscript{1189} and NtrC\textsubscript{4261}, were phosphorylated with radiolabeled AcP (lanes 5 and 6).
CrdA (Fig. S5), indicating that CrdA is not a promiscuous phosphoacceptor. Together, these data support the conclusion that CrdS is the cognate kinase for CrdA.

**Phosphotransfer requires conserved residues CrdS(H371) and CrdA(D53).** Sequence alignments indicated that CrdS(H371) and CrdA(D53) are the conserved residues required for phosphorylation. As described above, we generated amino acid substitutions in the putative sites of phosphorylation. We purified CrdA(D53A) and CrdS_{soluble}(H371A) constructs and assayed their ability to undergo autophosphorylation and display phosphotransfer in vitro. A change of the conserved histidine to alanine inhibited CrdS_{soluble} autophosphorylation, indicating that CrdS(H371) is the probable site of phosphorylation (Fig. 4B). When wild-type (WT) CrdS_{soluble}~P was incubated with CrdA(D53A) or CrdA(D53E), we observed no CrdS_{soluble}~P turnover or appearance of a phosphorylated target, indicating that D53 is the likely site for phosphorylation in CrdA. Furthermore, the half-life for CrdS_{soluble}~P decreased by approximately 50-fold when incubated with either CrdA(D53A) or CrdA(D53E) (Table 1). These results are comparable to the half-life for CrdS_{soluble}~P when incubated with the noncognate proteins NtrC_{1189} and NtrC_{4261}. This 50-fold decrease is not significant compared to the 1 million-fold reduction in the CrdS_{soluble}~P half-life when incubated with wild-type CrdA. The results are consistent with a model in which CrdS(H371) and CrdA(D53) are the probable sites of phosphorylation.

**CrdA is phosphorylated by acetyl-P and dephosphorylated by CrdS and CheA3.** In the above-described phosphotransfer assays, CrdA~P displayed rapid turnover due to either CrdS phosphatase activity or inherent lability of CrdA~P. In order to differentiate between these two possibilities, we determined the half-life for CrdA~P using [32P]AcP. Results indicate that CrdA, like many NtrC homologs, is phosphorylated in the presence of AcP (Fig. 4C) (33, 34). CrdA~P displayed a half-life of 53.5 ± 6.3 minutes (Table 2). This value is similar to those published for other NtrC homologs (35, 36). As a control, we tested the CrdA(D53A) mutant protein and observed no incorporation of label, indicating that the conserved aspartate is required for phosphorylation. Furthermore, incubation of CrdA with unlabeled AcP inhibited labeling by [32P]AcP. These results indicate that CrdA is likely phosphorylated at D53 and that the rapid turnover of CrdA~P in our phosphotransfer assays is due primarily to phosphatase activity by CrdS.

Some TCS kinases display phosphatase activity, which plays a significant role in the overall regulation of RR phosphorylation (35). The data described above indicate that CrdS likely acts as a phosphatase, and previous work indicated that CheA3 may also act as a phosphatase on CrdA~P (18). We therefore tested CheA3 and CrdS for phosphatase activity on CrdA~P. Upon incubation of CrdS_{soluble} with CrdA~P, the half-life of CrdA~P decreased from 53 to 0.7 minutes, indicating that CrdS_{soluble} does possess significant phosphatase activity. As a control, CrdA~P was incubated with either NtrB homolog HK_1190 or HK_4262 and displayed no significant decrease in radiolabeling (Table 2). These results provide further confirmation that CrdS and CrdA represent a cognate TCS. In addition, upon incubation with CheA3, we also observed a decrease in CrdA~P stability by a factor of 5-fold (Table 2), without a detectable transfer of phosphoryl groups to CheA3. This result is consistent with those published recently for *Rhodobacter sphaeroides* CheA3, which was shown to act as a specific phosphatase on CheY6-P (37). It is worth noting that *M. xanthus* CheA3 was not able to autophosphorylate using ATP or AcP under the conditions of our assays. Furthermore, *M. xanthus* DifE (CheA2) was unable to affect CrdA~P turnover (Table 2). Thus, in combination with the phenotype characterization, these data indicate that CrdSA comprises a cognate TCS and that CheA3 negatively regulates phosphorylation of CrdA.

