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The orphan response regulator EpsW is a substrate of the DifE kinase and it regulates exopolysaccharide in *Myxococcus xanthus*

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Here we attempted to identify the downstream target of the DifE histidine kinase in the regulation of exopolysaccharide (EPS) production in the Gram-negative bacterium *Myxococcus xanthus*. This bacterium is an important model system for the studies of Type IV pilus (T4P) because it is motile by social (S) motility which is powered by T4P retraction. EPS is critical for S motility because it is the preferred anchor for T4P retraction in this bacterium. Previous studies identified the Dif chemosensory pathway as crucial for the regulation of EPS production. However, the downstream target of the DifE kinase in this pathway was unknown. In this study, EpsW, an orphan and single-domain response regulator (RR), was identified as a potential DifE target first by bioinformatics. Subsequent experiments demonstrated that *epsW* is essential for EPS biosynthesis *in vivo* and that EpsW is directly phosphorylated by DifE *in vitro*. Targeted mutagenesis of *epsW* suggests that EpsW is unlikely the terminal RR of the Dif pathway. We propose instead that EpsW is an intermediary in a multistep phosphorelay that regulates EPS in *M. xanthus*.

Myxococcus xanthus is a model bacterium for the studies of multicellular behavior in prokaryotes^{1,2}. During vegetative growth, this bacterium moves as multicellular groups over solid surfaces. This is known as social swarming, which allows large groups of cells to move as a pack for the utilization of complex organics as nutrients in a cooperative manner³. Under starvation, hundreds of thousands of *M. xanthus* cells use their surface motility to aggregate and develop multicellular structures known as fruiting bodies^{4,5}. Within these structures, cells of this Gram-negative bacterium eventually differentiate into metabolically dormant myxospores which are resistant to environmental stresses such as heat, desiccation and UV radiation. Both vegetative swarming and developmental aggregation are social behaviors that requires the collective action of many cells.

Integral to the social behavior of *M. xanthus* is its ability to move or glide over solid surfaces^{1,5,6}. The motility of *M. xanthus* entails two genetically and mechanistically distinct systems known as adventurous (A) and social (S) motility⁷. The A-motility system allows cells to move even when they are well separated. The S system, which is the focus of this study, is only functional when cells are in close proximity. It is now known that S motility is powered by the retraction of the Type IV pilus (T4P) as is bacterial twitching motility⁸. In the current model for T4P-dependent motility, the distal end of a T4P is first tethered to a solid surface. The ensuing retraction of the T4P pulls the cell forward to result in a net translocation over the surface. The tip of a *M. xanthus* T4P attaches preferentially to exopolysaccharides

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(EPS) produced by its kin. These EPS are either deposited on a gliding substratum by or associated with the cell surface of a neighboring *M. xanthus* cell. This mechanism explains the social nature of T4P-mediated motility in *M. xanthus* because other cells are typically required as a source of EPS to activate the retraction or the motor function of T4P.

EPS production is regulated in part by a pathway consisting of the Dif chemosensory proteins in *M. xanthus*⁸. DifA is homologous to the methyl-accepting chemotaxis protein (MCP), DifC to the bridging or coupling protein CheW and DifE to the CheA-type histidine protein kinase (HK)⁹. These three proteins, which form a membrane signaling complex^{10,11}, are required for EPS production¹². DifD and DifG, respective homologues of CheY and CheC, are not required for EPS production¹³. They are negative regulators of EPS instead because their absence results in an overproduction of EPS. DifD is a single-domain response regulator (RR) that can be phosphorylated by the DifE kinase¹¹. Like CheC^{14,15}, DifG is a phosphatase that accelerated the dephosphorylation of phosphorylated DifD (DifD~P)¹¹. Interestingly, DifD does not function downstream of DifE in EPS regulation and it likely acts to divert phosphate from DifE as a phosphate sink^{16,17}. This led to a model wherein *M. xanthus* DifE autophosphorylates on a strictly conserved histidine residue and the phosphate is then transferred to a conserved aspartate on a hypothesized RR directly downstream of DifE in EPS regulation. In other words, DifE is proposed to phosphorylate at least one additional RR besides its known target DifD.