**DISCUSSION**

In this study we have identified an additional signaling protein in the Che3 pathway and further defined a complex signal transduction mechanism involving the histidine kinase CrdS, the transcription factor CrdA, and CheA3, which together regulate entry into development in *M. xanthus*. Our *in vitro* biochemical and *in vivo* phenotypic data allow us to propose a model whereby both CrdS and CheA3 cooperatively regulate the phosphorylation state of CrdA (Fig. 5). CrdA~P thereby alters transcription, affecting developmental gene expression (18). In our model, CrdS is able to act as a dual kinase/phosphatase, directly regulating the phosphorylation state of CrdA. When CrdS senses the appropriate signal, it undergoes autophosphorylation at histidine 371 and subsequently phosphorylates the response regulator CrdA on conserved aspartate residue 53. CrdS is then able to act directly on CrdA~P, leading to a dramatic change in the stability of the phosphoryl group, resulting in rapid dephosphorylation of CrdA. CrdS-mediated dephosphorylation of CrdA is predicted to utilize a mechanism similar to that proposed for NarX-mediated dephosphorylation of NarL (8, 26). Additionally, we have shown that purified CheA3 is able to dephosphorylate CrdA, consistent with our *in vivo* analysis and previous results suggesting that Che3 negatively regulates CrdA during development (18). Although we have not observed CheA3 kinase activity in our assays, we have demonstrated that CheA3 acts to alter CrdA~P stability. This does not exclude the possibility that CheA3 may also act as a kinase under conditions not yet identified. For instance, *M. xanthus* FtzE (CheA1) is active only when both CheW and MCP proteins are present *in vitro* (38). If CheA3 can also function as a kinase under some conditions, this would lead to a more complex regulatory mechanism by which CheA3, like CheS, could act both as a kinase and as a phosphatase.

Identification of CrdS as a putative kinase for CrdA was accomplished by comparing the genomes of *M. xanthus* and other members in the *Myxococcales* order. Because crdS, crdA, and crdB cooccur with similar gene neighborhoods, we hypothesized that CrdS and CrdA comprised a cognate histidine kinase-response regula-

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**TABLE 2 CrdA~P stability is significantly reduced in the presence of CrdS_{soluble} and CheA3**

| Protein(s) | Half-life (min) |
|------------|----------------|
| CrdA~P     | 53.5 ± 6.3     |
| CrdA~P and CrdS | 0.7 ± 0.3   |
| CrdA~P and CheA3 | 8.8 ± 1.3 |
| CrdA~P and CheA2 | 53.3 ± 9.8 |
| CrdA~P and HK_1190 | 50.0 ± 0.7 |
| CrdA~P and HK_4262 | 44.5 ± 6.5 |

* Half-lives were determined as indicated in Materials and Methods. Values given are standard errors. CrdA~32P was generated by incubation of CrdA with radiolabeled acetyl phosphate. Free acetyl phosphate was removed, and CrdA was incubated with equal molar amounts of each target protein. The decrease in the CrdA~P half-life indicates that both CrdS and CheA3 possess phosphatase activity.
tor pair. Thus, the presence of the che3 gene cluster appears to be a recent addition for *M. xanthus* and its close relative, *Stigmatella aurantiaca*. Phenotypic analysis of *crdS*, *crdA*, and cheA3 mutants provided in vivo evidence that both CrdS and CheA3 regulate CrdA. Mutations in *crdA* are delayed in aggregation, displaying a phenotype similar to that observed for mutations in cheA3. In contrast, overproduction of CrdS in the otherwise wild-type parent strain is observed to aggregate prematurely, similar to the cheA3 mutant. Additionally, the alternative targets provided, NtrC_1189 and NtrC_4261. The results indicate high fidelity for the CrdS-CrdA phosphotransfer reaction.

Perhaps our most important observation is that CheA3 can act as a CrdA phosphatase, as indicated by the significant decrease in the half-life for CrdA–P from 54 minutes to 9 minutes when incubated with CheA3. No such difference was observed when an alternative CheA homolog, DifE (or CheA2), was provided in vitro. Thus, it appears that CheA3 in *M. xanthus* may serve a role similar to that of CheA3 in *Rhodobacter sphaeroides*. In *R. sphaeroides*, CheA3 acts as a phosphatase capable of affecting CheY6–P stability (37). Interestingly, both CheA3 in *M. xanthus* and CheA3 in *R. sphaeroides* decrease the half-life of the phosphorylated RR by approximately 4–5-fold (37). While the overall effect of RR dephosphorylation appears to be similar, the underlying mechanism of CheA3-dependent CrdA dephosphorylation is not understood and is currently being investigated.

Many organisms contain complex signaling cascades to control critical, energy-intensive processes such as development. Thus, it is not surprising that *M. xanthus* possesses a complicated mechanism to regulate CrdA phosphorylation. However, it is not known how CrdA fits into the overall developmental program. Recent results illustrate that several NlrC-like activators participate within a complex cascade to regulate development for *M. xanthus* (39). No interaction between those NLAs and CrdA has been demonstrated. Additionally, it is not known how CrdS and CheA3 cooperate to regulate CrdA activity. One possibility is that CrdS and components upstream of CheA3 detect similar or related stimuli. CrdB contains a peptidoglycan-binding OmpA domain and requires CheA3 to process signals (18; S. Müller and J. Kirby, unpublished data). Similarly, the crdS gene cluster encodes a putative Pbp1a peptidoglycan-binding protein. Thus, it is possible that CrdB and CrdS respond to envelope stress to regulate the overall status of CrdA phosphorylation within the cell to affect development.

**MATERIALS AND METHODS**

**Bacterial growth.** All strains utilized in this study are listed in Table S1 in the supplemental material. *M. xanthus* was grown in charcoal-yeast extract (CYE), with kanamycin (80 μg/ml) and oxytetracycline (7.5 μg/ml) added when appropriate. *E. coli* strain DH5α was used for routine cloning, with antibiotic concentrations of 40 μg/ml kanamycin, 15 μg/ml tetracycline, and 100 μg/ml ampicillin added when selection was required.

**Construction of mutants.** The *crdS* deletion constructs were generated by allelic exchange and counterselection as previously described using pBJ114, which carries *galK* for counterselection on galactose medium (18). Potential mutants were verified by PCR and sequencing. CrdS expression constructs were generated by fusing the *pilA* promoter to a soluble fragment of CrdS (CrdS soluble; aa 346 to 578) and incorporated into the Mx8 phage attachment site (21). Point mutations in *crdS* and *crdA* were generated by PCR-based site-specific mutagenesis (Invitrogen).

**Developmental assays.** For all developmental assays, *M. xanthus* cells were harvested at between 100 and 150 Klett units (KU) and washed two times with water. Cells were resuspended in water to the final density of 250 KU. Ten-microliter spots were plated on CF media and grown at 32°C. Pictures were taken at the indicated times with a Nikon SMZ1500 microscope and a QImaging MicroPublisher 5.0 RTV charge-coupled-device (CCD) camera, processed with QCapture Pro software, and edited in Photoshop.

**Protein overexpression and purification.** All proteins were expressed from IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible vectors and cloned into the appropriate *E. coli* strains to support protein production. For typical overexpression, 1 liter of each strain was grown at 37°C with
the appropriate antibiotics until the optical density at 600 nm (OD_{600}) reached 0.4 to 0.6. Cells were induced upon addition of 0.5 mM IPTG and grown overnight at 20°C with shaking. Cells were pelleted by centrifugation and stored at −20°C until purification.

For purification of CrdA, CrdS, NtrC-2461, NtrC-1189, HK-4262, and HK-1190, frozen cell pellets were first suspended in 25 ml of buffer A (25 mM Tris at pH 7.6, 250 mM NaCl, 0.1% [vol/vol] Triton X-100, 1 mg/ml lysozyme, EDTA-free protease inhibitor [Roche]) and lysed with sonication on a Branson Sonicator for 3 × 40 s. Lysate was clarified at 10,000 × g centrifugation and passage through a 0.45-µm filter disk. The resulting lysate was loaded on a Hi-Trap HP immobilized-metal affinity chromatography (IMAC) column (GE), washed with five column volumes of buffer A, and eluted with a 15-ml linear gradient to 100% buffer B (25 mM Tris pH 8.0, 250 mM NaCl, 50% [vol/vol] glycerol, 1 mM dithiothreitol [DTT], 0.1% [vol/vol] Triton) and stored at −20°C until assays were performed.

Purification of CheA3 constructs began with cell pellets harvested from 4 liters of cells grown in Terrific broth as detailed above. Cells pellets were suspended in 25 ml CheA3 resuspension buffer (25 mM HEPEs at pH 7.6, 100 mM NaCl, 0.1% [vol/vol] Triton X-100, 5 mM MgCl₂, 50% [vol/vol] imidazole) and lysed by addition of CellLytic Express (Sigma). Lysate was loaded on a Hi-Trap HP (GE) column and then washed with five column volumes of CheA3 resuspension buffer. Proteins were eluted by a 15-ml linear gradient to 100% CheA3 elution buffer (25 mM HEPEs at pH 7.6, 100 mM NaCl, 0.1% [vol/vol] Triton X-100, 5 mM MgCl₂, 500 mM imidazole). Fractions containing CheA3 were immediately placed in CheA3 dialysis buffer (50 mM Tris at pH 7.6, 50 mM NaCl, 0.1% [vol/vol] Triton, 5 mM MgCl₂, 50% [vol/vol] glycerol, 1 mM DTT) and dialyzed overnight. Purification of DifE was done as described previously (31). All purified proteins were stored at −20°C until assays were performed. Proteins were purified to approximately 95% purity, as determined by Coomassie blue staining (see Fig. S3 in the supplemental material). Protein concentrations were determined using the Bradford assay.