There are known instances where a HK phosphorylates multiple RRs in two-component signal transduction pathways. In bacterial chemotaxis, a CheA kinase typically phosphorylates CheY as well as CheB, a methylesterase with a RR domain^{18,19}. CheA also phosphorylates CheV in *Bacillus subtilis* and an additional CheY in *Sinorhizobium meliloti*. In an extreme case in *Rhodobacter sphaeroides*, CheA3 interacts with five RRs in chemotaxis regulation. Such examples of one HK to multiple RRs are also found in canonical two-component systems^{20,21}. The response regulators NarL and NarP are both substrates of the same HKs NarX and NarQ in the regulation of anaerobic respiration with nitrate and nitrite as electron acceptors²². In the cyanobacterium *Synechococcus elongatus*, NblS was found to phosphorylate both RpaB and SrrA in the regulation of photosynthesis and metabolism^{20,23}. However, despite the many HKs and RRs in a cell, signal transduction pathways are well insulated from one another by specific interactions between HKs and RRs. That is, whether a HK has one or multiple cognate RRs, it only phosphorylates its target specifically and there is no evidence of nonspecific interactions between HKs and RRs^{20,21}. The structural determinants of the high specificity between HKs and RRs have been studied extensively by co-variant analysis^{24–28}. These analyses identified certain residues in RRs as critical determinants for their specific interaction with their cognate HKs. Mutations of these residues have been shown to alter and rewrite the specificity between RRs with HKs^{29,30}, validating bioinformatics as a useful approach to identify RRs, especially orphan ones, as target for specific HKs. In this study, DifD was used as a template to search for other RRs as potential targets of DifE phosphorylation by sequence analysis first. Subsequent studies both *in vivo* and *in vitro* led to the conclusion that EpsW, an orphan single-domain RR, is a target of DifE phosphorylation and part of the EPS regulatory pathway in *M. xanthus*.

Results

Bioinformatics analysis identified EpsW as a possible substrate of DifE kinase. Previous co-variant studies^{24–28} indicated that certain residues in RRs are critical determinants of specificity with regard to their interactions with their cognate HKs. These residues in RRs can be divided into two sets with the first one consisting of five residues and the second one seven²⁷. The first or the primary set contributes directly to the physical interactions between a RR-HK pair whereas the second does so indirectly. These residues as defined in RR468, a single-domain RR from *Thermatoga maritime*³¹, are shown in Fig. 1²⁷. In this RR, the primary residues are V13, L14, I17, F20 and N21 and the secondary ones are M55, K85, E88, E91, L95, S96 and R100. DifD is a single domain RR that is phosphorylated by DifE¹¹. Its specificity determinants were therefore used to identify additional DifE substrates. Alignment with RR468 indicated that the five primary residues in DifD (Fig. 1) are F13, M14, M17, D20 and I21 and the secondary ones are V56, L84, E87, V90, I94, E95 and S99.

Next, DifD was used to search the *M. xanthus* genome sequence³² producing alignment with over 100 RRs³³. These RRs were manually enumerated to identify those with at least two identical residues with DifD in the primary group. This narrowed the number to eight ORFs. They are MXAN_2050, MXAN_4049, MXAN_4232, MXAN_4463, MXAN_5053, MXAN_6627 and MXAN_7396 and MXAN_7420. Among them, only MXAN7420 or EpsW³⁴ matched with DifD (Fig. 1) three out of the five primary residues (F13, M17 and D20 in DifD; and F16, M20 and D23 in EpsW) with one additional conserved substitution (M14 in DifD and F17 in EpsW). For the seven secondary sites, DifD and EpsW share one identical residue (E85 in DifD and E101 in EpsW) and 2 somewhat conserved ones (V56 and S99 in DifD; T60 and N105 in EpsW) (Fig. 1). The observed conservation of specificity determinant residues here suggested one or more of these eight RRs, especially EpsW, could be substrates for DifE phosphorylation.

epsW is required for EPS production. These eight RR genes were targeted for mutagenesis. *epsW* is a small gene encoding a single-domain RR while the other seven genes all encode larger proteins with additional domains³². As a consequence, an in-frame deletion was constructed for *epsW* whereas other RR genes were mutated by simple insertions. Except the *epsW* mutation, none of the others led to an EPS phenotype (data not shown) and this manuscript will only focus on *epsW* hereafter. As shown in

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RR468 ---MSKKVLLVDDSAVLRKIVSFNLKKEGYEVIEAE-NGQIALEKLS-EFTPDLLIVLDIMMPVMDGF 62
DifD ---MAKRVLVDDAIERNIKIFASGGFEVVGEAANGLEAVEKEYK-ELKPDLTMTDIMPFKSGI 63
EpsW MESDLHTVLLVEDAPERKLGDLYLRDMGFKEVVVLPVSGRAALKHMETAAARPDVLCLDLLPDISGY 67
CheY MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDGVDALNKLQ-AGGYGFVISDWNMPNMDGL 66

RR468 TVLKKLQEKKEEKRIPIVIVLTAKGGEESLLSLGARKVMRKPFSPSQFIEEVKHLLE----- 122
DifD EATREIIKADSSA--VVIMCSALGQESLVMEAIFAGASDFIVKPFRAEDVLAVVKKVIGET----- 122
EpsW DLCEHIRRSATMADVPVVLVSARDLPEDKAHEFAGANGYLGKPFTQEEFTRRVQSLKSTGARRAP 134
CheY ELLKTIRADGAMSALPVLMTAEAKKENIIAAAQAGSGYVVKPFTATLEEKLNKIFEKLGM---- 129

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Figure 1. Alignment of RR4468, DifD, EpsW and CheY. The primary specificity-determinant residues in RR468, DifD and EpsW are in bold and underlined whereas the secondary ones are in bold only. The identical specificity-determinant residues in DifD and EpsW are highlighted in red and the conserved or semi-conserved ones in green. The three CheY residues in red can be mutated to generate constitutively active proteins and their counterparts in EpsW (also in red) were selected for specific mutagenesis in this study.

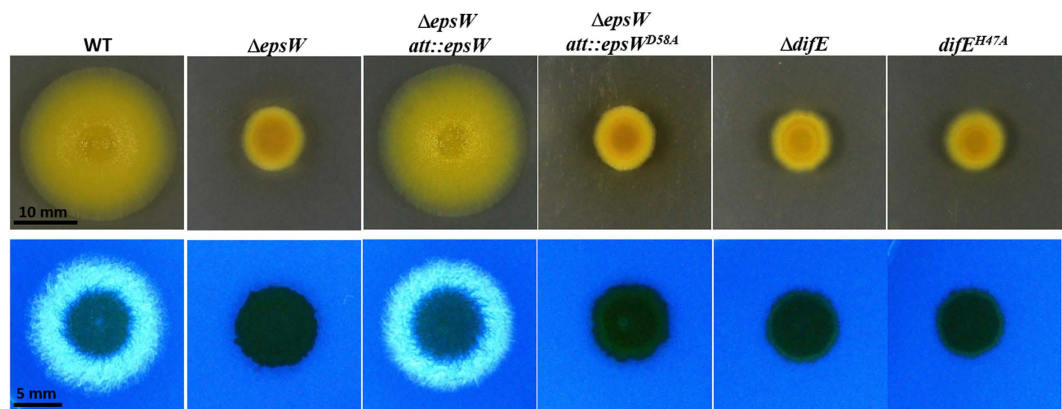


Figure 2. Examination of S motility and EPS production. Upper Panel. Examination of S motility on soft (0.4%) agar plates. Lower Panel. Analysis of EPS production on plates containing Calcofluor white. The genotypes of the strains are indicated on the top of the figure. The strains are DK1622 (wild-type, WT), YZ1830 ($\Delta epsW$), YZ1831 ($\Delta epsW att::epsW$), YZ1833 ($\Delta epsW att::epsW^{D58A}$), YZ603 ($\Delta difE$) and YZ1369 ($difE^{H47A}$). See Methods for details.

Fig. 2, the $\Delta epsW$ mutant colony did not fluorescence on plates supplemented with a fluorescent dye as an EPS indicator (See Methods), showing that the $\Delta epsW$ mutant is defective in EPS production. In addition, this mutant was defective in swarming on soft (0.4%) agar, indicating S-motility deficiencies³⁵. The wild-type *epsW* complemented $\Delta epsW$ in both S motility and EPS production assays (Fig. 2). These results demonstrate clearly that EpsW, a single-domain RR, plays an indispensable role in *M. xanthus* EPS regulation. It should be noted that *epsW* was so designated because it is associated with known EPS biosynthetic genes at the *eps* locus³⁴, but no *epsW* mutation had been constructed and examined previously. EpsW is considered an orphan RR because no HK gene is found in the immediate vicinity of *epsW*³⁴.

It is known that T4P functions upstream of the DifE pathway and T4P mutants are EPS^{-16,36,37}. A possible explanation for the above observations (Fig. 2) was that the $\Delta epsW$ mutation eliminated or attenuated either T4P expression or assembly and this T4P defect in turn resulted in the observed EPS phenotype of the $\Delta epsW$ mutant. To examine this possibility, we tested whether the deletion of *epsW* affected T4P expression and/or assembly. First, whole cell lysates were analyzed by immunoblotting with anti-PilA antibodies³⁸. The $\Delta epsW$ mutant was found to express PilA at a similar level as other strains (Fig. 3) known to express PilA at the wild-type level^{12,39}. These results indicate that the $\Delta epsW$ mutation does not lead to defects in PilA expression. Next, the fraction that contains surface pili³⁹ was subjected to immunoblotting with the same antibodies (Fig. 3). The $\Delta epsW$ mutant was found to be competent in the assembly of surface T4P as compared to the piliated *difE* mutant¹². In this experiment, the *pilB* mutant was used as a negative control because it cannot assemble T4P despite its ability to express PilA^{39–41}. The function of EpsW in *M. xanthus* EPS regulation is therefore not upstream of T4P. Instead these results are consistent with EpsW being downstream of DifE in *M. xanthus* EPS regulation as a substrate for its phosphorylation.