**Kinase assays.** Purified proteins were diluted to 5 µM in 1× kinase buffer (20 mM Tris at pH 8.0, 250 mM NaCl, 10 mM MgCl₂, 10 mM CaCl₂, 1 mM 2-mercaptoethanol), and ATP was added to start the reaction (250 µM ATP, 3 µM [γ-32P]ATP). Aliquots were removed and stopped by addition to an equal volume of 2× SDS-loading buffer. Samples were resolved by electrophoresis on 10% SDS-polyacrylamide gels. The dye front, containing unincorporated ATP, was removed. Gels were exposed for 4 to 6 h on a phosphor screen and then visualized using a Typhoon imager. ImageQuant was used to determine pixel density.

**Determination of CrdS autophosphorylation kinetics.** The kinetic determination of CrdS autophosphorylation was performed using a gel-based assay (28). CrdS\textsubscript{soluble} was diluted to 5 µM in 1× kinase buffer, and aliquots were divided into several tubes. Reactions were started by adding labeled ATP mixes (250 µM ATP, 0.3 µM [γ-32P]ATP) at eight different concentrations (250, 175, 100, 75, 50, 25, 10, and 5 µM ATP). Five-microliter samples were removed at 15, 30, 45, and 60 s, and the reactions were stopped by addition of an equal volume of SDS-loading buffer. To determine the quantities of CrdS\textsubscript{soluble}−P, a standard curve was generated by spotting known quantities of [γ-32P]ATP. Samples were run and visualized as detailed above. Velocities were determined using linear regression by plotting CrdS\textsubscript{soluble}−P quantities versus time. Enzyme activity was determined by best-fit Michaelis-Menten curves using Prism statistical software (GraphPad version 5).

**CrdS phosphotransfer to CrdA.** CrdS\textsubscript{soluble} was diluted to 10 µM in 1× kinase buffer and allowed to autophosphorylate for 30 minutes as previously described. Without removal of free ATP, the CrdS\textsubscript{soluble} sample was added to an equal volume of CrdA such that the final concentration of both proteins was 5 µM. Five-microliter samples were taken and quenched as described above at time points (5, 10, 15, 30, 60, and 120 s). Samples were electrophoresed on polyacrylamide gels under denaturing conditions, and the resulting phosphorimaging band intensities were quantified. Experimental samples were compared to a control CrdS\textsubscript{soluble} sample prior to addition of CrdA, which was arbitrarily set at 100% CrdS\textsubscript{soluble}−P.

**Calculation of half-life for CrdS phosphorylation.** For half-life determinations, CrdS\textsubscript{soluble} was allowed to autophosphorylate as described above for 30 minutes. Free ATP was removed by centrifugation over a Zeba 7K MWCO desalting column (Thermo Scientific), which was pre-equilibrated in 1× kinase buffer. Reactions were stopped at the indicated time points. To calculate half-lives, plots were generated by taking the natural log of pixel intensity versus time. Calculations for CrdS\textsubscript{soluble} and CrdA half-lives were determined using the equation $t_{1/2} = \ln(2)/k$.

**Phosphatase experiments using AcP-labeled CrdA.** CrdA was labeled in vitro using the high-energy phosphor-donor acetyl phosphate (AcP). [32P]AcP was synthesized as described previously, in reactions using purified acetate kinase (Sigma) from E. coli and [γ-32P]ATP (PerkinElmer) (40). Labeling of CrdA was performed by incubating 5 µM CrdA in 1× kinase buffer with freshly synthesized [32P]AcP in a 100-µl total reaction volume for 1 h. Unincorporated [32P]AcP was removed by running the sample over a Zeba 7K MWCO desalting column equilibrated in 1× kinase buffer. Phosphatase assays were performed by mixing 5 µM AcP-labeled CrdA with 5 µM target kinase/phosphatase. Reactions were stopped by addition of equal volumes of 2× SDS loading buffer and resolved on 10% SDS-polyacrylamide gels. Relative levels of labeling were compared to samples taken before addition of the kinase/phosphatase. Calculations of CrdA−P half-lives were calculated as previously described for CrdS.

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**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00110-11/-/DCSupplemental.

Table S1, PDF file, 0.899 MB.
Figure S1, PDF file, 0.582 MB.
Figure S2, PDF file, 0.258 MB.
Figure S3, PDF file, 0.451 MB.
Figure S4, PDF file, 0.237 MB.
Figure S5, PDF file, 0.213 MB.

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