EpsW is phosphorylated by the DifE kinase *in vitro*. We determined if EpsW is a target for DifE phosphorylation using purified proteins expressed in *Escherichia coli* (Fig. 4). EpsW with a 6 × His tag at its N-terminus was purified as described in Methods. DifE was purified and phosphorylated using

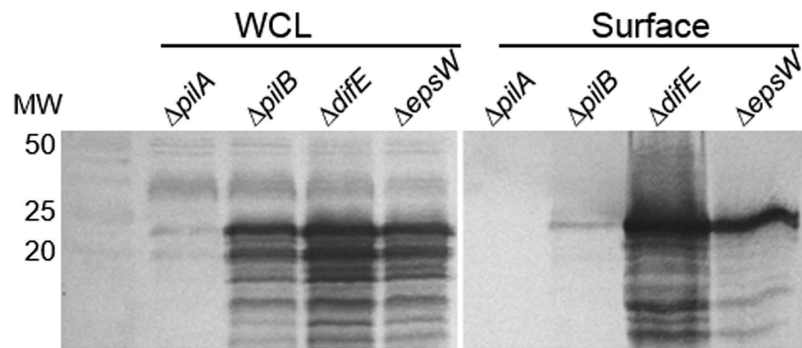


Figure 3. Examination of PilA expression and T4P assembly. To examine PilA expression, whole cell lysates (WCL) were prepared from 5×10^7 cells. For the examination of T4P assembly, the fraction of surface pili (Surface) were prepared from 5×10^8 cells as described in Methods. Samples were analyzed by immunoblot analysis using anti-PilA antibodies. The first lane contained molecular weight standard (MW) with the weight in KD indicated on the left. Strains analyzed were DK10407 ($\Delta pilA$), DK10416 ($\Delta pilB$), YZ603 ($\Delta difE$) and YZ1830 ($\Delta epsW$). See Methods for more details.

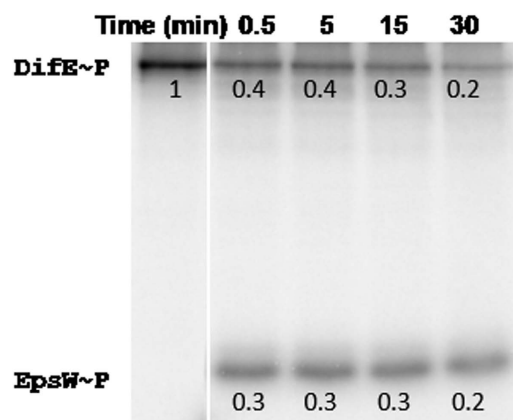


Figure 4. *In vitro* phosphotransfer from Dife~P to EpsW. Prephosphorylated Dife~P labeled with [γ - 32 P] ATP was mixed with equimolar amounts of EpsW and incubated for the indicated times in minutes (min). Samples were separated by SDS-PAGE and analyzed by phosphorimaging as described in Methods. The number below each band is the relative radioactivity as normalized to that of Dife~P without EpsW as loaded in the first lane. The position of each protein is indicated on the left.

[γ - 32 P] ATP as previously described¹¹. Phosphorylated Dife (Dife~P) was then mixed with an equimolar EpsW and incubated for 0.5, 5, 15 and 30 minutes before the reaction was terminated. These samples were separated by SDS-PAGE and phosphorylation was monitored by phosphorimaging. As shown in Fig. 4, Dife~P lost ~60% of its phosphate to EpsW in half a minute with the concurrent appearance of EpsW~P. Over the course of 30 minutes, the amount of Dife~P gradually decreased although no further increase in EpsW phosphorylation was observed. It is known that Dife~P by itself is stable as has been observed for other phosphorylated HKs¹¹. These results therefore indicated that additional phosphate transfer from Dife~P to EpsW occurred and the lack of further increase in the level of EpsW~P may be explained by dephosphorylation. The results here clearly demonstrate that EpsW is a substrate of Dife phosphorylation. Because DifeD is also phosphorylated by Dife~P¹¹, this is therefore a case where one HK phosphorylates multiple RRs^{20,21,42}. These results (Figs 1–4) are consistent with the notion that EpsW regulates *M. xanthus* EPS production in the same signal transduction system as Dife and DifeD.

Phosphorylation of EpsW and Dife is likely critical for their function. The phosphorylation of and phosphate transfer between Dife and EpsW are presumed to be important for their role in *M. xanthus* EPS regulation. To examine this experimentally, the conserved histidine residue (H47) for autophosphorylation in Dife^{39,43} was mutated to an alanine. This H47A mutant allele of *dife* (*dife*^{H47A}) was used to replace its wild-type counterpart at its chromosomal location (see Methods). As shown in Fig. 2, this *dife*^{H47A} mutant resembled a *dife* deletion strain as both showed a lack of S motility and EPS production.

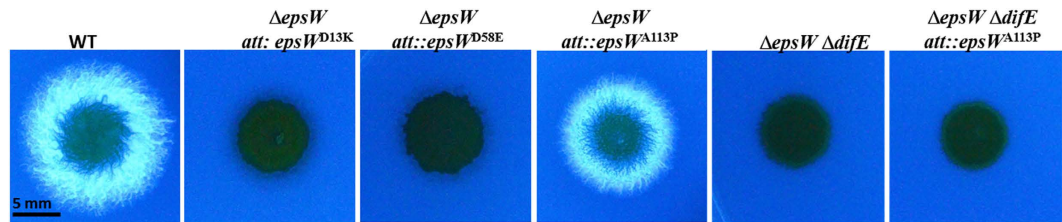


Figure 5. EPS production of *epsW* point mutants. EPS production was analyzed as in Fig. 2. Strains used were: DK1622 (wild type, WT), YZ1832 ($\Delta epsW$ att::*epsW*^{D13K}), YZ1834 ($\Delta epsW$ att::*epsW*^{D58E}), YZ1836 ($\Delta epsW$ att::*epsW*^{Q113P}), YZ1840 ($\Delta difE$ $\Delta epsW$), YZ1842 ($\Delta difE$ $\Delta epsW$ att::*epsW*^{Q113P}).

This observation shows that the autophosphorylation of DifE, thus its histidine kinase activity by extension, is likely critical for its function as a regulator of EPS production in *M. xanthus*.

Next, aspartate 58 (D58) of EpsW, the consensus phosphorylation site (Fig. 1), was mutated to an alanine (A) to examine if the phosphorylation of EpsW is required for EPS production. The plasmid with the D58A mutation of *epsW* (*epsW*^{D58A}) was examined for complementation of $\Delta epsW$. As shown in Fig. 2, unlike the wild-type, the *epsW*^{D58A} mutant allele failed to complement $\Delta epsW$ in S motility and EPS. These mutagenesis results argue that the phosphorylation events involving DifE and EpsW play key roles in EPS regulation and that the phosphorylated DifE and EpsW are the active forms of these proteins in EPS regulation in *M. xanthus*.

EpsW may function as an intermediary in a multistep phosphorelay. Our results thus far demonstrate that EpsW accepts phosphate from and functions downstream of DifE in the EPS regulatory pathway. Because EpsW is a CheY-like single domain RR, it may regulate the activity of an EPS biosynthetic enzyme by direct protein-protein interaction as does CheY with motor switch proteins^{44–46}. As discussed above, the *epsW*^{D58A} mutation led to the same EPS⁻ phenotype as the $\Delta epsW$ null allele. The phosphorylation therefore would presumably convert EpsW to a signaling conformation to activate its target enzyme by physical interaction. If this is true, a mutation may convert EpsW to an active conformation without the need for phosphorylation. Alternatively, EpsW~P may pass its phosphate to another protein in a multi-step phosphorelay⁴⁷. In this scenario, EpsW would act as an intermediary in a multistep phosphorelay to deliver the phosphate from its kinase to a phosphate acceptor downstream. As a consequence, the phosphorylation of EpsW would be required to signal downstream in this case.

For a RR not involved in multistep phosphorelay such as CheY, CheB or NtrC, replacing its phosphorylated aspartate residue with a glutamate (E) is known to convert it to a constitutively active form^{48–51}. This would be the equivalent of a D58E mutation in *epsW* (Fig. 1). In addition, the equivalents of D13K and Q114P mutations of *epsW* produced constitutively active CheY proteins that favor clockwise (CW) flagellar rotation in *E. coli*⁵¹. In other systems, these RR mutations can also bypass the requirement for their cognate kinases⁵¹. These three substitutions were made in EpsW and they were examined first for complementation of the $\Delta epsW$ mutation (Fig. 5). Somewhat unexpectedly, both the *epsW*^{D13K} and *epsW*^{D58E} alleles failed to restore EPS production to the $\Delta epsW$ mutant, indicating that they are not gain-of-function mutations leading to constitutively active proteins. The *epsW*^{Q114P} allele did restore EPS production to the $\Delta epsW$ strain (Fig. 5). When this allele was transformed into a $\Delta difE$ $\Delta epsW$ double mutant, however, the resulting strain mirrored the EPS⁻ phenotype of the parental strain. The phosphorylation of EpsW^{Q114P} is therefore likely required for its function in *M. xanthus* EPS regulation. These results here suggest that EpsW is likely not the terminal acceptor of phosphates from its kinase in EPS regulation. Instead, it may function as an intermediary to donate phosphates downstream in a multistep phosphorelay.

Discussion

In this study, we searched for the previously postulated RR that functions downstream of DifE as the substrate of this kinase. Bioinformatics analysis using the specificity determinant residues in DifD led to EpsW as a possible candidate (Fig. 1). Genetics experiments demonstrated that *epsW* is indispensable for EPS production in *M. xanthus* (Fig. 2). Studies with purified proteins showed that EpsW accepted phosphate from DifE~P (Fig. 4). These results indicated that EpsW is part of the Dif pathway for the regulation of EPS in *M. xanthus*. Interestingly, observations with specific *epsW* mutations suggest that EpsW is unlikely the terminal phosphate acceptor in the Dif pathway (Fig. 5). Here we propose that EpsW is downstream of the DifE kinase as a target of its phosphorylation. Instead of the terminal RR of the pathway, EpsW is more likely an intermediary in a multistep histidine-aspartate phosphorelay that regulates EPS production in *M. xanthus*.

There are a few lines of evidence that EpsW is a target of DifE and functions downstream of this kinase. The rapid dephosphorylation of DifE~P and the simultaneous phosphorylation of EpsW (Fig. 4) clearly demonstrate that EpsW is an authentic and cognate substrate for the DifE kinase^{21,42}. As a substrate, EpsW could function downstream of DifE or as another phosphate sink like DifD^{11,16}. The deletion

of *epsW* led to EPS⁻ (Fig. 2), the opposite of the EPS overproduction phenotype of a *difD* mutant¹³; this supports EpsW being downstream of DifE. Additional corroborations come from the observation that EpsW does not affect T4P production (Fig. 3) and that the *epsW*^{Q114P} mutation supported EPS production only in a *difE* wild-type background (Fig. 5). Mutagenesis of the consensus phosphorylation residues in DifE and EpsW (Fig. 2) lends support to the notion that the phosphorylation involving DifE and EpsW is key to their role in EPS regulation and that their phosphorylated forms apparently promote EPS production. We propose that signals from T4P¹⁶ stimulate DifE autophosphorylation which leads to the increase in the levels of phosphorylated EpsW to enhance EPS production in *M. xanthus*.

There are at least two possible scenarios how EpsW, a single-domain RR, may signal EPS downstream. Like CheY in chemotaxis⁵¹, EpsW could function as the terminal phosphate acceptor of the Dif regulatory pathway. That is, the activated form of EpsW, i.e., EpsW~P, would directly interact with a target protein to affect EPS production without further phosphate transfer. Alternatively, EpsW~P may pass its phosphate to proteins downstream in a phosphorelay as exemplified by the sporulation regulatory phosphorelay in *B. subtilis*^{47,52}. It is well known that a terminal RR can be converted to a constitutively active form by the substitution of either of two amino acid⁵¹. However, their equivalent mutations in EpsW, D13K and D58E, led to the lack of EPS instead of EPS overproduction (Fig. 5), arguing against EpsW as a terminal RR. On the other hand, the observations are easily explained if EpsW is in the middle of a phosphorelay. The *E. coli* CheY equivalent of EpsW^{D13K} is poorly phosphorylated and CheY^{D57E}, the EpsW^{D58E} equivalent, lacks phosphorylation all together⁴⁸. If the respective phosphorylation of EpsW^{D13K} and EpsW^{D58E} is poor and nonexistent, the flow of signals downstream will be attenuated/terminated in a phosphorelay, explaining the EPS⁻ phenotype of the *epsW*^{D13K} and *epsW*^{D58E} mutants (Fig. 5). The results with the *epsW*^{Q114P} allele is also consistent with a model of a multistep phosphorelay. It is known that CheY^{A113P} is phosphorylated by CheA to a similar level as the wild-type CheY in *E. coli*⁴⁸. If EpsW^{Q114P} behaves in a similar manner, it will still be phosphorylated and able to transmit signals downstream in a phosphorelay in a *difE*-dependent manner. This explains the EPS⁺ phenotype of the *epsW*^{Q114A} single mutant and the EPS⁻ phenotype of the *epsW*^{Q114A} Δ *difE* double mutant (Fig. 5). It is cautioned, however, that inferences with these point mutations is by no means conclusive. The results here cannot eliminate EpsW as the terminal RR of the pathway without further experimental validation.

Methods

Growth Conditions. *M. xanthus* strains were grown and maintained at 32°C on Casitone-yeast extract (CYE) agar plates or in CYE liquid medium⁵³. XL1-Blue (Stratagene), the *E. coli* strain used for plasmid construction and protein expression, was grown and maintained at 37°C on Luria-Bertani (LB) agar plates or in LB liquid medium⁵⁴. Unless noted otherwise, agar plates contained 1.5% agar. Kanamycin and ampicillin were added to media at 100 µg/ml when appropriate.

Construction of Plasmids and *M. xanthus* Strains. pWB435 and pMY45N, two plasmids for allelic replacement of *epsW* and *difE*, were constructed based the vector pMY7. To construct pMY7, a PCR fragment containing 26 base pairs (bp) upstream and 24 bp downstream of the coding sequence of *galK* was amplified from *Aeromonas hydrophila* str. 1719:1⁵⁵ (Stephen A. Smith, Virginia Tech). This fragment was inserted at the tandem ApoI sites of pZerO-2 (Invitrogen) downstream of and in the same orientation as the kan^R gene. This vector allows gene replacement in *M. xanthus* through positive and negative selection using kanamycin and galactose, respectively^{56,57}. An *epsW* in-frame deletion fragment was generated by a two-step overlap PCR as described previously^{13,58}. This fragment, which deleted codons 23–121 of *epsW*, was cloned into the EcoRV site of pMY7 to create pWB435. To create a plasmid for allelic exchange of *difE* with the *difE*^{H47A} mutant allele, a two-step overlap PCR technique utilizing overlapping mutagenic primers⁵⁹ was used to amplify a fragment approximately 650 bp upstream and downstream of the 47th codon of *difE*. This fragment was cloned into the HindIII and XbaI sites of pMY7 to create pMY45N.

Five plasmids with the wild-type and various mutant alleles of *epsW* were constructed using pWB425 as a vector^{58,60}. pWB425 is able to integrate at the Mx8 phage attachment site and express genes ectopically in *M. xanthus*. pWB427 contains within the EcoRV site of pWB425 a PCR fragment 50 bp upstream and 3 bp downstream of the coding region of the wild-type *epsW*. pWB427 was then used to construct pWB441, pWB442, pWB444 and pWB447 which contain the D13K, D58A, Q113P and D58E substitutions in *epsW*, respectively. These mutations were introduced by a two-step overlap PCR technique⁵⁹.

All strains used in this study are isogenic to the wild-type strain DK1622⁶¹. YZ603 (Δ *difE*), DK10407 (Δ *pilA::tet*) and DK10416 (Δ *pilB*) have been described previously^{13,38}. The Δ *epsW* deletion mutant YZ1830 was constructed from DK1622 using pWB435 and a positive-negative selection method^{13,57,62}. The Δ *difE* Δ *epsW* double deletion strain YZ1640 was constructed similarly using pWB435 and YZ603 (Δ *difE*) as the parental strain. To create YZ1369 (*difE*^{H47A}), pMY45N was used to replace the wild-type *difE* allele in DK1622. The strains YZ1831 (Δ *epsW attB::epsW*), YZ1832 (Δ *epsW attB::epsW*^{D13K}), YZ1833 (Δ *epsW attB::epsW*^{D58A}), YZ1834 (Δ *epsW attB::epsW*^{D58E}), YZ1836 (Δ *epsW attB::epsW*^{Q113P}) were constructed by electroporation of pWB427, pWB441, pWB442, pWB444 and pWB447 into YZ1830 (Δ *epsW*), respectively. YZ1642 (Δ *difE* Δ *epsW attB::epsW*^{Q113P}) was constructed by electroporation of pWB447 into YZ1640 (Δ *difE* Δ *epsW*).

Examination of S motility and EPS Production. Strains to be examined for S motility and EPS production were grown in CYE liquid medium, harvested during exponential growth and resuspended in MOPS (morpholinopropanesulfonic acid) buffer (10 mM MOPS [pH 7.6], 2 mM MgSO₄) at 5 × 10⁹ cells/ml. For analysis of S motility, 5 μl of this cell suspension was spotted onto CYE plates containing 0.4% agar and allowed to incubate at 32 °C for 5 days before documentation. For examination of EPS production, plates containing the fluorescent dye Calcofluor white were utilized^{63,64}. Briefly, 5 μl of cells at 5 × 10⁹ cells/ml was spotted onto CYE plates supplemented with the dye Calcofluor white at 50 μg/ml which EPS⁺ strains bind. Fluorescence upon exposure to ~365 nm UV was documented after incubation at 32 °C for 5 days.

Analysis of PilA expression and assembly of T4P. For the examination of PilA expression, whole cell lysates from 5 × 10⁷ cells were separated by 15% SDS-PAGE and analyzed by immunoblotting with anti-PilA antibodies³⁸ as the primary antibody and goat-anti-rabbit antibodies conjugated to alkaline phosphatase as the secondary antibody (Santa Cruz Biotechnology). To examine strains for the assembly of T4P, pili isolated from 5 × 10⁸ cells were prepared by shearing and a differential precipitation as previously described^{12,39}. Briefly, surface pili were sheared from cell suspensions by vortexing for 3 minutes. Intact cells were removed by two rounds of centrifugation at 16,000 × g for 10 minutes. MgCl₂ was added to the supernatant at a final concentration of 100 mM. After incubation on ice for 60 min, the pilus fraction was collected by centrifugation at 16,000 g for 15 min and resuspended in SDS-PAGE loading buffer prior to SDS-PAGE and immunoblotting analysis with anti-PilA antibodies.

Protein expression and purification. To express and purify EpsW, plasmid pWB717 was constructed by cloning the entire coding region of *epsW* (except the start codon) into the BamHI and HindIII sites of pQE30 (Qiagen). This fused an N-terminal 6 × His tag to EpsW. For the purification of 6 × His-EpsW, a procedure similar to that of 6 × His-DifD was employed¹¹. Briefly, XL1-Blue containing pWB717 was grown at 37 °C to an OD₆₀₀ of 0.5–0.6 in 1 L of LB media with ampicillin. IPTG (isopropyl-β-D-1-thiogalactopyranoside) was added to 0.1 mM to induce at 19 °C for approximately 15 hrs. Cells were harvested by centrifugation and stored at –80 °C until needed. Cells suspended in 20 ml of binding buffer (20 mM NaH₂PO₄, 500 mM NaCl, 25 mM imidazole, 10% glycerol, pH 7.4) were lysed by two consecutive passes at 18,000 p.s.i. through a French press (Thermo Scientific). Cellular debris was removed by centrifugation at 16,000 × g for 30 minutes, the supernatant was centrifuged at 100,000 × g for 60 minutes. The supernatant was filtered (0.45 μm) and loaded on a 5 ml HisTrap FF (GE Healthcare) column using an Akta Prime chromatography system (GE Healthcare). Elution was performed using a 25–500 mM imidazole gradient of 50 ml. Fractions were analyzed by SDS-PAGE and those judged to contain EpsW at greater than 90% purity were buffer exchanged into storage buffer (10 mM Tris-Cl [pH 7.5], 25 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM β-mercaptoethanol [β-ME], and 10% glycerol) using a HiTrap desalting column (GE Healthcare). Protein concentration was measured using the Bio-Rad protein assay with BSA as the standard. Protein stocks were adjusted to 10 mM in storage buffer and stored at –80 °C until needed. 6 × His-DifE were purified as previously described¹¹.

Phosphotransfer assays. Phosphotransfer assays were performed similarly as previously described¹¹. Briefly, DifE was prephosphorylated in 1 × kinase buffer (10 mM HEPES [pH 8], 25 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM β-ME and 10% glycerol) with 15 μCi of [γ-³²P]ATP (3,000 Ci/mmol, 10 mCi/ml) for 45 min at room temperature. The reaction was quenched by addition of 10,000-fold excess of unlabeled ATP to a final concentration of 0.5 mM. DifE~P was mixed with EpsW in 1 × kinase buffer to give final concentrations of 1 μM for each protein and allowed to incubate for the specified times. The reactions were stopped by addition of 4 × SDS-PAGE loading buffer (250 mM Tris-Cl [pH 6.8], 8% SDS, 0.004% bromophenol blue, 40% glycerol, 20% β-ME, 200 mM EDTA). Samples were then separated by 15% SDS-PAGE and the gel was dried on filter paper and exposed to a phosphor screen for approximately 15 hr. Imaging of the phosphor screen was performed using a Typhoon Trio (GE Healthcare) scanner. Image analysis and quantification was performed using ImageQuant TL (GE Healthcare).

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Author Contributions

W.P.B. and Z.Y. designed research; W.P.B., L.W. and M.Y.D. performed research; W.P.B. and Z.Y. wrote the manuscript which all authors reviewed.

Additional Information

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